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Volume 676

Polyploidization and Cancer

Edited by
Randy Y.C. Poon

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DEDICATION

For my parents

PREFACE

Limiting genome replication to once per cell cycle is vital for maintaining genome stability. Although polyploidization is of physiological importance for several specialized cell types, inappropriate polyploidization is believed to promote aneuploidy and transformation. A growing body of evidence indicates that the surveillance mechanisms that prevent polyploidization are frequently perturbed in cancers.

Progress in the past several years has unraveled some of the underlying principles that maintain genome stability. This book brings together leaders of the field to overview subjects relating to polyploidization and cancer. The importance of polyploidization in the evolution of cancer is discussed by Merlo, Wang, Pepper, Rabinovitch, and Maley. Proper execution of mitosis is controlled by the spindle-assembly checkpoint and is paramount in preventing mitotic slippage and polyploidization. Ito and Matsumoto discuss our current understanding of this checkpoint. Cytokinesis failure is another important route to polyploidization. A discourse on the mechanisms that lead to cytokinesis failure and their relationship to genome instability is provided by Normand and King. The evidence of a role of DNA damage in polyploidization is also discussed (Chow and Poon). In normal cells, polyploidization is prevented by p53-dependent mechanisms. Salient features of these pathways are described by Talos and Moll. As discussed by Duensing and Duensing, defective mitosis caused by supernumerary centrosomes is increasingly being recognized for their roles in causing polyploidy and cancer. Furthermore, important examples of polyploidization including hematopoietic cells (Nguyen and Ravid) and liver cells (Celton-Morizur and Desdouets) serve to illustrate the pivotal role of polyploidization in cancers and senescence. Last but not least, state-of-the-art methodologies of how ploidy can be measured are detailed by Darzynkiewicz, Halicka, and Zhao.

I thank the various authors for their invaluable contribution. Much remains to be learned about the regulation of mitosis, cytokinesis, centrosome duplication, checkpoints, and their relationship to polyploidization and tumorigenesis. It is hoped that these articles will serve as a resource for further progress of this important area of cancer research.

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tetraploidy were commonly held to be incidental to tumor evolution. However, within the past several decades the importance of chromosomal abnormalities and chromosomal instability has risen. When aneuploid DNA content became readily identifiable by flow cytometry, it was noted that this finding was more common in higher grade cancers and that aneuploid tumors of many kinds had a more aggressive clinical behavior than their diploid counterparts.[6,7] Today, some authors argue that the evidence points to aneuploidy playing a pivotal role in the chromosomal instability that generates tumor diversity, clonal evolution and malignant phenotypes.[8]

While aneuploidy could in principal be generated by progressive additions to the diploid DNA content by accumulated chromosomal gains, as by mitotic nondisjunction, it would then be puzzling that aneuploid tumor DNA contents are most commonly in the triploid to tetraploid range.[9] Boveri's hypothesis allows that a tetraploid intermediate is a common precursor to aneuploidy and that subsequent chromosomal evolution by loss of superfluous chromosomes or chromosome segments results in the aneuploid chromosomal complement. A conceptual model of the role of the tetraploid intermediate in carcinogenesis was formalized by Shackney et al.[10] Supporting experimental evidence comes from observations of a tetraploid intermediate during murine carcinogenesis.[11,12] Furthermore, when diploid and tetraploid mouse cells from a common mammary precursor were directly compared, the tetraploid cells had greater chromosomal instability and only the tetraploid cells gave rise to malignant tumors when transplanted into nude mice.[13] Perhaps most significantly, tetraploidy has been demonstrated to be a precursor of aneuploidy in several human cancers, including Barrett's esophagus (see below) and cervical carcinoma.[14] The mechanisms that underlie generation of the tetraploid state are now recognized to include the failure of cytokinesis and, in particular, failure of checkpoint control during mitosis.[15] Loss of p53 function plays an important role in augmenting this process, as failure of p53-dependent G1 checkpoint and DNA repair commonly result in G2/M checkpoint arrest; failure of this latter checkpoint, or accommodation or "slippage," allows cells to reenter the cell cycle with a failure of cytokinesis, resulting in tetraploid G1 cells.[15,16]

Tetraploidy and Aneuploidy in Barrett's Esophagus

It is difficult to determine the role of polyploidy and aneuploidy in the development of cancer because most cancers cannot be studied longitudinally. When we detect a neoplasm we either remove it or, if it has metastasized, treat it systemically (which may generate additional aneuploid cells). The same is true for most premalignant neoplasms. This prevents us from studying the effects of ploidy changes on the further development of the neoplasm and from making direct observations of the ordering of events in progression. An important exception is Barrett's esophagus (BE).

Barrett's esophagus is a premalignant neoplasm[17] that predisposes for the development of esophageal adenocarcinoma (EA).[18] Characterized by the presence of specialized intestinal epithelium in the esophagus, it can be recognized endoscopically as a salmon-colored epithelium just above the gastro-esophageal sphincter. Only about 0.5% of people with BE progress to EA per year and most people with BE will die of some other cause.[19] Unlike other premalignant neoplasms, such as an adenomatous polyp in the colon, BE is not removed when detected. Esophagectomies have an 8%-23% mortality rate[20] and thus the risk of progression to EA does not justify the risk of removal of the BE segment. Instead, the standard of care is surveillance with periodic endoscopic biopsies for the early detection of cancer. If EA is detected in an intensive surveillance program, it is often caught prior to metastasis and patients can be treated surgically. For these purely clinical reasons, BE presents a scientific opportunity to study the genetics of how a neoplasm changes over time as it progresses to cancer.

We study BE as a model of neoplastic progression in solid tumors. Aside from the danger of removing it and ease of biopsying it, BE is similar to many other conditions that predispose to carcinogenesis in a variety of respects. Like inflammatory bowel disease, hepatitis, pancreatitis, prostatitis, *H. pylori* infection in the stomach and *Schistosomiasis* infection in the bladder, BE is characterized by chronic inflammation.[21] Similar to other premalignant conditions, only a minority of patients with BE progress to cancer. In addition, neoplastic progression in BE is characterized by some of the most common genetic lesions across all cancers: loss of the tumor suppressor genes p16 (INK4A/CDKN2A) and

p53 (TP53) and the development of tetraploidy and aneuploidy. Studying BE provides us the major advantage of observing the development of these lesions over time. What have these longitudinal studies taught us about the role of polyploidy and aneuploidy in neoplastic progression?

p16

The first genetic and epigenetic lesion commonly observed in BE is loss of the p16 tumor suppressor gene. Tlsty and colleagues have argued that loss of p16 leads to decoupling of the synthesis of DNA and centrosomes in the cell cycle, such that if either is delayed, the cell might enter mitosis with the wrong number of centrosomes or the wrong amount of DNA and aneuploidy could result.[22] It is unclear if this happens in BE. To date, the association between the loss of p16 and ploidy abnormalities has not been adequately studied. We do know that patients can live for many years lacking p16 in their BE neoplasm but never develop aneuploidy.

p53

The genetic lesion that has been associated with the development of both tetraploidy and aneuploidy in BE is loss of the p53 tumor suppressor.[23,24] Our current hypothesis is that although aneuploid cells may arise in a p53 wildtype clone, they normally trigger the p53-dependent DNA damage checkpoint which either leads to senescence or apoptosis and so the aneuploid clone never grows large enough to be sampled. Once the p53 checkpoint is compromised, aneuploid clones are free to proliferate without check. This is why we believe that the loss of p53 precedes the development of both tetraploidy and aneuploidy. Loss of heterozygosity at the p53 locus is also the strongest single predictor of progression and is associated with a 16-fold increased risk of progression to EA[24] as well as a 6-fold increased risk of developing tetraploidy and a 7.5-fold increased risk of developing aneuploidy.

Tetraploidy

Tetraploidy, defined in this case as greater than 6% of cells with 4N DNA content, is also a predictor of progression associated with a 12-fold (95% CI: 6.2-22) increased risk of progression to EA.[25] Sometimes this may be an indication of cells being stalled in the G2 phase of the cell cycle. Other times, the presence of 8N cells in cell cycle analysis suggests that there are viable tetraploid cells in the neoplasm. FISH studies have found that loss of heterozygosity in p53 as detected by microsatellite analysis could be caused by deletion of one allele of p53 or, more often, by duplication of the genome followed by deletion of multiple p arms of chromosome 17 where p53 resides.[26]

Aneuploidy

Most cases of aneuploid clones in BE have DNA content between diploidy and tetraploidy further suggesting that tetraploidy is an intermediate stage of progression followed by selective loss of parts of the genome. This appears to be true of other cancers as well.[9,27-29] We have compiled a survey of 57 esophageal adenomas that were surgically removed prior to therapy and analyzed for DNA content in our study (Fig. 1). This new data agrees with our previously published data[25] that hypodiploids and supratetraploids are rare.

The detection of an aneuploid clone in BE is associated with a 9.5-fold (95% CI: 4.9-18) increased risk of progression.[25] However, the presence of both tetraploidy and aneuploidy is an indication of greater risk of progression than either alone[25,30] and may be a sign of more extensive genomic instability.

It should be noted that in BE, at least, the loss of p53 and the development of aneuploidy is not sufficient to cause cancer. In contrast, loss of p53 is thought to cause malignancy in colorectal carcinogenesis.[31] Although BE patients with both a p53 lesion and a ploidy lesion (either tetraploidy or aneuploidy) are at a very high risk of progressing to cancer, that process can still take years.[30] So there must be other loci that are being targeted by the gains and losses during the further evolution of aneuploid clones. Hopefully, genome-wide analyses of aneuploid BE and EA will reveal the final genetic lesions that cause invasion and metastasis.

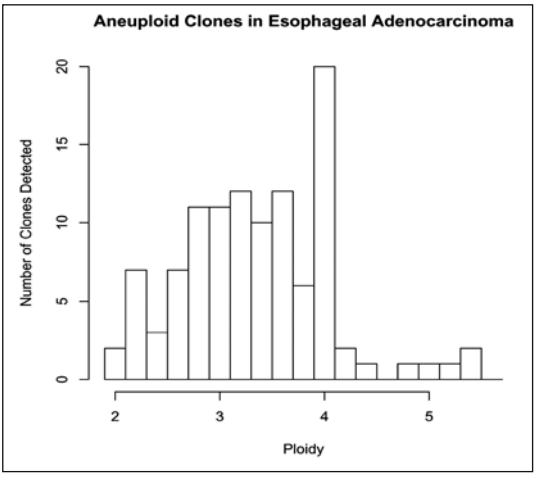

Figure 1. 55 out of 57 esophageal adenocarcinomas in the Seattle Barrett's Esophagus cohort were aneuploid or tetraploid. We detected 107 different aneuploid clones (and 2 diploid clones) in these 57 neoplasms. The frequencies of the different ploidies (2 = diploid, 4 = tetraploid) are shown in the histogram.

Not All Aneuploids Are Equal

Every aneuploid clone is unique. Each contains its own set of gains and losses, some of them probably random, others selected because they gave the clone a competitive advantage in the microenvironment of the neoplasm. Thus, it may be misleading to speak of aneuploidy as a "state". We should expect different aneuploid clones to behave differently during progression and therapy.

It is tempting to think of polyploidy as a state that should not substantially affect the fitness of a cell because the gene dosage is amplified equally across the entire genome. However, that would only be true if the expression of genes was a linear function of concentrations of regulatory proteins. Instead, genetic regulatory interactions, including posttranscriptional modifications, multi-mer regulatory complexes and the interactions of enhancers and inhibitors, are all highly nonlinear. Thus, a gene's expression level may actually decrease with the doubling of the genome if it is overwhelmed by the doubling of an inhibitor gene.[32,33] The effect of increasing the ploidy of a cell on its protein concentrations may be equivalent to genome-wide sequence mutation events. These nonlinear interactions may explain the lethal developmental abnormalities associated with ploidy irregularities in human embryos. Though in most cases gene expression levels do seem to be correlated with gene copy number.[34,35] In fact, the story may be even more complicated because the phenotype of the cell may depend on how it became polyploid. A study in a BE cell line that reliably produces a tetraploid sub-population showed evidence that the cells had somehow passed through mitosis without actually dividing and expressed genes characteristic of both mitosis and G1.[36]

Clones that become malignant may derive from tetraploid cells, for the reasons described earlier, including the fact that tetraploid cells have the genetic buffer that allows them to lose large chunks of their chromosomes and thereby select for different gene dosage effects while retaining enough copies of essential genes to maintain the viability of the cell. Diploid cells that start losing large portions of their genome are probably more likely to suffer a fatal genetic lesion.

If this view is correct, then aneuploidy is a crude mechanism to change gene expression levels. It is crude because copy number alterations often affect large regions of a chromosome, sometimes whole chromosomes and so affect many genes at once. We would predict that some aneuploid states would produce gene product levels that result in a higher fitness for an aneuploid cell than a tetraploid cell. Specifically, we would predict that an aneuploid clone should have a higher

fitness and expand faster than a tetraploid clone from the same neoplasm. This may explain the apparent transient nature of tetraploidy in BE and could be tested in competition experiments in tissue culture.

Because the regions of copy number change affect so many genes in parallel, it is difficult to determine which among those genes are responsible for any increase in fitness of a clone. While gene mapping studies can help pinpoint specific regions commonly amplified or deleted (e.g., location of p16, p53), in general we need better tools to measure the fitness effects of gene deletions and amplifications to clarify the selective effect of copy number changes.

Amplifications and deletions of a few specific genetic regions are known to be associated with cancer, but thus far a census of copy number changes across cancers has not been compiled. There are a variety of challenges associated with such a task. First of all, it is not clear how to calculate the frequency of a copy number change at a locus. Should it be the frequency across cancer patients, in which case lesions in the most common cancers would dominate the results, or should it be the frequency across sub-types of cancer, such that a lesion that appeared in 50% of a rare type of cancer would be weighted equally with a lesion that appeared in 50% of a common type of cancer? Different sub-types of cancer, even within a particular organ system, are likely to have different patterns of copy number changes across the genome. Many CGH studies have not segregated sub-types of cancer since we are still in the process of distinguishing new sub-types. Defining these different sub-types is important because cancers originating in different cell types and organs experience different microenvironments and thus different evolutionary pressures that select for copy number changes in different parts of the genome. Experimentally, if a cancer is aneuploid it is critical to normalize CGH arrays by the number of cells not the amount of DNA, else the average fluorescence reading in a CGH study would appear to be diploid while true diploid regions would be identified as deletions. CGH array studies are also sensitive to the amount of contaminating stroma than can obscure copy number changes in the cancer. Of course, different studies have used different platforms, so any compilation of current studies probably could not resolve copy number changes below the chromosome arm level. If the cancer genome project is successful, it should solve the problems of low resolution and incompatible technological platforms. In fact, the ability to find consistent patterns of genetic alterations in cancer is one of the justifications for the cancer genome project. A further important result of a comprehensive census of copy number changes in cancers would be a determination of which amplifications and deletions are not seen in cancers. These alterations are likely to be deleterious to a neoplastic clone and might lead to the development of new therapies.

Why Do Cancer Cells Survive with Such Massive Alterations to Their Genome?

The genomic instability that characterizes neoplastic progression often involves gains and losses at the scale of chromosome arms and even whole chromosomes. Thus, hundreds of genes are often duplicated or deleted in a single event. In most biological (and technological) systems, a mutation in a functional part is deleterious. How can a cell survive the massive alterations seen in cancers?

Amplification of a gene, while often increasing expression level of the gene, does not necessarily increase the protein level of that gene. There is evidence that if a gene is normally part of a protein complex and the other members of the complex are not over-expressed, the over-expressed, uncomplexed protein may be quickly degraded, resulting in normal protein levels.[34] Thus, cells may be robust to many amplification events.

Devoting metabolic effort for the benefit of the organism and suppressing cellular proliferation are disadvantageous for a neoplastic cell. Those cells that can reproduce faster, avoid differentiation, suppress apoptosis, stabilize their telomeres and stimulate angiogenesis when hypoxia becomes a problem will proliferate faster than competitors that lack those hallmarks of cancer.[37] At the initiation of a neoplasm, cells may not be adapted to the selective pressures of the neoplasm. It is quite possible that many mutations would be beneficial rather than deleterious because they result in the dismantling of the genetic machinery that keeps proliferation in check in a multicellular body.

Perhaps most of the genome of a multicellular organism is devoted to building and maintaining the organism rather than the individual cells[38] and so even large scale copy number changes may not be deleterious for a neoplastic cell.

This hypothesis, that large scale deletions in cells are much more likely to be beneficial to the cell than to an embryo, could be tested experimentally. One could experimentally delete chromosome arms by using the Cre-lox system of site-directed recombination[39-41] (and perhaps even whole chromosomes if one deleted the centromere). The fate of the cell as an orthotopic injection or as an embryonic stem cell could then be compared to wildtype cells. Expression of Cre under an organ-specific promoter would help test the effects of deletions in different organ contexts. It would be important to do the complementary experiment, testing the selective effects of amplifying whole chromosomes or chromosome arms. A recent study generated yeast strains with extra chromosomes through abortive nuclear fusions during mating and showed that an extra copy of a chromosome caused a decrease in fitness, regardless of which chromosome was duplicated.[34] We are not aware of an experimental method to carry out an equivalent study in mammalian cells, though fibroblasts from Downs syndrome patients (trisomy of chromosome 21) grow more slowly than fibroblasts from age-matched controls.[42]

Aneuploidy in Development

Certainly during development most inherited copy number changes are deleterious. The selective effect of ploidy changes appears to be fundamentally different in cancer as compared to ploidy abnormalities present in the germ line. During development, ploidy abnormalities are nearly always lethal. Trisomy (3n) of the entire genome, usually caused by the fertilization of a single egg by two sperm, is lethal, as is tetraploidy (4n), generally caused by the failure of the zygote to divide. Monosomy of individual chromosomes is invariably lethal at a very early stage in gestation, as is trisomy of all but a few chromosomes.[43]

Aneuploidy is common in human embryos and is the leading cause of miscarriage.[44] For unknown reasons, humans have a 10-fold higher rate of aneuploidy compared to other mammals.[45] A few duplications do lead to viable offspring, occurring as rare genetic disorders. Trisomy of chromosomes 13, 18 and 21 are the only whole-autosome trisomies compatible with survival until birth and only trisomy of chromosome 21 (Downs syndrome) allows for survival into adulthood. All are associated with significant developmental abnormalities. Trisomy of chromosome 13 (Patau syndrome) is associated with limb and facial abnormalities, heart and kidney defects and neurological abnormalities including failure of the brain to divide into halves during gestation (holoprosencephaly). Trisomy of 18 (Edwards syndrome) also leads to severe developmental abnormalities.[46] In addition, there are a variety of other conditions associated with ploidy abnormalities (loss or duplication) of various chromosome arms or smaller sections of chromosomes, including trisomy of chromosome arms 10q, 16p and q and monosomy disorders such as 4p- (Wolf-Herschhorn syndrome) and 5p- (cri-du-chat syndrome). While many of these abnormalities have been described, only a few have been genetically characterized. For instance, a 17p- monosomy known as Smith-Magenis syndrome is specifically caused by deletion of the RAI1 (retinoic acid induced 1) gene. The exact function of the gene is unknown, but it is believed be a transcriptional regulator critical for neurological development with disease symptoms the result of haploinsufficiency.[47]

So what is different about cancer that allows for rampant aneuploidy? A copy number change that affects every cell in the body may be likely to have catastrophic effects in at least one organ. In contrast, after development if a new copy number mutation occurs within a single organ, it may be unlikely to be immediately fatal. In fact, cells in any particular organ may not utilize many genes in the genome and so large deletions and amplifications may often be selectively neutral, rather than deleterious. So, large scale copy number changes may only be important during development. Of course, cancer is disadvantageous to the organism as a whole, so perhaps some copy number changes are lethal during development because they essentially cause cancer before the body can even form. One way to test this idea in a model species would be to determine if embryonic cells with extra chromosomes divide at different rates than normal embryos.

Polyploidy in the Evolution of Species

Polyploidization has been hypothesized by some to be a substantive force in genome evolution in both plants and animals.[48] There are a variety of hypotheses for the benefits of polyploidy in genome evolution, though few of these would explain any immediate benefit that would occur on the time scale of a developing cancer. Polyploidy in plants is common, particularly in angiosperms, which raises the question of why polyploidy is detrimental in animal systems but not in plants. It may be a more common phenomenon in plants for the following reasons. First, many plants are self-fertilizing and thus do not have to contend with the problem of reproduction with an individual with the standard chromosome number. Second, polyploidization in animals is thought to be developmentally lethal because of imbalances in protein levels; humans face this issue with their dimorphic sex chromosomes and the X-inactivation mechanism is required for dosage compensation. One would presume that plants would face the same challenges, though perhaps plants have better mechanisms for dosage compensation. In animals, ploidy abnormalities often lead to sterility, a problem not encountered by plants. Plants are also less susceptible to cancer than animals since they lack the circulatory system, migratory (potentially metastatic) cells and vulnerability to organ failure common in animals.

Despite these differences, some insights may be gained by examining the major hypotheses for the frequency of polyploidization in plant systems.[49] Polyploid plants tend to be larger and more robust. This may explain why many domesticated crops are polyploid and why polyploidy is more likely in plants found in extreme environments. Polyploids also have the advantage of gene redundancy: disadvantageous recessive alleles are masked by wild-type alleles and mutations occurring in duplicated alleles may not reduce the total fitness of the organism.[50] In plants, polyploidy also can lead to a loss of self-incompatibility, which may improve reproductive ability. Many animal genomes also show evidence of ancient polyploidization events.[48] Similar to the hypothesized diploid-tetraploid-aneuploid progression in cancer,[16,51] it is thought that genomes may be initially unstable after a polyploidization event and then detrimental duplicated genes are removed or diverge through the process of selection.[52]

Why Is Aneuploidy Common in Neoplastic Progression?

Setting aside the issue of what proportion of copy number changes are beneficial to a neoplastic cell, there is still a question of why aneuploidy and polyploidy are so common in cancer. There are at least three categories of possible explanations for the prevalence of aneuploidy in carcinogenesis: 1) Aneuploidy is a common side effect of lesions that occur during carcinogenesis but is otherwise evolutionarily neutral. 2) Aneuploid clones, by dint of their genetic instability, often generate specific genetic lesions that provide the clone with a competitive advantage over other clones, regardless of their ploidy. 3) Aneuploidy itself provides a competitive advantage over diploid clones.

A Competitive Advantage of Aneuploidy

Several lines of evidence seem most compatible with the third hypothesis: Aneuploidy is observed at high frequencies in many cancers and many different types of cancer.[53] Aneuploidy is more common in higher grade cancers and is associated with more aggressive clinical behavior.[6,7] Aneuploidy does not seem to be logically required for carcinogenesis. Diploid cells can silence tumor suppressor genes by methylation, sequence mutations or localized deletions. They can also activate oncogenes by sequence mutations, local amplifications or over-activation of up-stream genes in the same pathway. This line of reasoning also suggests that aneuploidy may be common because it often provides a direct competitive advantage over diploid cells. Why might this be true?

We argued above that nonlinear feedback effects in gene regulation may make large scale amplifications act like wide spread mutations, up- and down-regulating expression of genes across the genome. Our question then becomes: Given the conventional wisdom that random mutations are expected to reduce fitness, why do the 'mutation packages' resulting from aneuploidy apparently go to high frequency, as if under positive somatic selection? There is a crucial difference between the expected fitness effects of mutations in organisms versus in metazoan cells. Organismal selection has

favored any alleles that increase organismal survival and reproduction. Thus, organisms are typically on or near a peak in their fitness landscape so that most random moves in phenotype space (most mutations) are detrimental to organism fitness.

In contrast, during the progression of a neoplasm, cells are subject not to organismal selection, but to somatic selection among cells.[54] Through their evolutionary history of organismal selection, genomes have not been shaped for optimal fitness in terms of somatic selection. Indeed the opposite may be closer to the truth. Many of the genes in a metazoan genome may function to constrain cellular competition.[55] For example, a history of organismal selection has shaped cell genotypes that limit cell reproduction and increase cell mortality (e.g., through apoptosis).[54] This implies that many mutations may provide a competitive advantage for the mutant cell.[38] Organismal selection is also expected to create in cells mechanisms of 'antiredundancy' or hypersensitivity to the deleterious effects of mutations which help the organism by culling mutant genomes from its cell population.[56]

The combined effect of evolved constraints on cellular competition and evolved antiredundancy mechanisms is that in terms of somatic competition within an organism, cells are expected to be located at or near the bottom of a 'pit' in their fitness landscape.

For organisms located on a fitness peak, random mutations are expected on average to be deleterious. In contrast, for cells located in a fitness pit, random mutations are expected on average to be beneficial.

Since the vast majority of aneuploid and polyploidy cells are supradiploid (greater than 2N chromosomes, Fig. 1), at the very least they carry the burden of having to replicate more DNA during the synthesis phase of the cell cycle than a diploid cell. This should require more cellular resources and perhaps more time than a diploid cell. If the process of genome synthesis is sufficiently parallel and resources are not limiting, then the extra DNA content of a supradiploid cell may not reduce the reproduction rate of the cell. One might be able to test this by generating a tetraploid cell through fusion and comparing its cell cycle time to the diploid cells that were used to generate it. Aneuploids generated in yeast appear to have reduced proliferation rates and require increased glucose uptake to fuel the protein synthesis generated by extra copies of genes which are mostly active.[34] Furthermore, experimental suppression of CENP produces aneuploid cells with a reduced frequency of viable daughter cells but without a measurable change in the net proliferation rate.[57]

One possible explanation for a supradiploid competitive advantage over diploid cells is that supradiploid cells are likely to be more robust to deletions and gene inactivation because they have more copies of essential genes than diploid cells. If an essential gene is haploinsufficient, then a deletion of a single allele in a diploid cell will be fatal. However, a deletion of a single allele in a tetraploid cell would leave three viable alleles and the tetraploid cell would survive. Thus, we would expect environments that cause frequent deletions to select for supradiploid cells. Conditions of chronic inflammation, including Barrett's esophagus, produce oxygen and nitrogen radicals that can result in such DNA damage.[58-60]

Aneuploidy May Generate Advantageous Lesions

The fact that experimentally derived aneuploid cells tend to suffer a fitness disadvantage relative to wildtype cells[34,57] suggests that aneuploidy, as a form of a mutator phenotype,[61,62] may not provide a direct advantage but may increase the probability that one of the genetic variants it generates has a competitive advantage due to the inactivation of a tumor suppressor gene or the amplification of an oncogene. Mutators gain a competitive advantage over nonmutators in at least three cases. First, if beneficial mutations are more likely than deleterious mutations, a lineage that increases its mutation rate will also, on average, increase its fitness. Second, even if deleterious mutations are more common than beneficial mutations, if the occasional beneficial mutation has a dramatic effect, the mutator lineage may generate a variant that out-competes all other lineages even if most of the variants it had produced until that point had been burdened with deleterious mutations. Weaver et al found that although their aneuploid cells produced

fewer viable daughter cells than wildtype cells, aneuploid cells produced more colonies capable of anchorage independent growth.[57] Finally, if the selective environment—including both the microenvironment and the genetic make-up of the competitors—changes frequently, a lineage that produces a diverse set of offspring is more likely to produce at least one that can survive in the changing environment compared to a lineage that produces a homogenous set of offspring. In this case, a mutator lineage is more likely to adapt to the changing environment than a nonmutator. All of these possibilities may apply to neoplastic progression. Experiments to test the fitness effects of various mutations could test the first two cases. Whether an aneuploid cell evolves faster to a changing environment than a diploid cell could be tested in vitro with any number of changing exposures. It is interesting to note that spontaneously occurring mutator strains of *E. coli* do not appear to have a higher fitness than the nonmutator strains.[63,64] We should not be too quick to accept the idea of an indirect benefit of aneuploidy as a mutator phenotype.

Aneuploidy May Be an Evolutionarily Neutral By-Product of Carcinogenesis

The final alternative is that aneuploidy is evolutionarily neutral. It may be a common phenomenon if many genetic and epigenetic lesions can produce aneuploidy or polyploidy by disrupting the cell cycle machinery. Erosion of telomeres leads to bridge-breakage-fusion cycles and so aneuploidy may be a consequence of extensive proliferation in neoplasms.[65,66] If we assume that deletions are more likely to be deleterious than amplifications, then we would predict that most aneuploids and polyploids should be supradiploid. In any case, because the aneuploid clone is hypothesized to be neutral, it should only expand (and contract) by genetic drift, that is, very slowly and then only by chance. If these lesions are common, it is much more likely that a new aneuploid clone would emerge before a previous aneuploid clone could take over the neoplasm. On average, it requires N cell generations for a neutral clone to expand to fill a neoplasm of N cells. If a cell generation takes one day and we assume 1 cm^3 neoplasm (~10^9 cells) has a frequency of 10^{-4} stem cells,[67-72] then an evolutionarily neutral aneuploid stem cell should take approximately 300 years to fill that neoplasm.

The neutral hypothesis of the evolution of aneuploidy predicts that neoplasms will accumulate many aneuploid clones that coexist but mostly derive from different diploid progenitors. Thus, the aneuploid clones should not be closely genetically related. In contrast, the hypothesis that aneuploidy provides a selective advantage predicts that the aneuploid clone should expand in the neoplasm and, if there are multiple clones they will likely be closely related as the aneuploid lineage spins off genetic variants. These hypotheses could be distinguished by taking multiple biopsies from a neoplasm and characterizing their genetics to determine the size and relationships between clones. In BE, we typically see the later pattern, with either a single or closely related aneuploid clones in the neoplasm. This suggests that aneuploidy provides or leads to a selective advantage in BE. However, for unknown reasons, it is rare for an aneuploid clone to take over the entire neoplasm.[17]

Whether aneuploidy and more generally chromosomal instability, provides a competitive advantage for a neoplastic clone may have clinical importance. If aneuploidy provides a competitive advantage, then any surviving aneuploid cells after therapy are likely to replenish and dominate the neoplasm at relapse. If aneuploidy is evolutionarily neutral, a diploid surviving clone is just as likely to grow back to fill the void caused by the intervention. We would predict that aneuploid neoplasms would contain more genetic variants than diploid neoplasms and be more likely to harbor a therapeutically resistant clone. However, neoplasms dominated by genetic drift are more likely to accumulate genetic diversity than neoplasms in which one clone has a competitive advantage and can drive other clones extinct. The current amount of genetic diversity and thus the likelihood of resistance, depends on the interplay between the frequency with which new clones are generated and the homogenizing effects of clonal expansion.[53,73]

DNA Damage Sensing by Linkage

There is a much literature on the mechanisms of DNA damage sensing in the cell cycle, much of it centered on the role of p53.[74-76] This work has been more competently reviewed by others.[77,78] Here we focus on a hypothesis for an alternative and perhaps more primitive, mechanism for sensing DNA damage: linkage between tumor suppressor genes and essential genes.

Some regions of the genome may be protected by the presence of genes that are necessary for the survival of the cell. If enough deletions occur in that region to knock out all alleles of such genes, the cell dies and those genetic lesions are not propagated in the tissue. There is another set of genes, including tumor suppressor genes, which if lost decreases the fitness of the organism but not necessarily the cell. That is, if a clone loses enough alleles of tumor suppressor genes, it might expand but eventually kill its host. Thus, selection at the organism level may have increased the fitness of organisms by shuffling the gene order in a genome to place genes essential for the survival of a cell near to genes essential for the survival of the organism. In this way, if a deletion knocks out the tumor suppressor gene, it is also likely to knock out the nearby essential gene and the cell will die before it can generate a cancer. Thus genes essential for the survival of the cell may act like a crude form of DNA damage sensing, specific to a particular locus in the genome, that triggers apoptosis.

We predict that there has been selection for linking tumor suppressor genes with genes essential for cell survival. Such a linkage would bias against the discovery of the tumor suppressor gene. Linkage with an essential gene would tend to prevent those tumor suppressor genes from being knocked out in neoplasms with chromosomal instability and those tumor suppressor genes would be more likely to be identified in cases where the tumor suppressor gene was silenced by methylation or sequence mutations, leaving the nearby essential gene intact. The observation of at least 245 rearrangements between the human and mouse genomes may be a signature of selection for this kind of linkage.[79] This hypothesis provides one of the first explanations for a selective pressure that may be driving genome rearrangements in species.

Ancient and Recent Cancer Genes

Evolution is often described as a process of accretion. New features are added to old structures. Genes are duplicated and then diverge, adding interactions to an existing gene network. Thus, ancient genes are likely to be in the middle of such a network with many other genes depending on them. Recently evolved genes are likely to be at the periphery with fewer genes depending on them.[80] This leads to the prediction that copy number changes that affect recently evolved genes should be less likely to be deleterious than copy number changes that affect ancient genes. Careful analysis of CGH data from neoplasms might be used to test this prediction. Alternatively, an in vitro system for testing fitness effects[81] might be used to test the prediction in conjunction with the targeted deletion or amplification of either ancient or recently evolved genes.

Conclusion

Aneuploidy is a feature of almost all cancers and many premalignant conditions, including Barrett's esophagus. There are a few exceptions to this rule[27,82] including microsatellite instable colorectal cancer which can generate many genetic lesions without requiring copy number abnormalities.[83] Despite its prevalence, the significance of aneuploidy in cancer is poorly understood. By considering aneuploidy in an evolutionary context and examining its selective effects, we may better be able to understand how this phenomenon contributes to cancer progression. Evolutionary theory predicts that most mutations are deleterious, yet the prevalence of aneuploidy indicates that cancers are able to tolerate an enormous load of mutations and still utilize the body's resources and proliferate faster than normal cells. Evidence in Barrett's esophagus correlates the incidence of tetraploidy and aneuploidy with increased risk of progression. By designing experiments to examine the effects of aneuploidy in cancer and normal cells, we can help determine how aneuploidy develops and spreads in the hopes of developing predictive tools to allow us to better treat patients and prevent cancer.

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Chapter 2

Molecular Mechanisms and Function of the Spindle Checkpoint, a Guardian of the Chromosome Stability

Daisuke Ito and Tomohiro Matsumoto*

Abstract

For equal segregation, chromosomes, which are distributed randomly in the nucleus of interphase, must be aligned at the spindle equator in mitosis before the onset of sister chromatid separation. The spindle checkpoint is a surveillance mechanism that delays the onset of sister chromatid separation while each chromosome is on the way to the spindle equator. Failure in the function of the checkpoint results in aneuploidy/polyploidy, which would be a cause of cancer. Here, we review chromosome dynamics in mitosis, molecular mechanisms of the spindle checkpoint and finally tumorigenesis triggered by missegregation of chromosomes.

Introduction

Mitosis is the final stage of the cell cycle in which chromosomes duplicated in the preceding S phase are segregated equally to two daughter cells. Aneuploidy is a state of the cell with an abnormal chromosome number and is thought to be a cause of congenital hereditary disorders such as Down's syndrome and a trigger for tumorigenesis. It is generally believed that aneuploidy is caused by a failure in chromosome segregation during cell division including both mitosis and meiosis. In the mitosis in vertebrate cells, chromosomes are highly condensed in prophase; following the nuclear membrane breakdown, the condensed chromosomes are captured by spindle microtubules in prometaphase. After the chromosomes are attached to the spindle, they congress on the metaphase plate and are segregated to each daughter cell. In this process, premature separation of a chromosome which is not correctly attached to the spindle microtubules from both poles (called bipolar attachment) leads to a catastrophic consequence: each daughter cell possesses extra or fewer numbers of chromosomes. Usually a surveillance mechanism prevents missegregation of chromosomes during cell division. The spindle checkpoint (also referred to as spindle-assembly checkpoint) is a mechanism that ensures the accurate segregation of chromosomes in mitosis by delaying the onset of anaphase until all the kinetochores of chromosomes are fully attached to the spindle. In this chapter, we provide an overview of chromosome dynamics in mitosis and the molecular mechanism of the spindle checkpoint from the aspect of both its activation and silencing. We also discuss how aneuploidy/polyploidy can trigger tumorigenesis.

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Bipolar Attachment and Chromosome Congression

Chromosomes, which are distributed randomly in the nucleus at the onset of mitosis, must be placed on the metaphase plate before anaphase. To be more precise, all sister chromatids must be bi-oriented (i.e., they attach to the spindle via two kinetochores, each of which interacts with the spindle radiated from one of the two poles) and are positioned on the spindle equator (Fig. 1), a mid point between the two poles. Interaction between the spindle and chromosomes plays a key role in determining the position of each chromosome in mitosis.

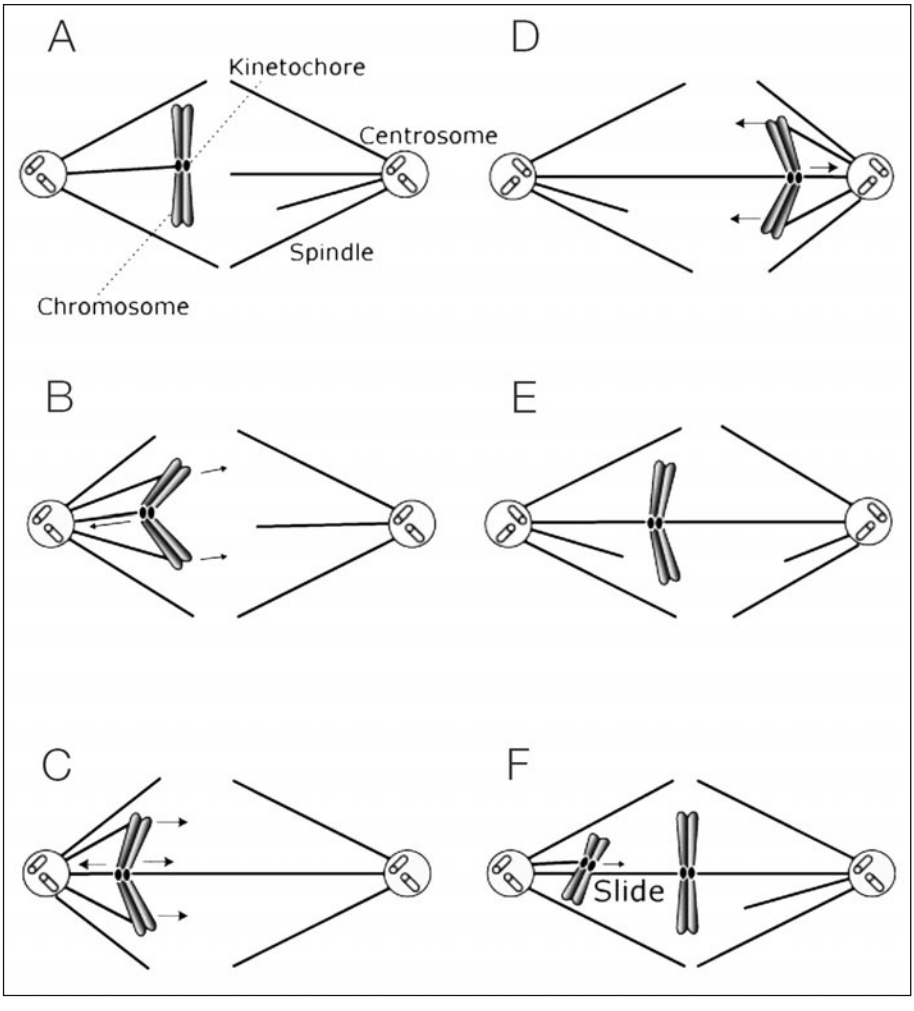

Figure 1. Chromosome dynamics in mitosis. At an early stage of mitosis, sister chromatids interact with the spindle from one pole via one kinetochore (A). These sister chromatids are pulled toward the pole and the chromosome arms are pushed away by interaction between kinesin-related protein and microtubules (B). Upon attachment to the spindle from the other pole, the mono-oriented sister chromatids become bi-oriented (C) and start congression and then they are pulled by the other pole (D). The sister kinetochores switch on and off the poleward and polar ejection force (E). Eventually the sister-chromatids are positioned at the spindle equator (F). In addition, mono-oriented sister chromatids laterally attach to the matured K-fiber between another kinetochore and the pole and slide to the metaphase plate (F).

At an early stage of mitosis, each sister chromatid interacts with the spindle radiated from one pole via one kinetochore (Fig. 1A). This mono-oriented sister chromatid is pulled toward the pole by the attached spindle. At the same time, its arm is pushed away, likely by interaction between kinesin-related proteins, Kid[1,2] and microtubules (Fig. 1B). The two opposing forces, the poleward force and the polar ejection force, act on the leading kinetochore. As it moves closer to the attached pole, the polar ejection force increases. It has been proposed that the leading kinetochore switches off the poleward force when it senses the increasing polar ejection force and thereby allows pole-away movement.[3] When the sister chromatid moves away from the pole and the polar ejection force decreases, the leading kinetochore switches on the poleward force again and moves toward the pole. By repeating this switching process, the leading kinetochore allows oscillation, the movement of the sister chromatid going back and forth around the pole.

Upon the attachment to the spindle radiated from the other pole during the period of oscillation, mono-oriented sister chromatids become bi-oriented (Fig. 1C) and start congression, the movement toward the spindle equator. The kinetochore closer to its attached pole encounters the poleward force through the attached spindle and polar ejection force through both the arm and the sister kinetochore. As the polar ejection force increases, the kinetochore switches off the poleward force. Its sister kinetochore then becomes the leading kinetochore and moves the sister chromatid toward the other pole (Fig. 1D). When the sister chromatid passes the spindle equator, the new leading kinetochore switches off the poleward force due to an increase in the ejection force. The sister kinetochore then switches on the poleward force and pulls the sister chromatid back to its attached pole (Fig. 1E). Repeating these processes results in congression and the sister chromatid is eventually placed at the spindle equator (Fig. 1F).[3,4] Although bi-orientation followed by congression is a major process to place sister chromatids at the metaphase plate, a recent study demonstrated that mono-oriented sister chromatids can migrate to the metaphase plate.[5] A mono-oriented sister chromatid oscillating near the pole may need a long time to find the spindle radiated from the other pole. The kinetochore of such a sister chromatid laterally attaches to the K-fiber that is formed between the pole and another kinetochore (Fig. 1F) and slides to the metaphase plate with the aid of a kinetochore motor, CENP-E. This cooperative process rescues mono-oriented sister chromatids and greatly contributes to placing all sister chromatids at the metaphase plate within a limited time.

Nonetheless, congression is a major event bringing each sister choromatid to the metaphase plate. It is initiated and progresses independently at each sister chromatid. As a result, some sister chromatids arrive at the spindle equator while others are yet unattached or monooriented. Sister chromatids which arrived at the spindle equator earlier do not separate until all sister chromatids arrive at the metaphase plate. A surveillance mechanism termed the spindle checkpoint is responsible for preventing premature sister chromatid separation. Kinetochores not attached to the spindle or attached abnormally activate the spindle checkpoint.

Molecular Basis of the Spindle Checkpoint

The mechanism by which the spindle checkpoint inhibits the premature separation of sister chromatids has been intensively studied until now. As shown in Figure 2, the outline of the spindle checkpoint mechanism is as follows. In the presence of kinetochores which are not attached to the spindle microtubules emanating from the opposite poles, the spindle checkpoint is activated to prevent the premature onset of anaphase. The functional components localize on the unattached kinetochore (reviewed in ref. 6). In particular, one of the most important components, Mad2, forms a complex with Cdc20/Slp1,[7,8] an activator of the APC/C (Anaphase Promoting Complex or Cyclosome) which is an E3 ubiquitin ligase targeting Securin for destruction. As long as the checkpoint is activated, Mad2 stays with Cdc20 and prevents activation of Cdc20-APC/C. Consequently, Securin remains stable and continues to bind to Separase. Securin is a stoichiometric inhibitor of Separase, a protease to cleave the cohesin complex which holds the sister chromatids together.[9] This way, the spindle checkpoint maintains sister chromatids held together until all the kinetochores fulfill the bi-polar attachment. When all the kinetochores fulfill the bi-polar

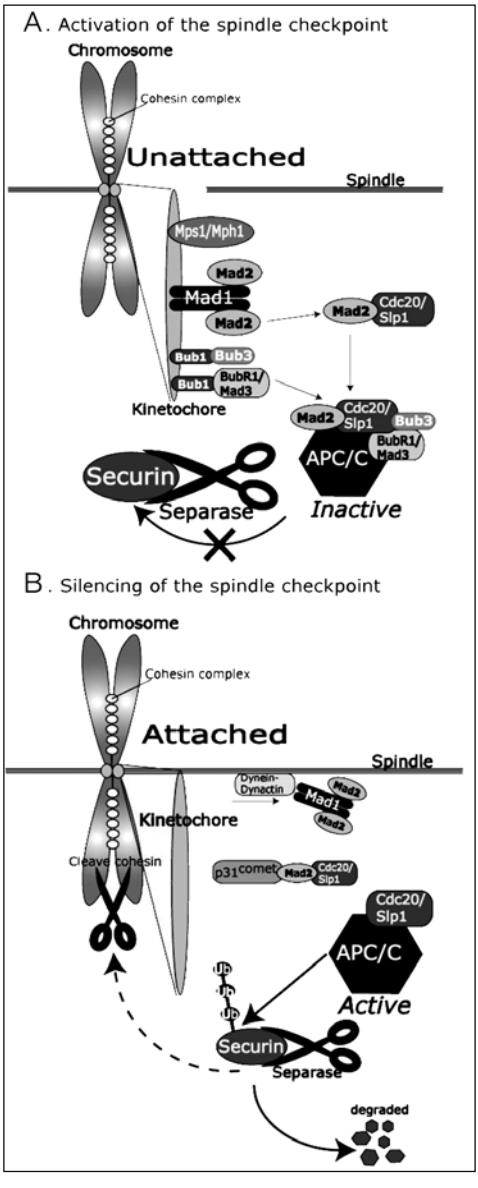

Figure 2. Molecular mechanism of the spindle checkpoint. A) Activation of the spindle checkpoint: In the presence of an unattached kinetochore, the spindle checkpoint is activated to prevent premature onset of anaphase. When the spindle checkpoint is activated, the functional components including Mad1, Mad2, BubR1/Mad3, Bub1, Bub3 and Mps1/Mph1 localize on the unattached kinetochore. In particular, Mad2 forms a complex with Cdc20. Presumably, Mad2, BubR1, Bub3 and Cdc20 form a mitotic checkpoint complex (MCC) to inhibit the activity of APC/C to ubiquitinate Securin. Consequently, Securin continues to inhibit Separase which cleaves the cohesin complex. B) Silencing of the spindle checkpoint: When the last kinetochore is attached to the spindle, the functional components disappear from the kinetochore with the help of the factors such as $p31^{comet}$ and Dynein-Dynactin. Then the APC/C achieves its activity and ubiquitinates Securin.

attachment with the spindle microtubules, the functional components disappear from the kinetochore and Mad2 no longer forms a complex with Cdc20/Slp1. Then APC/C achieves its activity and ubiquitinates its target Securin. Subsequently, 26S proteasome selectively degrades poly-ubiquitinated Securin so that Separase can acquire its activity to cleave the cohesin complex between sisiter-chromatids.[9]

The mechanism for spindle checkpoint activation has been largely elucidated in the past ten years, mainly in experimental systems such as yeast, *Xenopus* egg extracts and mammalian cultured cells. The factors involved in the spindle checkpoint have been identified by genetic screens in the budding yeast *Saccharomyces cerevisiae*.[10,11] The proteins identified include Mad (Mitotic-arrest deficient) 1, 2, 3 and Bub (Budding uninhibited by benzimidazole) 1, 3, which are widely conserved among eukaryotes (Mad3 is BubR1 in higher eukaryotes). In addition to these proteins, Mps1 kinase has been identified as a factor involved in the spindle checkpoint function, which was first discovered in budding yeast as a factor required for the duplication of spindle pole body (SPB), an equivalent organelle to the centrosome in higher organisms.[12,13] Some other proteins involved in the function of spindle checkpoint have been characterized only in higher eukaryotes. These include Rod (Rough deal)-Zw10 (zeste white-10)-Zwilch (RZZ) complex,[14] p31^{comet} (previously known as CMT2)[15] and a minus-end directed microtubule motor protein, CENP-E.[16]

Activation of the Spindle Checkpoint Signaling

When the chromosome is not properly attached to the spindle, the spindle checkpoint is activated to inhibit the progression from metaphase to anaphase. In the spermatocyte of mantids, the cell is arrested in metaphase forever in the presence of an improperly attached free X chromosome. However, when the misattached kinetochore was placed under tension by a micromanipulation needle, the cell entered anaphase.[17] In mammalian PtK1 cells, laser ablation of the last unattached kinetochore relieved the metaphase arrest and the cell entered anaphase.[18] These experiments showed that a single kinetochore not under tension generates a sufficient signal to inhibit the onset of anaphase.

The spindle checkpoint is thought to detect kinetochore-occupancy of the attached spindle and/or tension on the kinetochore/spindle. Because attachment is stabilized by tension,[19] it is difficult to experimentally determine whether a lack of attachment or tension can activate the spindle checkpoint. The components of the spindle checkpoint are known to specifically localize on the kinetochores in response to the spindle checkpoint activation. Mad1 and Mad2 localizes to the unattached kinetochore, not to the attached kinetochore lacking tension.[20] On the other hand, Bub1 and BubR1/Mad3 are recruited to the attached kinetochore lacking tension.[21] However, the mechanism how the components are specifically recruited to the kinetochore remains to be elucidated.

Some of the kinetochore proteins are required for the recruitment of spindle checkpoint components. Localization of Mad1 and Mad2 to the unattached kinetochore depends on Hec1 and Nuf2, components of the outer kinetochore.[22,23] However, the direct physical interaction between the spindle checkpoint proteins and the kinetochore components remains to be clarified.

We have seen that the spindle checkpoint components are recruited to the kinetochore in response to a defect in kinetochore-spindle microtubule association. Now we focus on the downstream events. The downstream target of the spindle checkpoint is the anaphase-promoting complex (APC/C) (reviewed in ref. 9). Mad2 forms a complex with Cdc20 and this association is essential for the function of the spindle checkpoint: inhibit activity of APC/C.[7,8] In mammalian cells, spindle checkpoint components Mad2, BubR1, Bub3 and Cdc20 form a large complex, designated mitotic checkpoint complex (MCC). MCC is a more potent inhibitor of APC/C than only Mad2.[24]

Mad2 Template Model

As mentioned above, Mad2 is one of the most important components of the spindle checkpoint since it binds to Cdc20 to inhibit the activity of APC/C. Protein structural analyses have revealed that Mad2 possesses two conformations, open-form (O-Mad2, also known as N1-Mad2) and closed-form (C-Mad2, or N2-Mad2).[25-27] In this chapter, we use the terms C- or O-Mad2 in attempt for better understanding. The "Mad2 template model" hypothesis is proposed to explain the significance of the two structural states of Mad2.[28] Mad2 holds closed conformation when it is bound to Mad1 or Cdc20 and open conformation when it is free in cytoplasm.[25-29] Cytoplasmic free O-Mad2 changes its conformation to C-Mad2 upon binding to Mad1-bound C-Mad2 at the unattached kinetochore so that it is capable of forming a C-Mad2-Cdc20 complex (Fig. 3). In this model, it is proposed that Mad1-bound C-Mad2 acts as a "template" for the conformation conversion. In this respect, it is hypothesized that the C-Mad2-Cdc20 complex also catalyzes the conformation changes of cytoplasmic O-Mad2 and that consequently the activation signal of the spindle checkpoint is amplified.[28] This hypothesis could reasonably explain why a single unattached kinetochore is sufficient to activate the spindle

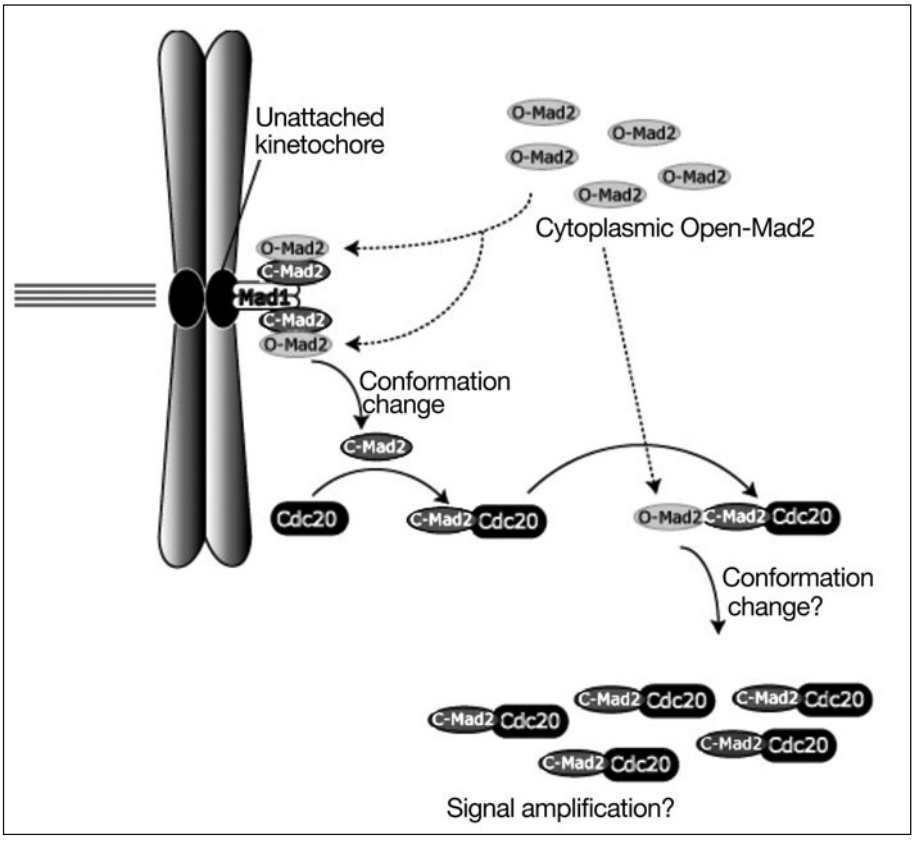

Figure 3. Mad2 template model. Mad2 holds closed conformation when it is bound to Mad1 or Cdc20 and open conformation when it is free in cytoplasm. Cytoplasmic-free O-Mad2 changes its conformation to C-Mad2 upon binding to Mad1-bound C-Mad2 at the unattached kinetochore and forms a C-Mad2-Cdc20 complex. In this model, Mad1-bound C-Mad2 acts as a "template" for the conformation conversion. It is hypothesized that the C-Mad2-Cdc20 complex also catalyzes the conformation changes of cytoplasmic O-Mad2.

checkpoint. Further investigation is required to clarify the mechanism how the Mad1-bound C-Mad2 catalyzes the conformation changes.

Phosphorylation and Spindle Checkpoint Function

Some protein kinases such as Mps1, Bub1, BubR1 and Aurora are involved in the process of spindle checkpoint activation. Thus, it is likely that phosphorylation of certain proteins is a key event for signaling cascade of the spindle checkpoint. Aurora kinases are implicated in many events in the cell cycle: centrosome separation and maturation, spindle assembly and stability, chromosome condensation, congression and segregation and cytokinesis.[30] Its role in spindle checkpoint activation has also been reported. It is proposed that budding yeast Aurora kinase Ipl1 activates the spindle checkpoint in response to tension-defective kinetochore by correcting improper attachment.[31]

Mps1 and Bub1 kinases are known to phosphorylate Mad1 in vitro; however, its significance remains unclear.[32,33] In budding yeast, overexpression of Mps1 causes spindle checkpoint activation without disturbing the formation of the mitotic spindle and leads to the hyper-phosphorylation of Mad1.[32] In the fission yeast *Schizosaccharomyces pombe*, the Mph1 (Mps1p-like pombe homolog) kinase has been identified as a spindle checkpoint component and its overexpression causes a mitotic arrest in a Mad2-dependent manner.[34] In our recent study, we attempted to elucidate the mechanism by which Mph1 kinase activates the spindle checkpoint in fission yeast. *mph1* overexpression did not cause a growth defect attributable to a mitotic arrest in the strains lacking spindle checkpoint components. In addition, when *mph1* was overexpressed, strong Mad2-GFP foci were observed on condensed chromosomes (Fig. 4), which suggested that Mad2 presumably accumulated on the kinetochores upon the activation of spindle checkpoint (unpublished data). These findings indicate that Mph1 acts upstream of the examined spindle checkpoint components and facilitate spindle checkpoint signaling.

It has been shown that a component of the kinetochore is phosphorylated when it is not under tension. The phosphorylation of an unidentified protein is recognized by the 3F3/2 antiphosphoepitope antibody and the phosphorylation is required for the recruitment of Mad2 to the kinetochore.[35-37]

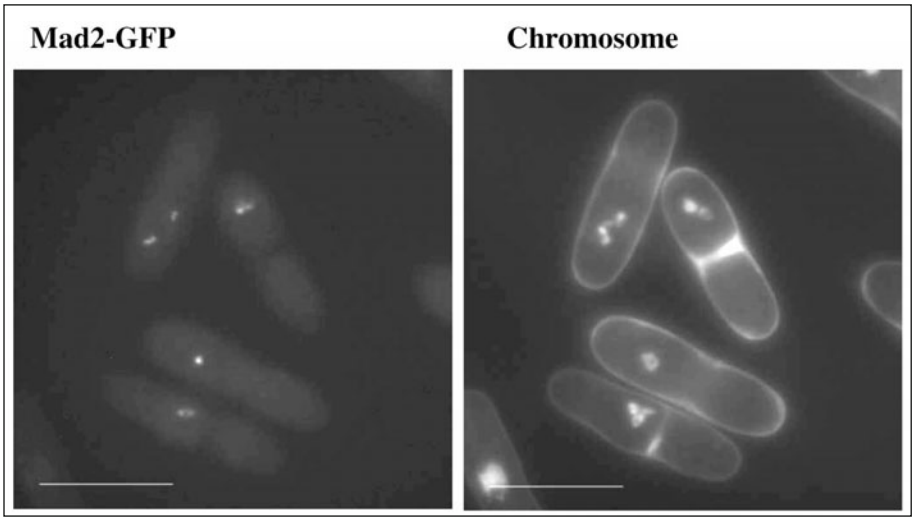

Figure 4. Mad2 on the kinetochore. Mad2-GFP localized on kinetochores when *mph1* was overexpressed. Left panel, Mad2-GFP. Right panel, condensed chromosomes visualized with Hoechst 33342. Scale bars indicate 5 μm.

In order to understand the significance of protein phosphorylation in spindle checkpoint activation, further studies are obviously needed to clarify the substrates of the kinases.

Silencing the Spindle Checkpoint

After all kinetochores are properly attached to the spindle, the spindle checkpoint is turned off within minutes and the chromosomes are synchronously segregated toward each spindle pole.[38] Compared to the activation of the spindle checkpoint, the mechanisms for silencing the checkpoint remain to be ambiguous. To date, through the yeast two-hybrid assay using Mad2 as a bait, Mad2-binding protein p31^{comet} (formerly CMT2) has been identified. In HeLa cells, overexpression of p31^{comet} abrogated the function of the spindle checkpoint and overrode the mitotic arrest.[15] In addition, the association of p31^{comet} with Mad2 coincided with the dissociation of Mad2-Cdc20, which indicated that p31^{comet} plays an important role in silencing the spindle checkpoint.

Recently, two groups reported the role of ubiquitination in spindle checkpoint silencing in mammalian cells. It was shown that the addition of UbcH10, an APC-specific ubiquitin-conjugating enzyme (E2), overrides the mitotic arrest induced by nocodazole.[39] In this process, multi-ubiquitination of Cdc20 by APC/C leads to the dissociation of Mad2 and BubR1 from Cdc20 and this promotes the progression to anaphase. To prevent premature activation of APC/C, deubiquitinating enzyme USP44 deubiquitinates Cdc20 and thereby antagonizes the ubiquitination by APC/C.[40]

In higher eukaryotes, cytoplasmic dynein plays a role in silencing the spindle checkpoint by removing Mad2 and RZZ complex from the attached kinetochores.[41,42] Thus, dynein is thought to be involved in stripping and transporting outer domain proteins away from the kinetochore upon the microtubule attachment. Very recently, through the RNAi screen in *Drosophila*, a novel protein named Spindly has been identified as a factor essential for silencing the spindle checkpoint.[43] The human homologue of the protein was identified and had similar functions. Regarding the silencing mechanism of the spindle checkpoint, the questions such as how kinetochores detect the completion of proper attachments and convey a signal to silence the checkpoint remain to be elucidated.

Additional Surveillance System

The spindle checkpoint is backed up by other surveillance mechanisms in higher eukaryotes. These mechanisms prevent proliferation of cells with extra or missing chromosomes.

A recent study[44] addressed the question of what happens to aneuploids produced by missegregation of chromosomes in mitosis. The authors labeled the nucleus by GFP-tagged histone H2B and monitored its behavior by time-lapse microscopy. They subsequently fixed the cells to analyze chromosome segregation by FISH. The analysis indicated that cells in which chromosomes did not segregate equally became binucleated by furrow regression at a significantly high frequency (Fig. 5A). In contrast, they detected no missegregation events in cells that completed cytokinesis to form two mononucleated cells (Fig. 5B). The results suggest that a surveillance mechanism is responsible for counting the chromosome number at the end of anaphase/telophase and suppresses cytokinesis if two daughter cells contain different chromosome context. Although furrow regression results in production of a tetraploid cell, the chromosome context can be maintained normally. The authors also followed the fate of the tetraploid cells produced by the furrow regression. If diploid human keratinocytes immortalized with telomerase (N/TERT-1) became tetraploids, cell cycle progression was delayed and 50% of them remained in interphase. In contrast, the cell cycle progressed with no delay in the resulting tetraploid HeLa cells. Most of the HeLa tetraploids formed multipolar spindles, which could result in chromosome breakage as well as aneuploidy (Fig. 5A). HeLa cells, which were established from a cervical cancer tissue, may be much more tolerant of tetraploidy or have lost a surveillance mechanism to suppress the growth of tetraploids.

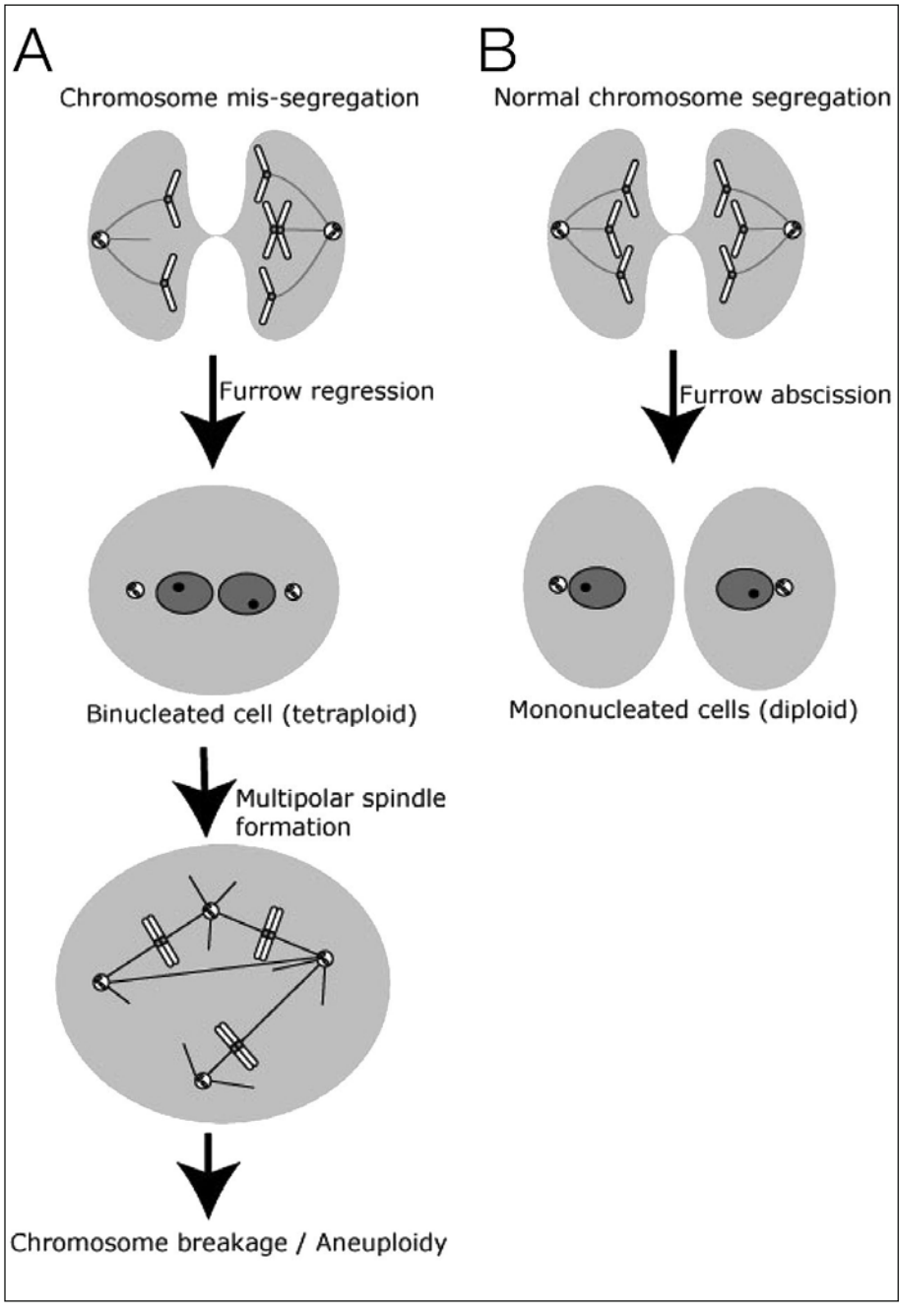

Figure 5. Furrow regression/tetraploidy induced by nondisjunction. A) The cell in which chromosomes are not equally segregated becomes binucleated by furrow regression. The binucleated tetraploid cell subsequently forms a multipolar spindle leading to chromosome breakage and aneuploidy. B) In contrast, the cell with normal chromosome segregation produces two diploid mononucleated cells.

A Trigger of Tumorigenesis

A German cell biologist Theodor Boveri proposed nearly hundred years ago that abnormalities of number and/or structure of the centrosome cause chromosome missegregation and that centrosome abnormalities are expected to affect cell shape, polarity and motility. Deregulation of centrosome number and function thereby may foster both chromosome instability and loss of tissue architecture, two of the most common characters in solid tumors.[45] According to his hypothesis, tetraploids/polyploids with multiple centrosomes would be an intermediate state, which eventually generate aneuploid cancer cells.

Boveri's proposal has been supported by a variety of evidence.[46,47] Importantly, it is supported by clinical studies of patients with a premalignant condition such as Barrett's esophagus (BE). Neoplastic progression in BE is characterized by the development of increased tetraploid population, in which p53 is concomitantly lost. This population, within a year or so, serves as an epicenter that generates a gross aneuploid cell population.[48] In a recent study with the mouse model system, it has also been demonstrated that p53-null tetraploids induced by treatment with dihydrocytochalasin B (an inhibitor of cytokinesis) promote tumorigenesis when transplanted into nude mice.[49]

Tetraploids can be produced by a defect in the spindle checkpoint. If the checkpoint misses a chromosome not attached to the spindle and allows premature sister chromatid separation, a daughter cell may not receive the accurate number of chromosomes. As mentioned earlier, such an event would activate a surveillance mechanism backing up the spindle checkpoint and result in regression of the cleavage furrow and tetraploidy.

A defect in silencing the spindle checkpoint also induces tetraploidy. Overexpression of Mad2 causes a delay in transition from metaphase to anaphase. High dosage of Mad2 would stabilize the Mad2-Cdc20 complex and prevent activation of Cdc20 for the onset of anaphase. In higher eukaryotes, overexpression of Mad2 induces tetraploidy, probably because cells eventually exit from mitosis without separating sister chromatids. In a recent mouse study, it has been shown that chromosome instability in tetraploidy/aneuploidy and tumorigenesis are induced in the transgenic mice overexpressing Mad2.[50]

As shown in Figure 5, in tetraploids mitosis with a multipolar spindle causes chromosome nondisjunction/breakage and thereby chromosome instability. It is, however, likely that chromosome instability in tetraplids/polyploids is induced for other reasons as well. In yeast model studies, it has been demonstrated that as ploidy increases, chromosome stability decreases.[51,52] Notably, a recent genome-wide screen has identified genes that cause ploidy-specific lethality. Loss of function of these genes does not affect growth of a haploid strain, but causes lethality in polyploids.[53] The ploidy-specific lethality genes are categorized into three functional groups—homologous recombination, sister chromatid cohesion maintenance and mitotic spindle function—providing clues to understand the underlying mechanisms to induce chromosome instability in polyploids. In yeast, tetraploids have a high incidence of syntelic kinetochore attachment to the mitotic spindle (a pair of sister kinetochores are attached to the same pole). It has been proposed that such an abnormal interaction is due to mismatches in the ability to scale the size of the spindle pole body (the equivalent structure of centrosome in higher eukaryotes), mitotic spindle and kinetochore. It is also plausible that the length of S phase, which is long enough for haploids to replicate their genome, may not be so for tetraploids. The geometric and/or time constraint in polyploids may be a cause of chromosome instability.

Conclusion

The spindle checkpoint is a unique signaling cascade in that the physical condition such as lack of tension and/or occupancy of the spindle at the kinetochore is a trigger for activation. To date, it has not been elucidated how the kinetochore recognizes the state of interaction with spindle microtubules and how the physical signal is converted to the biochemical signal to inhibit premature progression to anaphase. Future studies should attempt to identify and characterize a kinetochore protein complex responsible for the signal sensing/conversion.

Recently it has been shown that a defect in the spindle checkpoint causes tetraploidy/aneuploidy and leads to tumorigenesis in the mouse model studies. Thus, the spindle checkpoint can be

referred to as a guardian of chromosome stability and a barrier of tumor development/progression. Although spindle poisons such as taxol and nocodazole may be considered as effective anticancer drugs, the cellular effect of these drugs should be reexamined. In the presence of a spindle poison, cells lacking the spindle checkpoint would escape mitosis without separating sister chromatids and become polyploids, some of which could grow more aggressively.

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Chapter 3

Understanding Cytokinesis Failure

Guillaume Normand and Randall W. King*

Abstract

Cytokinesis is the final step in cell division. The process begins during chromosome segregation, when the ingressing cleavage furrow begins to partition the cytoplasm between the nascent daughter cells. The process is not completed until much later, however, when the final cytoplasmic bridge connecting the two daughter cells is severed. Cytokinesis is a highly ordered process, requiring an intricate interplay between cytoskeletal, chromosomal and cell cycle regulatory pathways. A surprisingly broad range of additional cellular processes are also important for cytokinesis, including protein and membrane trafficking, lipid metabolism, protein synthesis and signaling pathways. As a highly regulated, complex process, it is not surprising that cytokinesis can sometimes fail. Cytokinesis failure leads to both centrosome amplification and production of tetraploid cells, which may set the stage for the development of tumor cells. However, tetraploid cells are abundant components of some normal tissues including liver and heart, indicating that cytokinesis is physiologically regulated. In this chapter, we summarize our current understanding of the mechanisms of cytokinesis, emphasizing steps in the pathway that may be regulated or prone to failure. Our discussion emphasizes findings in vertebrate cells although we have attempted to highlight important contributions from other model systems.

Cytokinesis Occurs in Multiple Stages

The process of cytokinesis can be divided into four stages including specification of the cleavage plane, ingression of the cleavage furrow, formation of the midbody and abscission (Fig. 1). Each stage is dependent on the proper execution of the prior stage and thus interference with any stage may result in cytokinesis failure. The first stage of cytokinesis specifies the cleavage plane by recruiting a central regulator of cytokinesis, RhoA, to the site of cleavage. If this step is perturbed, cytokinesis will not initiate properly. In the second stage of cytokinesis, the cleavage furrow ingresses through formation of an actomyosin ring and myosin-dependent motor activity. Failure at this step may lead to a lack of furrow initiation or partial ingression of the furrow followed by regression. The third stage of cytokinesis is characterized by formation of the midbody and stabilization of the cytokinetic furrow. This stage requires proper function of proteins located in the central spindle, a microtubule-based structure that separates segregated chromosomes during anaphase, and on proteins that stabilize interactions between the actomyosin ring and the central spindle. A failure at this stage will lead to regression of the cleavage furrow. The final stage in cytokinesis, abscission, is the step in which the cytoplasmic contents are finally separated from one another. This event requires the presence of a functional midbody, but also additional proteins involved in vesicle trafficking and fusion. Failure at this stage may lead to regression of the cleavage furrow or to formation of a persistent connection between the two daughter cells. Cytokinesis is thus a series of linked processes and a problem at any step of this cascade may be sufficient to induce

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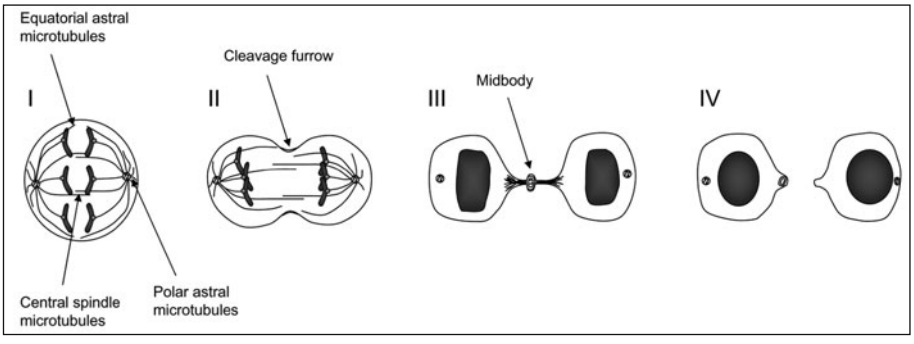

Figure 1. Multiple stages of cytokinesis. Three populations of microtubules first specify the site of cleavage by activating RhoA in a narrow zone between segregating chromosomes (I). Formation and activation of the actomyosin ring next leads to furrow ingression (II). The constricting furrow compacts the central spindle microtubules leading to midbody formation (III). Abscission of the furrow occurs by physically separating the cytoplasm of the daughter cells (IV).

failure. Some proteins participate in multiple steps in cytokinesis and thus perturbation of their abundance or activity may be especially prone to induce cytokinesis failure.

Stage I. Positioning the Division Plane and Initiating Cytokinesis

The Importance of Microtubules

Classic micromanipulation experiments determined that the mitotic spindle dictates the position of the cleavage furrow.[1,2] However, a bipolar spindle is not necessary for induction of a cleavage furrow,[3,4] suggesting that microtubules themselves play an essential role in initiating cleavage. Three separate populations of microtubules have been implicated in the regulation of cytokinesis (Fig. 1; reviewed by ref. 5). First, equatorial astral microtubules, which emanate from the spindle pole to the site of cleavage, may be stabilized in the equatorial cortical region[3] and deliver positive signals that stimulate formation and contraction of the cleavage furrow.[2] In contrast, polar astral microtubules, which emanate from the spindle pole to sites away from the site of the furrow, may help position the cleavage furrow by inhibiting cortical contractility,[6-8] perhaps by spatially biasing the pattern of myosin recruitment.[9,10] Finally, central spindle microtubules, which form an overlapping network between the spindle poles following anaphase, send positive signals that become especially important during later steps of cytokinesis. The signals sent by these distinct microtubule populations are partially redundant, ensuring that selection of the division plane is robust.[11,12]

The RhoA Pathway Plays an Essential Role in Furrow Initiation

What are the positive signals delivered by microtubules that initiate furrowing at the correct place in the cell? A central event is the localized activation of the small GTPase RhoA at the site of the future furrow (Fig. 2; reviewed by ref. 13). RhoA is essential for furrow formation in animal cells[14-17] and activated RhoA localizes to a narrow zone within the furrow.[18-22] Localized activation of RhoA within this narrow zone is thought to be important for efficient furrowing, as perturbations that broaden the zone of RhoA activation often lead to a failure of the furrow to form or to ingress.[19] A narrow zone of activation is established by tethering RhoA activators to the central spindle, delivering a strong yet spatially restricted signal for cytokinesis initiation.

An essential activator of RhoA is the guanine nucleotide exchange factor ECT2,[17,19,23-26] originally identified as a protooncogene.[27] ECT2 is sequestered in the nucleus during interphase (Fig. 2) and released following nuclear envelope breakdown in mitosis, but the protein remains inactive because it exists in an autoinhibited conformation.[24,28] In late anaphase, ECT2 localizes to the central spindle and associates with the centralspindlin complex, composed of the kinesin protein

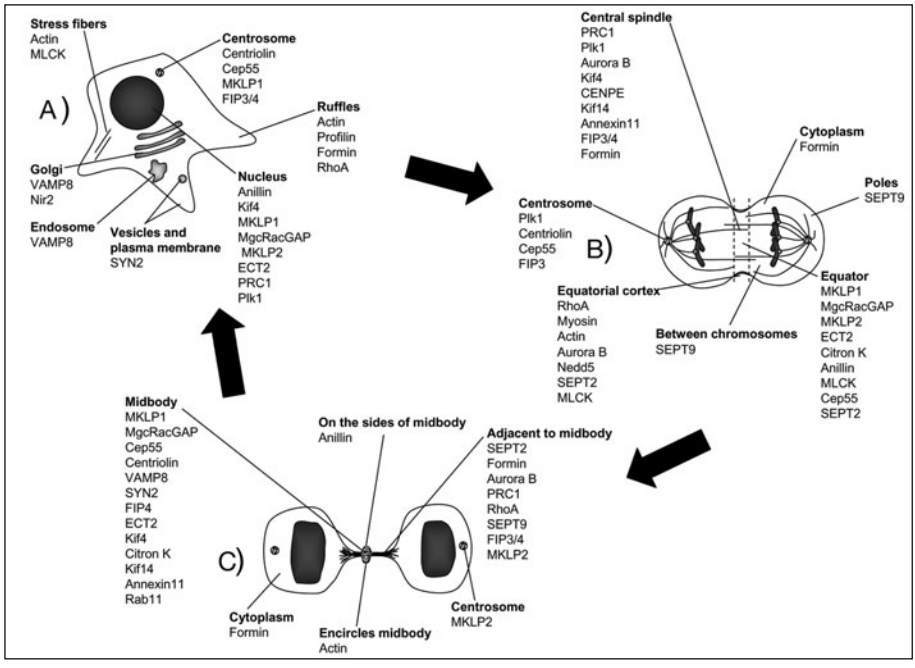

Figure 2. Localization of cytokinesis components. Interphase (A), anaphase (B) and late cytokinesis (C).

MKLP1 and the GTPase activating protein (GAP) MgcRacGAP.[17,19,29,30] MgcRacGAP binds to ECT2 and stabilizes it in an active conformation that permits it to interact with RhoA.[19] Tethering of the centralspindlin complex to the central spindle is thought to restrict activated ECT2 within a narrow zone, resulting in a narrow zone of RhoA activation.[19,23,25,29] Depletion of MKLP1[19] or disruption of the central spindle[31,32] leads to delocalization of ECT2 and MgcRacGAP from the central spindle, broadening the region of RhoA activation.[19] Cells containing a broad zone of RhoA activation fail to form a furrow.[19] Therefore, tethering of MgcRacGAP and ECT2 to the central spindle is not essential for RhoA activation, but is instead important for efficient furrowing by restricting the zone of RhoA to within a narrow zone at the equator of the cell.

These findings suggest that cytokinesis failure could result from failure to properly deliver RhoA activators to the cortex, causing insufficient activation of RhoA. Alternatively, cytokinesis could fail if RhoA is activated too broadly, in regions outside the cleavage furrow. Interestingly, ECT2 deregulation can lead to oncogenic transformation[27,28,33] although it is not clear whether perturbation of cytokinesis is an important component of this phenomenon, as ECT2 may participate in other processes such as spindle assembly[34] and regulation of the Ras/MAP kinase pathway.[35] Like many genes involved in cell division, ECT2 expression is induced by growth factors[36] in a manner that depends on the Rb/E2F pathway.[37] ECT2 is overexpressed in some tumors[38,39] where it could broaden the region of RhoA activation, perturbing proper initiation of cytokinesis. Alternatively, elevated ECT2 could perturb late stages of cytokinesis, as RhoA may need to be inactivated for cytokinesis to be completed.[25] In fact, overexpression of some fragments of ECT2 has no effect on cytokinesis initiation, but specifically blocks later stages of cytokinesis.[24,25]

Other proteins may regulate RhoA activity during cytokinesis.[40] These include additional Rho GEFs such as GEF-H1[41] and MyoGEF,[42] both of which are essential for cytokinesis in mammalian cells. Additional proteins may influence the location and timing of RhoA activation, including the armadillo protein p0071[43] and the Rho effector mDia1, which may sustain RhoA activation

in a positive-feedback loop.[44] In contrast, the protein HEF1, which is upregulated in tumor cells, may impair the RhoA activation cycle.[45]

GAP proteins are also important for controlling RhoA activation and inactivation. As stated earlier, RhoA may need to be inactivated during late cytokinesis to disassemble the cleavage furrow and thus hyperactivation of RhoA could block cytokinesis completion. Two GAP proteins that may inactivate RhoA during cytokinesis are MgcRacGAP and p190 RhoGAP.[46] Although MgcRacGAP plays a critical role in activation of RhoA by recruiting and activating ECT2, phosphorylation of MgcRacGAP by Aurora kinases may stimulate its ability to serve as a RhoGAP,[47] contributing to RhoA inactivation. As its name suggests, MgcRacGAP may also inhibit the GTPase Rac. The activity of Rac is suppressed in the spindle midzone[21] and constitutively activated Rac induces a mutlinucleation.[48] Thus in addition to activating RhoA by recruiting ECT2, MgcRacGAP may inactivate Rac in the furrow to support cytokinesis.[48-51]

Failure of Cytokinesis During Stage I

Together these studies emphasize the importance of microtubules in delivering signals that lead to localized activation of RhoA and possibly suppression of Rac, in the furrow. Recent studies suggest that cytokinesis failure may occur in cells in which spindle elongation or spindle positioning is perturbed, disrupting delivery of activation signals to the cortex. The first example is binucleation of cells in the liver, which may be regulated physiologically.[52,53] In humans, the number of polyploid cells averages 30-40% in the adult liver.[54,55] Studies in rat hepatocytes indicate that tetraploid cells arise from cytokinesis failure in which diploid, mononucleated cells undergo mitosis but do not form a contractile ring.[52] Cells do not undergo anaphase spindle elongation, perhaps because reorganization of the actin cytoskeleton is impaired.[53] Furthermore, astral microtubules fail to contact the equatorial cortex in cells that fail cytokinesis,[53] suggesting that the delivery of RhoA activators to the cortex is impaired. In rat liver, the number of binucleated cells increases following weaning, suggesting there may be important connections between liver physiology and cytokinesis regulation,[53] but how these pathways might impact microtubule organization remains unclear.

The second example is cytokinesis failure that occurs in cells that contain mutations in the APC (Adenomatous Polyposis Coli) tumor suppressor. Some APC mutations may induce cytokinesis failure by interfering with microtubule-dependent anchoring of the mitotic spindle.[56] Although APC has important roles in formation of the mitotic spindle and the spindle checkpoint,[57-60] cells expressing APC mutants become polyploid over time,[56,59,61] indicating that the protein is important for proper cytokinesis. Different APC alleles may have distinct effects on mitosis.[56] For example, in cells expressing a particular C-terminal truncation mutant of APC, microtubules make less contact with the cell cortex, spindles undergo considerable rotation during mitosis and cells do not efficiently initiate cytokinesis.[56] The physiological relevance of these findings was confirmed by the finding that the *Min* allele of APC gives rise to similar mitotic defects and that the frequency of tetraploid cells is greatly increased in *Min*[56]and APC knockout mice.[60] Although it is likely that tetraploidy can arise through multiple mechanisms in tumors carrying different APC alleles, these findings suggest that failure to properly anchor the mitotic spindle can be an important source of tetraploidy.

Stage II. Ingression of the Cleavage Furrow

In the second stage of cytokinesis, activated RhoA leads to recruitment and activation of effector proteins that organize the furrow and stimulate ingression (Fig. 3). RhoA stimulates actin polymerization through activation of formins and stimulates myosin activity by activating kinases such as Rho kinase (ROCK) and Citron kinase. Scaffolding proteins such as anillin and septins also play important roles in organizing the cleavage furrow and promoting cytokinesis. Here we discuss each of these processes and how they might be perturbed to result in cytokinesis failure.

Stimulation of Actin Filament Assembly

Formins are proteins that nucleate formation of unbranched actin filaments in response to stimulation by RhoA (for review, see ref. 62). In the absence of active RhoA, most of the formins

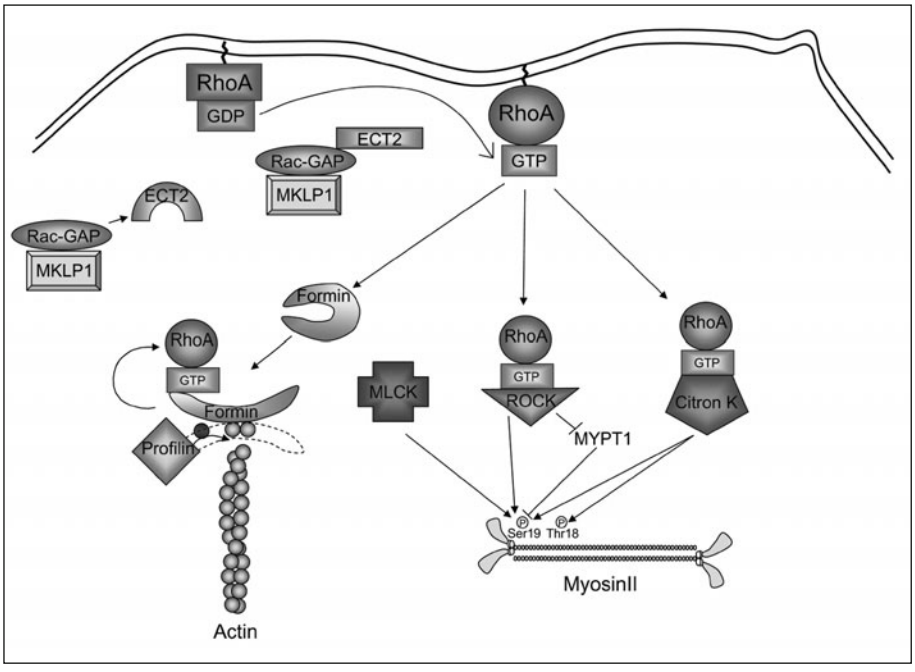

Figure 3. Role of the RhoA pathway in furrow initiation. Autoinhibition of ECT2 is suppressed by association of ECT2 with MgcRacGAP. ECT2 then activates RhoA by stimulating exchange of GDP for GTP. Active RhoA then activates formins to stimulate actin nucleation and binds to ROCK and Citron kinases, stimulating phosphorylation and activation of myosin.

that regulate cytokinesis are autoinhibited.[63] RhoA binding relieves autoinhibition to promote actin polymerization.[64,65] The mammalian formin mDia1 is activated downstream of Rho signaling,[66] and cytokinesis is blocked if mDia1 is inhibited by antibody injection.[67] However, deletion of mDia1 does not perturb cytokinesis in mouse embryonic fibroblasts,[68] suggesting redundant pathways for actin nucleation. Cytokinesis may also depend on the use of preexisting actin filaments that are nucleated outside the furrow.[69,70] Formins may be important in later stages of cytokinesis, as they have been implicated in regulation of Src activity, which has been shown to be important for completion of cytokinesis.[67,71]

Localization and Activation of Myosin

Myosin II (hereafter simply referred to as myosin) is the principle motor protein required for cytokinesis (for review, see ref. 72). Myosin is recruited to the cleavage furrow at early stages of cytokinesis in a RhoA-dependent fashion. Myosin activity and localization are regulated by phosphorylation of its regulatory light chain (myosin light chain or MLC). Because myosin motor activity is directly required for furrow ingression,[73] perturbation of myosin localization or its activity could result in cytokinesis failure.

Phosphorylation of serine 19 of MLC stimulates actin-activated ATPase activity of myosin,[74,75] whereas phosphorylation at threonine 18 promotes myosin assembly. Phosphorylation of myosin at these positions is important for proper localization of myosin to the furrow and for ingression.[76-79] In contrast, phosphorylation at serines 1, 2 and 9 of MLC inhibits myosin ATPase activity[80,81] During mitosis, MLC is phosphorylated at these positions by CDK1.[76,82,83] At anaphase, inactivation of CDK1, controlled by the degradation of mitotic cyclins by the Anaphase-Promoting Complex/Cyclosome (APC/C), is important for MLC dephosphorylation and myosin activation

during cytokinesis. Therefore, failure to degrade mitotic cyclins, or to fully inactivate CDK1, could perturb myosin activation and disrupt cytokinesis.

Three kinases contribute to myosin activation by phosphorylating positions 18 and 19 of MLC (Fig. 3). Two of these kinases, ROCK and Citron kinase, are activated by RhoA. ROCK localizes to cleavage furrows[84,85] and a small molecule inhibitor of ROCK slows cleavage.[84] Citron kinase also localizes to the cleavage furrow and is required for cytokinesis in several systems.[25,50,86-92] Citron kinase can phosphorylate MLC at both ser19 and thr18[93] and its overexpression causes unregulated contraction of the cortex, supporting its role as a positive regulator of myosin activity.[87] Mouse knockout studies suggest that Citron kinase may play an especially important role in neurogeneic and spermatogenic cytokinesis.[94-97] It is likely that ROCK and Citron kinase play partially overlapping roles, explaining why each protein is not essential for cytokinesis in all systems. There is no evidence to suggest that ROCK or Citron kinase is overexpressed or mutated in human tumors, but Citron kinase interacts with the kinesin protein KIF14,[89] which is overexpressed in several tumor types.[98-101] Whether overexpression of KIF14 perturbs Citron kinase function in cytokinesis remains unknown. Knockdown of KIF14 induces cytokinesis failure,[102] perhaps as a result of a failure to recruit Citron kinase to the cleavage furrow.

Myosin light chain kinase (MLCK) is the third and final kinase that has been implicated in direct phosphorylation of myosin light chain. This kinase is activated by calcium/calmodulin and some isoforms of MLCK and calmodulin localize to the cleavage furrow.[103-106] Inhibition of calmodulin or MLCK can disrupt cytokinesis in cultured cells,[106-108] but mice lacking MLCK develop normally, but die after birth, suggesting the kinase is not essential for cytokinesis in all tissues.[109] How myosin light chain kinase is regulated in the cleavage furrow is unclear, but hydrolysis of PIP2 may be important for IP3-induced calcium release, which could stimulate MLCK activity.[110] The mild and varied phenotypes associated with MLCK inhibition again suggest functional redundancy in MLC phosphorylation during cytokinesis.

The overall degree of MLC phosphorylation is also affected by the activity level of myosin phosphatase. This enzyme is inhibited by several mechanisms during cytokinesis to favor MLC phosphorylation. Myosin phosphatase is a heterotrimeric enzyme consisting of a targeting subunit that binds myosin (MYPT1 or MBS), a catalytic subunit (the delta isoform of PP1c) and an additional small subunit. Both ROCK and Aurora B may phosphorylate MYPT1 in the furrow to inactivate the phosphatase.[111-113] In addition, a number of other kinases including Raf-1[114] may negatively regulate myosin phosphatase (reviewed in ref. 72). Thus a number of signaling pathways could converge on myosin phosphatase to regulate cytokinesis.

Organization of Actin and Myosin in the Furrow

How actin and myosin are organized within the cleavage furrow and how contraction occurs, is not well understood (for a more detailed discussion, see refs. 115 and 116). Phosphorylated myosin localizes to the furrow in early anaphase[79] and localization of myosin requires RhoA activation[13,117] but not myosin ATPase activity.[73,118,119] Recruitment may require phosphorylation of MLC, as mutations or inactivation of kinases that perturb MLC phosphorylation also disrupt myosin localization. Other scaffolding components, such as anillin, may be important for maintaining myosin within the furrow, as discussed below.

Both actin and myosin are highly dynamic in furrows and dynamic actin is important for cytokinesis.[108,120] For example, the actin disassembly factor cofilin is necessary for cytokinesis.[121] Cofilin is negatively regulated by the kinase LIMK1 and thus upregulation of LIMK1, or loss of its negative regulator LATS1, is sufficient to enhance actin polymerization and induce cytokinesis failure.[122] Interestingly, inhibition of myosin slows disassembly of actin filaments of the furrow,[123] suggesting that myosin motor activity may help drive actin disassembly.

Scaffolding Proteins in the Furrow

Anillin

Another conserved furrow component is anillin, which may act as a scaffold protein that binds F-actin, myosin, septins and activated RhoA.[124-129] Although anillin localizes to the furrow at early stages of cytokinesis, it is not essential for ingression. Instead, it may stabilize the furrow and be important for later stages of cytokinesis including midbody formation and abscission.[92,127,130-132] However, anillin becomes essential for ingression if the central spindle is disrupted, suggesting it may make early steps of cytokinesis more robust.[128] Anillin interacts with RhoA[128] and its localization to the furrow requires activation of RhoA.[128,129,132,133] MgcRacGAP may also be directly involved in targeting anillin to the furrow,[134] providing a link between the centralspindlin complex and anillin localization.

Anillin contains domains that permit it to interact with phosphorylated myosin,[127] actin filaments and septins.[124,125,135] These features make anillin ideally suited to crosslink the actomyosin and septin cytoskeletons within the contractile ring. Anillin may enhance the robustness of early stages of cytokinesis by promoting the anchoring of myosin in the vicinity of activated RhoA, favoring myosin phosphorylation. Anillin may be essential for asymmetric ingression of the cytokinetic furrow, which occurs when the furrow ingresses from only one side of the cell, rather than circumferentially.[136] Asymmetric ingression may be important in epithelia[137] and embryos[136,138] where it may serve a mechanical function, enhancing the robustness of cytokinesis. Anillin is also important for completion of cytokinesis, as anillin remains in the cytoplasmic bridge even after myosin and actin have dissociated.[127] Interestingly, whereas overexpression of anillin seems to have little phenotype in Drosophila S2 cells,[129] overexpression of anillin in mammalian cells is very toxic,[132] suggesting anillin could have important functions independent of cytokinesis. Levels of anillin appear to be controlled by the ubiquitin-proteasome pathway, as anillin is targeted for ubiquitination and degradation during G1 by the APC/C.[132]

Like the liver, the heart also contains a large number of tetraploid cells that arise through cytokinesis failure. Although it was originally proposed that cytokinesis failure might be a consequence of failure to disassemble myofibrils within cardiomyocytes, recent work suggests this is unlikely to be the case.[139] Instead, cells that fail cytokinesis show complete disassembly of the myofibril, but show abnormal localization of anillin and failure of anillin to concentrate at the midbody.[139] However, these cells also show delays in furrow ingression, suggesting that earlier steps in cytokinesis may also be affected in these cells.

Septins

Septins represent a second class of scaffolding protein that may help to organize proteins within the cleavage furrow. Septins are GTP-binding proteins that can form filaments and localize to the cytokinetic ring.[140-144] Several human septins have been implicated in cytokinesis, including SEPT2 (Nedd5), SEPT9 (MSF) and SEPT12. SEPT12 localizes to the central spindle and midbody during anaphase and cytokinesis, respectively.[145] SEPT2 accumulates in the contractile ring and midbody[146-148] and microinjection of antibodies[146] or antisense downregulation[147]of SEPT2 interferes with cytokinesis. Inactivation of SEPT9 by antibody microinjection or siRNA also induces cytokinetic defects.[149,150]

Septins may participate in several aspects of cytokinesis, including regulation of actin and microtubule dynamics. SEPT2 associates with actin, forming filaments in association with actin bundles and focal adhesions,[146] whereas SEPT9 associates with the microtubule network.[150,151] Septins may also play a direct role in cytokinesis by interacting with anilllin.[124,125,135] Furthermore, SEPT2-containing filaments may provide a molecular platform for myosin and its kinases to ensure the full activation of myosin that is necessary for cytokinesis.[152] Finally, septins may form a barrier that restricts the diffusion of membrane proteins in the furrow,[153,154] thus helping retain activated RhoA within the narrow zone required for efficient initiation of cytokinesis. In mammalian cells, the p85 subunit of PI3 kinase may regulate SEPT2 in cytokinesis,[155] linking cellular signaling pathways with steps in cytokinesis. Septins may be deregulated in tumors, either through gene

fusions[156-158] or by overexpression.[159] The SEPT9 gene is amplified and overexpressed in mouse mammary tumors and human breast cancer cell lines[158] and high SEPT9 expression in human breast cancer cells is associated with oncogenic phenotypes and cytokinesis defects.[160]

Stage III. Formation of the Midbody

The central spindle, also referred to as the spindle midzone, plays an important role in keeping separated chromosomes apart prior to cytokinesis completion, because when microtubules are depolymerized in late anaphase, the nuclei collapse back together.[73] Microtubules in the midzone may be locally nucleated, as the minus ends of the midzone microtubules are decorated with gamma-tubulin.[161,162] As cytokinesis progresses, the constricting furrow compacts the midzone microtubule array. The furrow ingresses until a cytoplasmic bridge is formed that is 1-1.5 microns in diameter. Several kinesin-like motor proteins and chromosomal passenger proteins move along the midzone spindle towards the plus ends and accumulate in the overlapping region, forming a phase-dense structure referred to as the Flemming body, stembody, telophase disc, or midbody (reviewed in ref. 163). Disassembly of the actomyosin ring may be an important step at this stage of cytokinesis, as loss of F-actin accompanies and may trigger midbody formation.[164] Once the cytoplasmic bridge matures and abscission begins, the bridge becomes insensitive to the actin inhibitor latrunculin,[92] implying that the plasma membrane is linked to the midbody by a connection that does not involve dynamic F-actin. Scaffolding proteins such as anillin and septins may stabilize the bridge structure. In almost all systems, central spindle formation is essential for midbody formation, which in turn is necessary for abscission.[16,32,165] In this section, we discuss the components that are required for formation of the central spindle and midbody.

PRC1 is a microtubule bundling protein that is critical for midzone formation in mammalian cells.[32,166] PRC1 accumulates on the central spindle in anaphase and suppression of PRC1 expression causes failure of microtubule interdigitation.[32] In the absence of PRC1, astral microtubules can guide the equatorial accumulation of anillin, actin and chromosome passenger proteins, enabling cleavage furrow ingression, but abscission fails.[167] PRC1 has separate domains that independently target the protein to the midzone and bundle microtubules.[32] PRC1 is targeted to the midzone by the kinesin protein KIF4, which transports PRC1 to the ends of microtubules. Absence of KIF4 leads to a failure to accumulate PRC1 in the central spindle and abolishes central spindle formation.[168] PRC1 in turn recruits the centralspindlin complex and additional mitotic kinesins including CENP-E, MCAK[169] and KIF14.[89] PRC1 also serves as an important docking site for the kinase Plk1 in the central spindle.[170] PRC1 expression may be perturbed in cancer cells or in response to checkpoint signaling pathways. PRC1 upregulation in tumors[169,171] may be a consequence of p53 inactivation, as induction of p53 can inhibit PRC1 expression and interfere with cytokinesis completion.[172,173]

Although the centralspindlin complex (MKLP1 and MgcRacGAP) is important for cytokinesis initiation as described earlier, centralspindlin is also necessary for spindle midzone and midbody formation and ultimately for abscission.[31,165] Centralspindlin is recruited to the midzone by PRC1 (reviewed in ref. 174) and proper localization requires the presence of both members of the centralspindlin complex.[16] A splice variant of MKLP1, called CHO1, includes an additional domain that can interact with F-actin,[175] suggesting that CHO1 could link the actin and microtubule cytoskeletons. Injection of antibodies that target this domain induces failure in late steps of cytokinesis,[175] suggesting CHO1 may stabilize interactions between midbody microtubules and the ingressing cleavage furrow in late steps of cytokinesis. Centralspindlin is also important for recruiting additional proteins to the midbody that are required for abscission and both components of the complex appear to be regulated by phosphorylation. Each of these topics will be discussed in more detail below.

Proteomic approaches have identified a large number of proteins that concentrate at the midbody (Fig. 2)[176] and a functional role for some of these proteins in abscission has been supported by results of RNAi experiments.[92,162,177] Several of these proteins localize to the Golgi apparatus during interphase and are released from the Golgi during mitosis by phosphorylation.[178-180]

Inhibition of Golgi disassembly during mitosis perturbs cytokinesis,[181] perhaps by interfering with release of components that are essential for cytokinesis. Precisely how these proteins function in cytokinesis remains unclear, but one potential function is to recruit mitotic regulators such as Plk1 to the midbody.[179]

Additional proteins that localize to the midbody and are required for cytokinesis include LAPSER1, which may recruit the microtubule severing protein katanin to the midbody,[182] and annexin 11.[183] Annexins are Ca(2+)-binding, membrane-fusogenic proteins with diverse but poorly understood functions. Cells lacking annexin 11 fail to establish a functional midbody and instead remain connected by intercellular bridges that contain bundled microtubules but exclude normal midbody components such as MKLP1 and Aurora B.[183] These data suggest that despite its potential role in membrane fusion, annexin 11 seems to be required at an earlier step for recruitment of MKLP1 and Aurora B to the midbody.

Stage IV. Abscission

Once the midbody is formed, it subsequently organizes the final event of cytokinesis, termed abscission. By the time of abscission, the cytoplasmic bridge has narrowed to 0.2 microns in diameter. At these late stages, microtubule bundles become compacted and begin to disappear.[92,184] In this process, the cytoplasmic bridge is reorganized to permit separation of the daughter cells. A wide variety of proteins involved in vesicle and protein trafficking, membrane fusion and other processes are required for abscission, suggesting the final stage of cytokinesis is just as complex as earlier stages. Human cultured cells may remain connected by the cytoplasmic bridge for many hours before undergoing abscission.[185] In some systems, such as embryos, blastomeres often remain connected by intracellular bridges for many cell cycles. In spermatocytes, the cytoplasmic bridge is in fact stabilized[186] and cytokinesis completion does not occur, enabling communication between the cytoplasm of adjacent cells. Thus in certain circumstances abscission may be the target of physiological regulation.

Membrane Trafficking and Cytokinesis

Membrane trafficking plays a critical role in the process of cytokinesis (Fig. 4). Three pathways have been implicated in the process of cytokinesis. First, the secretory pathway, including Golgi-derived components, may contribute new membranes and proteins to the ingressing furrow and also participate in late steps of cytokinesis completion. Second, the endocytic pathway and recycling endosomes may remodel membranes in the cleavage furrow and also contribute vesicles that may participate in the final steps of cytokinesis. Finally, recent evidence suggests that components of the ESCRT machinery, best characterized for its role in multivesicular body formation, may also be essential for the final stages of cytokinesis. The relative contributions of each of these pathways in the process of cytokinesis is likely to be dependent on cell type.

The Role of the Secretory Pathway

In large embryos, such as *Xenopus* and Sea Urchins, furrow ingression is coupled to insertion of new membrane via microtubule-dependent exocytosis.[187-190] In smaller cells, such as mammalian tissue culture cells, it is less clear whether new membrane insertion is required. Brefeldin A (BFA), which disrupts ER-Golgi-dependent trafficking, blocks cytokinesis completion in some studies[191,192] but not others.[193-196] Further evidence for a role of Golgi-derived vesicles in cytokinesis completion has emerged from studies of the protein centriolin, which may help recruit secretory vesicles to the site of abscission at the midbody.[191] Centriolin was initially identified as a protein that localizes to the maternal centriole during interphase and accumulates on mature centrioles during metaphase.[197] Knockdown of centriolin in mammalian cells causes cytokinesis failure, with the two cells remaining connected by a cytoplasmic bridge.[197] Centriolin localizes to a ring-like structure within the midbody,[191] that also contains gamma-tubulin, GAP-CenA and the centralspindlin complex. Recruitment of centriolin to the midbody ring is dependent on the centralspindlin complex, explaining why centralspindlin may be essential for cytokinesis completion.[191]

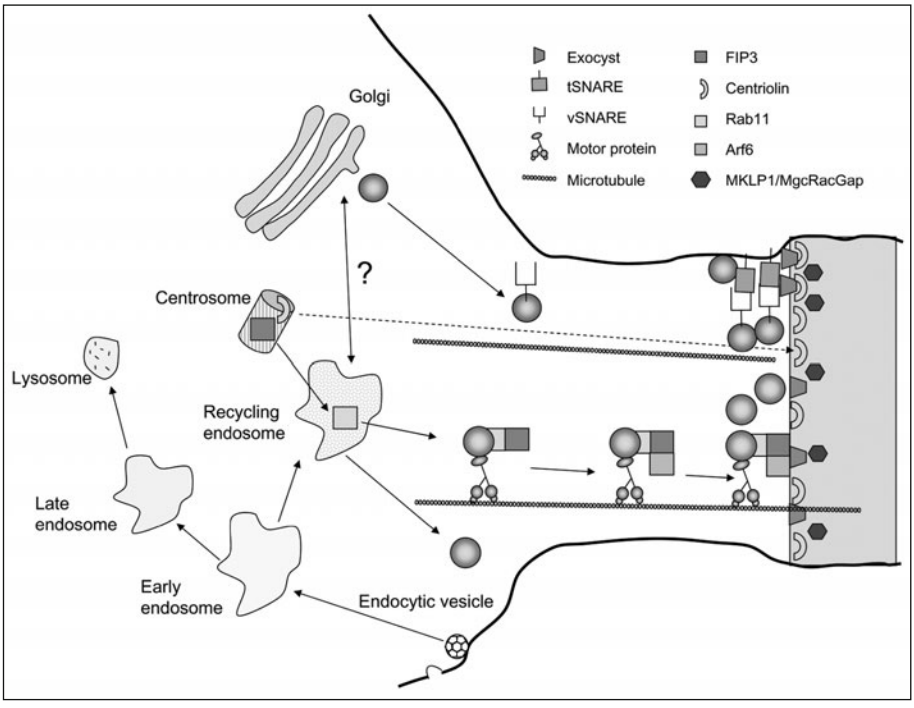

Figure 4. Membrane trafficking in cytokinesis. Secretory vesicles accumulate at the intercellular bridge in a centriolin-dependent manner by SNARE interaction and vesicle tethering by the exocyst complex. Vesicles originating from the recycling endosome and containing the complex Rab11/FIP3 move along microtubules. Interaction of FIP3 with both ARF6 and the exocyst permits vesicle targeting to the midbody. Vesicles then fuse with each other and the plasma membrane, physically separating the daughter cells.

Centriolin may facilitate cytokinesis completion by recruiting to the midbody proteins involved in vesicle tethering and fusion. Centriolin interacts with components of the exocyst,[191] a protein complex that tethers secretory vesicles to the plasma membrane (reviewed in ref. 198). Several components of the exocyst localize to the midbody ring in a centriolin-dependent manner and depletion of exocyst components by siRNA interferes with cytokinesis completion.[191] Centriolin also interacts with snapin, a snare-associated protein and centriolin is required for the recruitment of snapin and SNARE proteins to the midbody.[191]

Secretory vesicles, derived from the Golgi apparatus, accumulate at the intercellular bridge during late steps in cytokinesis.[191] At the time of abscission, the vesicles disappear, suggesting they undergo homotypic fusion with each other and also heterotypic fusion with the plasma membrane, releasing their contents.[191] Interestingly, vesicles seem to accumulate only on one side of the midbody, suggesting that delivery is asymmetric.[191] This finding is consistent with the fact that following abscission, the midbody remains attached to one of the two daughter cells where it may play additional roles in signaling or marking the age of the cell.[191] Why abscission typically occurs on only one side of the midbody remains unclear. It has been suggested that abscission may be triggered by arrival of the maternal centriole from one daughter cell,[184] but this event has not been observed consistently.[191] Asymmetric abscission may be important to enable the midbody to remain attached to a daughter cell, or it may provide an opportunity to regulate the timing of abscission. However, asymmetric abscission may be inherently more prone to failure than if abscission were to occur on both sides

of the midbody. The mechanism and significance of asymmetric abscission is an interesting topic for future investigation.

The Role of Endocytosis and the Recycling Endosome Pathway

Several lines of evidence suggest that the endocytic and the recycling endosome pathways play critical roles in cytokinesis completion. Endocytosis within the furrow may be important for remodeling the plasma membrane during ingression. In addition, endocytosis from other regions of the cell may serve as a source of vesicles destined for delivery to the cleavage furrow either directly or through the recycling endosome. For example, some endocytic vesicles internalized from the polar region are subsequently trafficked to the midbody area during later stages of cytokinesis.[199] Inhibition of proteins essential for endocytosis, including clathrin, dynamin and alpha-adaptin, perturb cytokinesis in several systems[200-204] and inhibitors of clathrin-dependent endocytosis block cytokinesis completion in mammalian cells.[195,199] In addition, there may be direct interactions between the endocytic machinery and proteins required for cytokinesis such as anillin.[205]

Small GTPases that regulate membrane trafficking have been directly implicated in cytokinesis completion. Arf GTPases initiate the budding of vesicles by recruiting coat protein complexes onto donor membranes, whereas Rab GTPases regulate the targeting and docking/fusion of vesicles with acceptor membranes.[206] Two different GTPases, Arf6 and Rab11, have been implicated in regulation of cytokinesis. Rab11 localizes preferentially to the recycling endosome (RE) and is required for proper RE organization and the recycling of vesicles to the plasma membrane. Both Arf6 and Rab11 concentrate near the cleavage furrow and are required for late steps of cytokinesis in mammalian cells.[199,207-210] Both GTPases interact with a common set of effector proteins that assist in delivery of endosomal vesicles to the cleavage furrow, termed FIP3 (Arfophilin-1) and FIP4 (Arfophilin-2).[207-209,211-214] FIP3-containing endosomes accumulate near the cleavage furrow and are required for successful completion of cytokinesis.[209] Recruitment of FIP3 to the midbody requires ARF6 and recruitment of ARF6 to the midbody requires FIP3.[215] Other studies show that Arf6 interacts with MKLP1, suggesting the centralspindlin complex is important for targeting Arf6 to the cleavage furrow.[207,216] The exocyst has also been implicated in targeting of vesicles derived from the recycling endosome. For example, Exo70, a component of the exocyst complex, colocalizes with Arf6 in Rab11-positive endosomes.[208] Exo70 interacts with FIP3 and FIP4 biochemically and depletion of Exo70 impairs FIP3 and Rab11 localization to the furrow and midbody.[208] Together these studies suggest the following model of delivery of endosomal vesicles to the midbody (Fig. 4). Rab11 first recruits FIP3 to endosomes. FIP3 in turn associates with ARF6 and together this complex localizes to the midbody via interactions with the exocyst and MKLP1.

Membrane Fusion During Abscission

Following vesicle targeting to the site of abscission, membrane fusion is necessary to complete cytokinesis. SNARE proteins are critical components required for membrane fusion (reviewed in ref. 217). Several SNARE proteins or associated components have been implicated in cytokinesis completion in different organisms.[92,218-220] In mammalian cells, two SNARE proteins, syntaxin 2 and endobrevin/VAMP-8, localize to the midbody during cytokinesis.[191,221,222] Expression of dominant negative mutants or depletion of SNAREs impairs abscission, but has no effect on ingression of the cleavage furrow, suggesting that SNARE-mediated fusion is required only in the latest steps of cytokinesis.[191,221] Septin proteins may assist in membrane fusion by restricting the diffusion of membrane-associated components such as the exocyst to the region of abscision.[153] Furthermore, septins may assist in abscission by directly recruiting the exocyst[223] and SNARE proteins.[224]

Role of the ESCRT Machinery

Recently, protein subunits of the Endosomal Sorting Complex Required for Transport (ESCRT) that are normally involved in late endosome to lysosome trafficking have also been implicated in abscission. These proteins are best known for their roles in multivesicular body formation (reviewed in ref. 225), where they are important for membrane invagination. ESCRT complexes also play important roles in the topologically equivalent process of viral budding. Because abscission

likely requires changes in membrane organization, a role for the ESCRT complex in cytokinesis is very intriguing. However, the precise mechanism of membrane invagination mediated by the ESCRT complex remains unknown and it is unclear whether the ESCRT pathway functions independently in abscission or whether it assists in secretory- or endosomal vesicle-mediated cytokinesis completion.

Components of the ESCRT machinery localize to the midbody and inhibition of some ESCRT complexes blocks late steps in cytokinesis. For example, CHMP3, a subunit of the ESCRT-III complex, localizes to the midbody and deletion of a C-terminal autoinhibitory domain of CHMP3 inhibits cytokinesis.[226] Other subunits of the ESCRT machinery implicated in abscission include tumor-susceptibility gene 101 (Tsg101), a subunit of the ESCRT-I complex and Alix, an ESCRT-associated protein.[222,227] Alix may interact with actin and microtubules,[228,229] establishing a link between the ESCRT machinery and cytoskeletal components that are present at the midbody. Alix and Tsg101 are recruited to the midbody by interaction with centrosome protein 55 (Cep55), a centrosome and midbody protein essential for abscission.[222,230,231] Interestingly, Tsg101 has been implicated in cancer and may have additional functional roles in cell cycle and transcriptional regulation (reviewed in ref. 225).

Regulation of Cytokinesis

Thus far we have outlined the core pathways and components essential for each stage of cytokinesis. In the remaining part of the chapter, we discuss how these components are regulated to ensure that cytokinesis occurs at the proper place and time. Many regulatory pathways impinge upon the cytokinesis machinery, suggesting that cytokinesis may be responsive to a variety of different cues within the cell. The complexity of cytokinesis regulation suggests that cytokinesis failure could result from alterations in the activity of these regulatory pathways.

Regulation of Cytokinesis by Protein Kinases

Cytokinesis is regulated by mitotic protein kinases, including cyclin-dependent kinases (CDKs), Polo kinase (Plk1) and the Aurora B kinase complex (Fig. 5). Mitotic CDK activity prevents cytokinesis onset until anaphase by phosphorylating cytokinesis components in a manner that inhibits their activity. For this reason, CDK1 must be inactivated for cytokinesis to proceed.[232] In fact, inhibition of CDK1 with a small molecule is sufficient to induce the initial events of cytokinesis,[233-235] suggesting that CDK1 inactivation is the trigger for cytokinesis initiation.

In contrast, Polo kinase and Aurora B kinase positively regulate the events of cytokinesis and must remain active for a period of time following CDK inactivation to promote cytokinesis. This period of the cell cycle, which lasts for about an hour in HeLa cells, has been referred to as "C-phase".[3,236] C-phase is initiated by inactivation of CDK1, mediated by cyclin destruction catalyzed by the APC/C. At later times following anaphase, the APC/C also ubiquitinates other proteins that are essential for cytokinesis, including anillin,[132] Polo kinase[237] and Aurora B.[238] Thus the APC/C may also be responsible for terminating C-phase, an idea that is consistent with the finding that treatment of cells with proteasome inhibitors doubles the duration of C-phase.[73]

Regulation of Cytokinesis by CDK Activity

Because CDK1 is a central negative regulator of cytokinesis, it is possible that failure to fully inactivate CDK1, perhaps as a consequence of failure to fully degrade mitotic cyclins, could inhibit cytokinesis at some step. One setting in which this might occur is in cells that become arrested in mitosis due to persistent activation of the spindle checkpoint, which normally inhibits the APC/C until chromosomes become properly aligned and attached at the metaphase plate.[239] Prolonged activation of the checkpoint may result in abnormal mitotic exit, resulting in incomplete activation of APC/C, or improper timing of degradation of different substrates, leading to cytokinesis failure. There is also evidence that APC/C activity may be spatially regulated within the cell, with the subpopulation of APC/C that is associated with the spindle poles remaining inhibited until later stages of mitosis.[240] It is therefore possible that perturbation of spindle organization could interfere with the timing of degradation of mitotic regulators, thus perturbing cytokinesis.

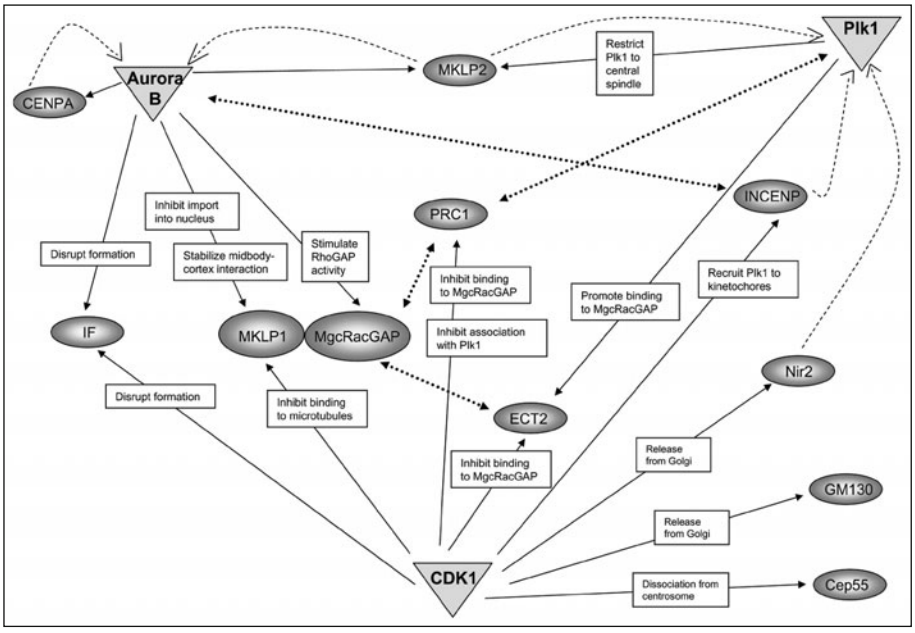

Figure 5. Regulation of cytokinesis by mitotic kinases. A major function of CDK1 is to prevent precocious cytokinesis before proper chromosome segregation. CDK1 thus negatively regulates some of the main players of cytokinesis. At the same time, CDK1 plays a positive role in cytokinesis by releasing cytokinesis proteins from the Golgi apparatus and by facilitating binding of Plk1 to its substrates. Plk1 and Aurora B phosphorylate substrates that are important for both early and late steps of cytokinesis. Solid arrows indicate phosphorylation; dashed arrows indicate changed protein localization; dotted arrows indicate protein interactions. IF, intermediate filaments.

CDK1 activity restrains multiple steps in cytokinesis. Cytokinesis initiation is inhibited because the RhoA pathway is kept inactive. This is a consequence of phosphorylation of ECT2 by CDK1 at a site that blocks its association with MgcRacGAP.[19,26] In addition, myosin light chain is phosphorylated by CDK1 at sites that inhibit myosin activation[82] and high CDK1 activity also inhibits cortical recruitment of myosin.[241] Central spindle formation is also inhibited by CDK1-dependent phosphorylation. Phosphorylation of PRC1 by CDK1 inhibits its ability to bundle microtubules[32,166,242] and its ability to interact with Plk1.[170] CDK1 also phosphorylates MKLP1, inhibiting its motor activity by reducing its affinity for microtubules.[243] Thus CDK1-dependent phosphorylation acts at many steps to block cytokinesis.

Though CDK1 restrains cytokinesis onset, CDK1-dependent phosphorylation is also essential for cytokinesis because it primes Plk1-dependent phosphorylation that occurs during early stages of cytokinesis. CDK1 activity is also important during mitosis to promote dissociation of cytokinesis proteins from cellular organelles that would otherwise sequester the protein. For example, the protein Nir2 is required for cytokinesis and must dissociate from the Golgi apparatus in order to participate in cytokinesis; dissociation is mediated by CDK1-dependent phosphorylation.[179] CDK1-dependent phosphorylation is also important for dissociation of Cep55 from the centrosome and its subsequent phosphorylation by Plk1.[231]

Regulation by Polo Kinase

Polo kinase is an essential positive regulator of cytokinesis in multiple organisms. In mammalian cells, Plk1 localizes to the midzone during anaphase and to the midbody during telophase

and cytokinesis[244] and plays an essential role in the initiation of cytokinesis.[245-248] Plk1 activity is required for recruitment of itself and ECT2 to the central spindle and inhibition of Plk1 with small molecule inhibitors abolishes RhoA GTPase localization to the equatorial cortex, suppressing cleavage furrow formation.[246-248] Plk1 also appears to be important for the interaction between ECT2 and MgcRacGAP.[247] Other evidence suggests that Plk1 may bind to ECT2 in a CDK1-dependent manner.[249] Another study using a distinct Plk1 inhibitor demonstrated that when Plk1 is inhibited, it spreads over the arms of chromosomes, resembling the localization of its binding partner PICH.[245] Therefore, Plk1 activity is required for its own proper localization during cytokinesis and also for recruitment and activation of RhoA.

Plk1 activity may be required for later steps in cytokinesis as well, as Plk1 is targeted to the central spindle by the motor protein MKLP2 and phosphorylation of MKLP2 by Plk1 is required for cytokinesis.[250] Phosphorylation of MKLP2 by Plk1 may be necessary for the spatial restriction of Plk1 to the central spindle during anaphase and telophase, although interaction with PRC1 also appears to be important for docking of Plk1 to the central spindle.[170] Plk1 may also interact with and phosphorylate MKLP1 during cytokinesis,[244,251] although others suggest that Aurora B may be the relevant kinase.[252] Cep55 also appears to be a Plk1 substrate whose phosphorylation is primed by CDK1 but the consequences of this phosphorylation remains unknown.[231] A recent proteomic screen identified a large number of proteins that bind to the Polo-box domain of Plk1, including the Rho kinase ROCK2,[253] where Plk1 and RhoA may function together to enhance ROCK2 activity. Another substrate of Plk1 that may be involved in regulation of mitosis and cytokinesis is NudC,[254,255] a dynein/dynactin associated protein that is essential for midzone formation and cytokinesis completion in C. elegans and mammalian cells.[254]

Plk1 is overexpressed in a broad range of human tumors (for review see ref. 256). Overexpression of Plk1 in HeLa cells leads to an increase of cells with large, often fragmented nuclei or multiple nuclei[257] as well as centrosome amplification,[258] suggesting that increased expression of Plk1 observed in some tumors may have an effect on cytokinesis completion as well as chromosome segregation. This finding has been corroborated in human primary cells.[259]

Regulation by Aurora B and the Chromosome Passenger Complex

The chromosome passenger complex (CPC) consists of the proteins Aurora B, INCENP, survivin and borealin. The complex plays many important roles throughout mitosis and has been implicated in the regulation of cytokinesis (see ref. 260 for review). At the metaphase-anaphase transition, the CPC relocalizes from centromeres to the spindle midzone and the equatorial cortex[261-263] and ultimately concentrates near the midbody, adjacent to the centriolin ring.[191] MKLP2, a kinesin-6 family motor protein, is required for relocalization of Aurora B and also Plk1, to the central spindle in human cells.[250,252,264,265]

Aurora B activity is necessary for several events in cytokinesis (Fig. 5). First, Aurora B is required for proper localization and function of MKLP1. Treatment of human cells with a small molecule inhibitor of Aurora B in early mitosis inhibits localization of MKLP1 (and its binding partner MgcRacGAP) to the central spindle.[266] However, addition of an Aurora inhibitor at later stages of mitosis inhibits phosphorylation of MKLP1 without disrupting its localization,[252,267] yet perturbs cytokinesis completion, indicating that MKLP1 must remain phosphorylated to permit abscission. How phosphorylation regulates MKLP1 is not completely clear, as MKLP1 is phosphorylated at multiple sites that may have distinct effects.[252,267] Phosphorylation of MKLP1 could be important for stabilizing interactions between the cortex and midbody, or be important for recruiting proteins such as centriolin that are necessary for abscission. In addition, phosphorylation of MKLP1 by Aurora B is important to prevent the protein from being sequestered back in the nucleus as cells enter interphase.[252] It is interesting to note that many components required for cytokinesis are located in the nucleus or associated with Golgi apparatus during interphase (Fig. 2) and thus localization of cytokinesis components to the midbody could require sustained phosphorylation that prevents the proteins from being resequestered by these structures as cells exit mitosis.

Another important substrate of Aurora B is MgcRacGAP, whose phosphorylation appears important for completion of cytokinesis.[47,268,269] Phosphorylation of MgcRacGAP has been proposed to stimulate its activity as a GAP for RhoA, which could be important for terminating RhoA activity in late stages of cytokinesis.[47] Another study indicates that phosphorylation of MgcRacGAP by Aurora B at a different site might activate the protein by stimulating release of the GAP domain from an inhibitory interaction with PRC1.[268]

There are several other important Aurora substrates including vimentin, an abundant intermediate filament protein. Intermediate filaments must be disassembled during mitosis to allow cell division and mitotic phosphorylation is important for filament dissociation, as expression of nonphosphorylatable mutants of vimentin leads to cells that show a persistent filamentous bridge.[270,271] Following mitotic exit and during later stages of cytokinesis, ROCK[272] and Aurora B[112,270,271,273] maintain vimentin phosphorylation after CDK1 is inactivated. Aurora B may also promote cytokinesis by inhibiting myosin light chain phosphatase.[112] Aurora B also phosphorylates CENP-A, which appears to play an important role in cytokinesis.[274] Cells expressing mutants of CENP-A that cannot be phosphorylated result in mislocalization of the passenger complex and cause a delay in the final stages of cytokinesis.

Although Aurora B is a critical positive regulator of cytokinesis in vertebrate cells, this role does not seem conserved in yeast, as the budding yeast ortholog Ipl1 and the fission yeast ortholog Ark1 are not essential for cytokinesis. However, in budding yeast, Ipl1 may negatively regulate late steps of cytokinesis in cells with spindle defects,[275,276] perhaps by regulating the localization of anillin-like proteins. This pathway may prevent abscission until segregating chromosomes have cleared the midzone. Whether a similar pathway operates in mammalian cells is not yet clear.

We are just beginning to learn about the mechanisms that regulate Aurora B activation. New work suggests that the TD60 protein may play an important role in activating Aurora B at centromeres.[277] In multiple organisms, components of the mitotic exit network, including the phosphatase CDC14, play important roles in regulating cytokinesis,[278-280] in part through regulation of targeting of the Aurora B complex.[281] Aurora B is also regulated by a Cul3-containing ubiquitin ligase, which is important for removing Aurora B from mitotic chromosomes and allowing its accumulation on the central spindle during anaphase.[282]

In vertebrate cells, Aurora B is expressed in a cell-cycle dependent manner, peaking in the G2/M phase of the cell cycle[283,284] and is highly expressed in a number of cancers.[263,284-291] However, in these tumors, expression of other proliferative markers, such as Ki-67, MCM2, geminin and Aurora A is also increased,[291,292] suggesting that Aurora B upregulation may be part of a broader upregulation of mitotic components in tumor cells. Because Aurora B is a positive regulator of cytokinesis, it is unclear whether its overexpression would perturb cytokinesis. Elevated levels of Aurora B may promote cytokinesis completion in cells that would otherwise undergo cytokinesis failure due to other abnormalities in the mitotic machinery. However, it is possible that perturbing Aurora B expression, or other components of the CPC, could alter the stoichiometry of the complex, perturbing cytokinesis.[293]

Regulation of Cytokinesis by Tyrosine Kinases

Tyrosine kinase signaling pathways may also regulate cytokinesis completion. Small molecule inhibitors of Src, including PP2 and SU6656, inhibit abscission in HeLa cells.[294] Src activity appears to be required in early mitosis, followed by delivery of tyrosine-phosphorylated proteins to the midbody via Rab11-driven vesicle transport.[294] Src colocalizes with the diaphanous-related formins mDia1 and mDia2 in endosomes and midbodies of dividing cells and inhibition of Src blocks cytokinesis.[67] Other tyrosine kinases, such as Fyn and its associated proteins are required for cytokinesis in lymphocytes,[295] through mechanisms that remain obscure.

Regulation of Cytokinesis by Lipids

Several studies indicate that phosphoinositide-containing lipids may be important for cytokinesis, with phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) playing a central role. In mammalian cells, PtdIns(4,5)P2 accumulates at the cleavage furrow and overexpression of proteins

that bind to PtdIns(4,5)P2 perturbs cytokinesis completion but not ingression of the cleavage furrow.[296] Overexpression of dominant negative kinases required for PtdIns(4,5)P2 generation also inhibits cytokinesis completion in mammalian cells.[296]

Hydrolysis of PtdIns(4,5)P2 by phosphlipasec(PLC) yields inositol trisphosphate (IP_3), which stimulates calcium release from internal stores. Inhibitors of PLC can interfere with cytokinesis,[297] which can in some cases be rescued by addition of calcium.[298] Alternatively, PtdIns(4,5)P2 may play a direct role in recruiting membrane proteins required for stability of the furrow. Several proteins required for cytokinesis, such as septin, profilin and anillin can bind to PtdIns(4,5)P2 and overexpression of a protein containing a PtdIns(4,5)P2-binding domain blocks cytokinesis completion by interfering with adhesion of the plasma membrane to the contractile ring at the furrow.[296]

Other studies have shown that the membrane lipid phosphatidylethanolamine (PE) is exposed on the cell surface of the cleavage furrow during late cytokinesis.[299] Addition of a cyclic peptide that binds tightly to PE inhibits cytokinesis completion,[299] perhaps by interfering with contractile ring disassembly.[300] Mutant cell lines that fail to synthesize adequate PE also show defects in cytokinesis completion that can be rescued by PE addition.[300] Proper PE organization may be essential for RhoA inactivation at late stages of cytokinesis, which may in turn be necessary for actin disassembly.[301]

Coupling of Cytokinesis to Other Cellular Pathways

The complexity of cytokinesis regulation provides opportunities for linking cytokinesis to other cellular pathways. Emerging evidence suggests interesting new connections between cytokinesis and the pathways involved in regulation of protein synthesis, DNA replication and DNA damage.

Cytokinesis and Protein Synthesis

Recent work suggests that proper regulation of protein synthesis may be essential for cytokinesis to proceed with high efficiency. Interestingly, of 214 genes identified in a genome-wide RNAi screen in Drosophila S2 cells, 22% were ribosomal proteins and another 5% were involved in translation.[177] Recent work suggests that the protein 14-3-3σ may play an important role in regulating protein synthesis during mitosis.[302] In normal cells, cap-dependent translation is suppressed during mitosis, whereas cap-independent translation is increased. Cells lacking 14-3-3σ do not make this switch, perturbing the pattern of proteins that are synthesized.[302] Downregulation of 14-3-3σ perturbs localization of Plk1 to the midbody and leads to cytokinesis failure.[302] These effects may be a consequence of failure to properly synthesize proteins containing an internal ribosomal entry site during mitosis, such as Cdk11.[302] Interestingly, 14-3-3σ expression is often reduced in tumor cells by targeted degradation or promoter hypermethylation. Loss of 14-3-3σ may in turn result in defective cytokinesis as a consequence of alterations in protein synthesis.

Cytokinesis and DNA Replication

Interestingly, one of the components of the Origin Recognition Complex (ORC), which is required for initiation of DNA replication, may also play a role in cytokinesis in metazoans. In vertebrate cells, Orc6 localizes to kinetochores and to a reticular-like structure around the cell periphery and ultimately to the cleavage furrow and midbody.[303] Elimination of Orc6 induces mutlipolar spindles and formation of multinucleated cells in both human cells[303] and *Drosophila*,[304] suggesting this function is conserved. In *Drosophila*, Orc6 interacts with a septin protein that may be important for cytokinesis. Domains of Orc6 required for DNA replication and cytokinesis appear separable, suggesting that Orc6 has evolved a domain that participates specifically in cytokinesis.[304] How Orc6 might couple the processes of DNA replication and cytokinesis completion remains unclear.

Cytokinesis and DNA Damage

Several lines of evidence suggest that cytokinesis may be regulated in response to DNA damage. Components required for DNA repair, such as BRCA2, may be directly involved in cytokinesis. Other evidence suggests that DNA damage pathways may regulate the expression of cytokinesis

proteins, or regulate their activity by posttranslational modification. Coupling of DNA damage pathways to cytokinesis regulation could be important for preventing the cleavage furrow from cutting damaged DNA that cannot be accurately segregated during mitosis. The existence of such pathways may explain why spontaneous chromosome missegregation is tightly coupled to cytokinesis failure in human cells.[185] In this model, DNA damage, or perhaps incompletely replicated DNA, may trigger pathways that prevent segregation of unreplicated or damaged sister chromatids and at the same time activate pathways that block cytokinesis completion.

BRCA2 is an example of a protein that may play direct roles in both DNA repair and cytokinesis. BRCA2 is required for recombination-based repair of DNA double-strand breaks.[305] However, BRCA2-deficient cells also show centrosome amplification that may be a consequence of defective cytokinesis.[306] BRCA2 localizes to the midbody and inactivation of BRCA2 in murine embryonic fibroblasts and HeLa cells interferes with cytokinesis.[307] BRCA2 may be regulated during mitosis, as it is a Plk1 substrate whose phosphorylation is inhibited in the presence of DNA damage.[308] Interestingly, downregulation of a BRCA2-interacting protein (BCCIP) also leads to defective cytokinesis.[309] Other proteins involved in DNA damage responses may influence cytokinesis regulation by interacting with cytokinesis components. For example, the DNA damage checkpoint kinase Rad53 has been shown to associate with septins in budding yeast.[310] In mammalian cells, Ku70, a DNA-binding protein required for DNA damage repair, forms a complex with ARF6 during mitosis,[311] suggesting a possible link between the DNA damage pathway and completion of cytokinesis.

Transcriptional controls may provide another mechanism for inhibiting cytokinesis in response to DNA damage. The expression of several cytokinesis proteins, including Plk1, ECT2, anillin and survivin, is repressed when DNA is damaged, in a manner that depends on an intact Rb pathway.[312] Other studies suggest that expression of cytokinesis proteins may be inhibited by activation of the p53 pathway.[313] For example, it has been shown that ECT2 expression is repressed by p53 via protein methyltransferases, suggesting that cytokinesis could be more likely to fail under conditions of p53 activation.[314]

Posttranslational modifications may also regulate cytokinesis in response to DNA damage. For example, Aurora B becomes highly poly-ADP-ribosylated when DNA is damaged, a modification that inhibits its kinase activity.[315] Poly(ADP-ribosyl)ation is an immediate cellular response to DNA strand breaks that is catalyzed by NAD^+-dependent enzymes, poly(ADP-ribose) polymerases (PARPs).[316] This effect is mediated by direct interaction between the BRCT domain of PARP1 and Aurora B.[315] Because Aurora B activity is essential for chromosome segregation and cytokinesis, induction of DNA damage could lead to errors in chromosome segregation and failure of cytokinesis.

Conclusion

Cytokinesis is a surprisingly complex process that requires the interplay of many components and regulatory pathways. Cytokinesis failure can arise through defects in any of the four stages in cytokinesis and as a consequence of inactivation or hyperactivation of any of a large number of different components (summarized in Fig. 6). Although many cytokinesis proteins have been identified, we are just beginning to understand how these proteins interact with one another and how they are regulated. Understanding the causes of cytokinesis failure is important, as it may set the stage for genetically unstable tetraploid cells that give rise to tumors.[317] However, cytokinesis failure also seems to occur physiologically in some tissues, even in those that are not tumor prone such as the heart. Understanding how cytokinesis is regulated physiologically in response to different signals, or under conditions of cell stress or damage, remains an important area for future research. Although cytokinesis failure may be accompany certain pathological states such as cancer, it is likely that pharmacologically-induced cytokinesis failure may be an important issue to consider as new medicines are developed. Inhibitors of Rho kinase are being developed for cardiovascular medicine[318] and inhibitors of Aurora kinase are under development as anticancer agents.[319] Because these compounds are likely to induce cytokinesis failure in normal tissues, it will be important to

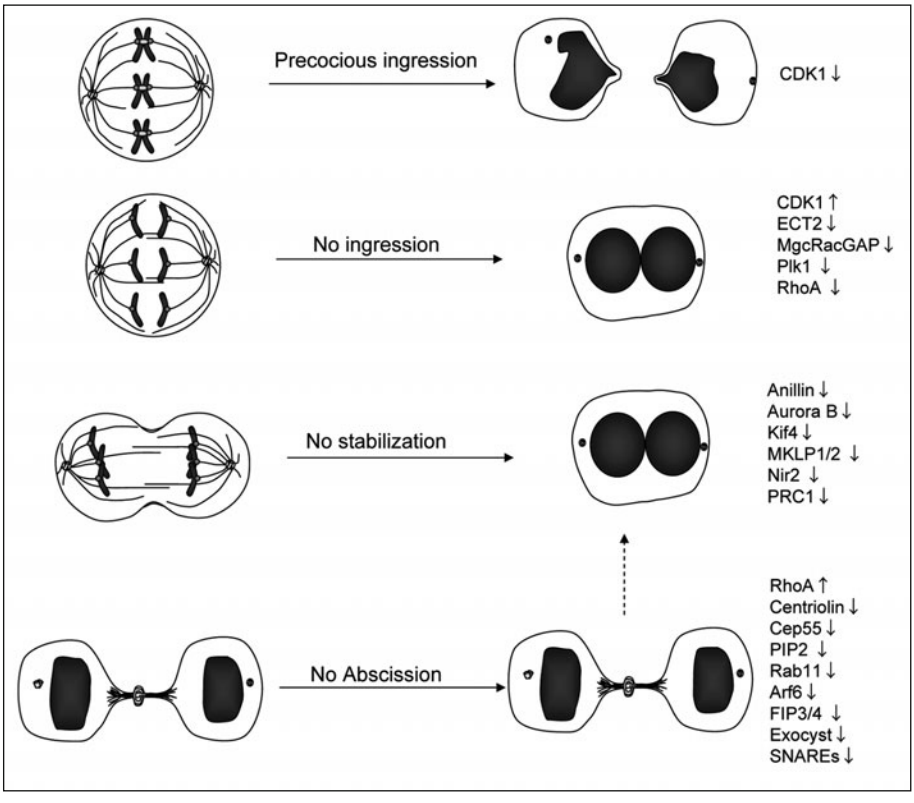

Figure 6. Summary of different phenotypes resulting from cytokinesis failure. Inhibition (downward arrow) or excessive activation (upward arrow) of different cytokinesis components can give rise to distinct phenotypes, including precocious ingression before the chromosomes have been separated, regression of the furrow giving rise to binucleated cells, or stabilization of the cytoplasmic bridge where daughter cells remain connected. The list is not comprehensive; see text for additional examples.

determine how sensitive various tissues are to cytokinesis failure and the consequences of production of tetraploid cells in different tissue types.

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Chapter 4

DNA Damage and Polyploidization

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Abstract

A growing body of evidence indicates that polyploidization triggers chromosomal instability and contributes to tumorigenesis. DNA damage is increasingly being recognized for its roles in promoting polyploidization. Although elegant mechanisms known as the DNA damage checkpoints are responsible for halting the cell cycle after DNA damage, agents that uncouple the checkpoints can induce unscheduled entry into mitosis. Likewise, defects of the checkpoints in several disorders permit mitotic entry even in the presence of DNA damage. Forcing cells with damaged DNA into mitosis causes severe chromosome segregation defects, including lagging chromosomes, chromosomal fragments and chromosomal bridges. The presence of these lesions in the cleavage plane is believed to abort cytokinesis. It is postulated that if cytokinesis failure is coupled with defects of the p53-dependent postmitotic checkpoint pathway, cells can enter S phase and become polyploids. Progress in the past several years has unraveled some of the underlying principles of these pathways and underscored the important role of DNA damage in polyploidization. Furthermore, polyploidization per se may also be an important determinant of sensitivity to DNA damage, thereby may offer an opportunity for novel therapies.

Polyploidization and Cancer

Tumorigenesis is a multistep process that arises from the accumulation of genetic alterations. These genetic changes can come in the form of point mutations that deregulate oncogenes or tumor suppressor genes. On the other hand, drastic gains or losses of whole chromosomes or chromosomal fragments (aneuploidy) are also the norm in cancer. Whether mutation of specific genes or aneuploidy is more critical for tumorigenesis is very much a contentious issue (reviewed in ref. 1).

In one school of thought, which gains prominent shortly after the discovery of oncogenes and tumor suppressor genes, states that these genetic modifications are the main driving force for tumorigenesis, with aneuploidy only as a byproduct of the process. In another school of thought, which origin can be traced to Theodor Boveri nearly 100 years ago, contests that aneuploidy might be a cause of tumorigenesis.[2] For example, weakening of the spindle-assembly checkpoint triggers chromosomal instability and aneuploidy, which appear to be an important stimulus in the initiation and progression of different cancers.[3,4] It is likely that a combination of specific gene mutations and chromosomal instability cooperate to induce tumorigenesis (reviewed in ref. 5).

Polyploidization can initiate chromosomal instability and aneuploidy (reviewed in ref. 6). Tetraploid cells are commonly found in early stages of tumors. Notable examples include Barrett's

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esophagus[7-10] and cervical carcinoma.[11] Several studies have provided evidence that tetraploidization increases chromosome instability in yeast[12,13] and in mammalian cells.[14,15] Moreover, tetraploidy may be an intermediate state in transformation. Cowell and Wigley (1980) found that during transformation of epithelial cells from mouse salivary glands, tetraploids are generated before undergoing a period of chromosome instability.[14] Many viruses can induce tetraploidy via cell fusions. While a direct demonstration between viral-induced cell fusion and human cancer is not yet available, several lines of evidence from in vitro and animal models suggest a link of the two events (reviewed in ref. 16).

It is believed that extra number of chromosomes in tetraploids may provide a buffer for chromosome loss and DNA repair. Moreover, it is generally accepted that the multistep progression of cancer—including initiation, progression, heterogeneity and drug resistance—is a product of evolutionary processes.[17] The extra set of chromosomes in tetraploids may act as a reservoir of genetic materials to allow clonal evolution of tumor. In this connection, parallels can be drawn with the role of whole genome duplication in evolution. Ohno (1970) proposed that whole genome duplication provides the primary source of redundant genes for new evolutionary opportunities.[18] He advanced that two rounds of whole genome duplication by tetraploidization occurred during the evolution of vertebrates, with the first occurred in early chordates ~500 million years ago and the second occurred at the stage of fish or amphibian ~430 million years ago. Although the details of time and number of duplications have been debated over the years,[19] it is generally accepted that polyploidization is able to promote adaptive evolutionary changes. Likewise, it is possible that tetraploidization also contributes to the evolution of cancer cells.

A seminal study by Fujiwara et al (2005) indicates that tetraploids can be generated by transient blocking of cytokinesis in p53-null mouse mammary epithelial cells. Importantly, tetraploidization promotes aneuploidy and tumorigenesis.[15] The presence of p53 normally suppresses the generation of tetraploid cells, presumably by activating the intrinsic apoptotic pathway.[20] Another study reported that chromosome nondisjunction (both copies of a chromosome segregate to the same daughter cells) leads to binucleated tetraploids by promoting cleavage furrow regression; the tetraploid cells then become aneuploidy through further divisions.[21] These and other studies provide strong evidence of the importance of tetraploidization as an early step in tumorigenesis.

How tetraploidization promotes chromosome instability remains incompletely understood. The extra centrosomes in tetraploids are likely to be critical determinants of chromosome instability (reviewed in ref. 22). Indeed, increased centrosome number is a common characteristic of several tumors. The cause of centrosome amplification in tumors is not known, but defects in the control of the centrosome replication cycle or cytokinesis are the likely underlying mechanisms (reviewed in ref. 23). Because centrosomes are microtubule organization centers, cells with supernumerary centrosomes form multipolar mitotic spindles and display other errors during chromosomal segregation. The uneven segregation of genetic materials into the daughter cells may result in different fates, including mitotic catastrophe, aneuploidy and transformation.

Although polyploid cells frequently contain multiple centrosomes, multipolar mitosis can be suppressed either by functional silencing of extra centrosomes or by centrosome clustering.[24-28] Indeed, Ganem et al (2009) found that the fraction of cells undergoing multipolar mitosis is markedly less than that possessing extra centrosomes in a variety of cancer cell lines.[29] A genome-wide RNA interference screen in *Drosophila* S2 cells revealed that a variety of proteins, including those that organize microtubules at the spindle poles and components of the spindle-assembly checkpoint, are required for centrosome clustering.[26]

Mechanisms of Polyploidization

Polyploidization may arise from diploid cells through a number of different mechanisms, including cell fusion, endoreduplication, mitotic slippage and cytokinesis failure. Before focusing on the role of DNA damage in polyploidization, we will first review the various mechanisms that can generate polyploid cells.

Multinucleated cells can be produced by fusion of different cells (called heterokaryon). Cell fusions are important in several physiological processes, including fertilization, development, immune system defense and tissue repair. Infections with many viruses can also induce cell fusions (reviewed in ref. 16). Enveloped viruses enter cells with the help of viral proteins that fuse biological membranes. A side effect of this mechanism is the ability of viruses to fuse different cells together. Cell fusions is believed to be important to cancer development and progression. Classic studies by Barski et al (1960) revealed that synkaryons (cells formed by fusion and subsequently display a single nucleus) formed in vitro could form tumor when implanted in mice.[30] Fusion of tumor cells with other cells in vivo has also been documented, for instance between human glioma cells with hamster cells[31] and tumor cells with myeloid cells.[32]

Tetraploid cells are also frequently generated after mitotic failure. Unscheduled exit from mitosis is normally prevented by the spindle-assembly checkpoint until all the kinetochores are properly attached to the spindles (reviewed in ref. 33). After prolonged activation of the spindle-assembly checkpoint, however, cells can exit mitosis precociously by a process termed mitotic slippage (also called adaptation).[34,35] In cells that undergo mitotic slippage, CDK1 is inactivated and the cells enter G_1 phase without chromosome segregation and cytokinesis. The nuclear envelope then randomly reforms around groups of chromosomes, generating cells that contain tetraploid DNA contents and two centrosomes. Although the exact mechanism of mitotic slippage is not known, the central event seems to be a slow but continuous degradation of cyclin B1.[36]

A p53-dependent "postmitotic checkpoint" is activated after mitotic slippage.[37] Activation of the p53-p21$^{CIP1/WAF1}$ axis leads to the inhibition of CDK2 and delays S phase entry. The prolonged block in mitosis prior to slippage ensures the accumulation of p21$^{CIP1/WAF1}$ before the synthesis of cyclin E-CDK2.[38] Other p53-independent mechanisms may also contribute to the postmitotic checkpoint. For example, expression of human papillomavirus E6 mutant that is defective in targeting p53 for degradation can partially induce polyploidy.[39] Proper function of the spindle-assembly checkpoint is also required for the postmitotic checkpoint. Vogel et al shows that spindle-assembly checkpoint-compromised HCT116 cells failed to arrest at the postmitotic checkpoint after nocodazole treatment.[40]

In addition of mitotic slippage, a failure in cytokinesis after anaphase also produces binucleated tetraploid cells. Successful cytokinesis requires the complete clearance of chromatin from the cleavage plane. Conditions including chromosome nondisjunction[21] and chromosomal bridge[41] can severely delay cytokinesis and promote cleavage furrow regression and tetraploidization. Such chromosomal segregation defects have been estimated to occur at a remarkably high frequency of ~1% in dividing somatic cells and at even higher incidence in transformed cells.[42,43]

A p53-dependent "tetraploidy checkpoint" has been proposed to prevent S phase entry in cells that have undergone mitotic slippage or aborted cytokinesis.[44] The checkpoint is believed to sense the increase in chromosome number and halt the cell in tetraploid G_1 state. However, the function of the tetraploidy checkpoint is contentious and its existence has been disputed.[15,45,46] One possibility is that the p53-dependent arrest after tetraploidization is mainly due to DNA damage or centrosomal stress during the aberrant mitosis (reviewed in ref. 6). Indeed, DNA damage can be readily detected in cells undergoing prolonged mitotic arrest.[47,48] Another possibility that has been proposed is that as transcription is turned off during mitosis, the lack of transcription during a protracted mitotic arrest can trigger subsequent stress and cell cycle arrest.[49] Irrespective of the precise signals that activate p53, cells with defective p53 pathway are expected to be prone to polyploidization following mitotic slippage or aborted cytokinesis.

While DNA reduplication is stringently prevented in the normal cell cycle, multiple rounds of genome reduplication, called endoreduplication, occur in cell types such as megakaryocytes and trophoblast giant cells. The mitotic CDK1 is typically inactivated to restrain mitosis during endoreduplication cycles. This has been observed in a wide range of endoreduplication

cycles, including maize endosperm,[50] *Drosophila*[51] and megakaryocytes.[52] Likewise, extensive genome reduplication can be triggered by disruption of CDK1 expression in mammalian cells.[53] Although the molecular basis of how CDK1 inactivation contributes to genome reduplication remains to be defined, the prevailing view is that APC/C plays a salient role in preventing rereplication. Unscheduled activation of APC/C reduces the concentrations of mitotic cyclins and geminin, resulting in rereplication.[54,55] To what extent does this pathway plays in the polyploidization of cancer cells remains to be deciphered. It is conceivable that DNA reduplication can occur in situations where CDK1 activity is inhibited for an extended period of time, such as after DNA damage. In fact, a connection between DNA damage and polyploidization is well established. Before describing the evidence of linkages between DNA damage and polyploidization, we will first review the current understanding of the DNA damage checkpoints in mammalian cells.

The DNA Damage Checkpoints

Surveillance mechanisms termed the DNA damage checkpoints prevent precocious entry into the cell cycle after DNA damage (Fig. 1). In essence, DNA damage activates sensors that facilitate the activation of the PI-3 (phosphoinositide 3-kinase)-related protein kinases ATM and ATR. ATM/ATR then activates CHK1 or CHK2, which in turn inactivates CDC25s (for the intra-S DNA damage checkpoint and the G_2 DNA damage checkpoint) or activates the p53-p21$^{CIP1/WAF1}$ pathway (for the G_1 DNA damage checkpoint), culminating in the inhibitory phosphorylation of CDKs and a halt in cell cycle progression (reviewed in ref. 56).

Following exposure to ionizing radiation or other genotoxic insults that elicit DNA double-strand breaks, ATM is autophosphorylated at Ser1981, leading to dimer dissociation and

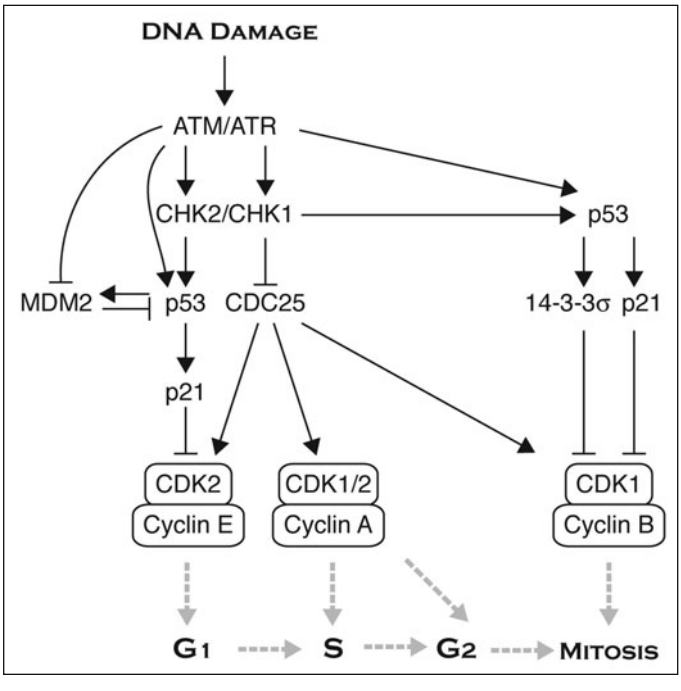

Figure 1. The DNA damage checkpoints. A simplified version of the major pathways of the DNA damage checkpoints is shown. Uncoupling the checkpoints promotes mitosis in the presence of DNA damage. See text for details.

activation of the kinase. ATR is activated by a broader spectrum of stress including ultraviolet irradiation, hypoxia and replication stress. ATM and ATR phosphorylate residues in the SQ/TQ domain of CHK1/CHK2, thereby stimulating the kinase activity of these effector kinases.

The upstream sensors that initiate the activation of ATM/ATR consist of an intricate network of large protein complexes, of which many components contain the BRCT domain. These include the RAD9-HUS1-RAD1 (9-1-1) clamp and the RAD17-RFC clamp loader that facilitate ATR-mediated activation of CHK1.[57] Another large complex that participates in ATM/ATR activation is the so-called BRCA1-associated genome surveillance complex composed of BRCA1, BLM and MRN (MRE11-RAD50-NBS1).[58,59] Stalled replication forks mainly activate the ATR-CHK1 pathway. Replication fork progression can be impaired by insufficient nucleotide supply or lesions and obstacles on the DNA. Several proteins including ATRIP (ATR-interacting protein), TopBP1 and Claspin appear to be required for recruiting ATR to single-stranded DNA present at stalled replication forks to phosphorylate CHK1.[60] The ATR-CHK1 pathway is essential even in the absence of exogenous stresses during unperturbed S phase, probably for maintaining high rates of replication fork progression.[61] Claspin is usually degraded by $SCF^{\beta\text{–}TrCP}$-mediated ubiquitination following the phosphorylation of Claspin by PLK1. This pathway is inhibited after DNA damage.[62] In response to genotoxic stress in G_2 phase, the phosphatase CDC14B translocates from the nucleolus to the nucleoplasm and activates APC/C^{CDH1}. This degrades PLK1 and consequently stabilizes Claspin, allowing the G_2 DNA damage checkpoint to be maintained.[63]

Once the ATM/ATR-CHK1/CHK2 cascade is activated, the G_2 DNA damage checkpoint is believed to be carried out by the inactivation of all three isoforms of the CDC25 family (CDC25A, CDC25B and CDC25C) by CHK1 and CHK2.[64] Phosphorylation of $CDC25C^{Ser216}$ by CHK1/CHK2 inactivates its phosphatase activity either directly or indirectly through the creation of a 14-3-3 binding site. Binding of 14-3-3 masks a proximal nuclear localization sequence and anchors CDC25C in the cytoplasm, preventing efficient access of CDC25C to cyclin B1-CDK1. Interestingly, phosphorylation of a proximal site (Ser214) by cyclin B1-CDK1 inhibits further phosphorylation of $CDC25C^{Ser216}$. This provides an elegant mechanistic explanation for the suppression of DNA damage-mediated CDC25C inactivation during mitosis.[65]

CDC25B is believed to possess a unique role in activating cyclin B1-CDK1 at the centrosome. CHK1 may shield centrosomal cyclin B1-CDK1 from unscheduled activation by CDC25B during normal G_2 phase and presumably also during the G_2 DNA damage checkpoint. The molecular basis of this activity may be due to CHK1-dependent phosphorylation of $CDC25B^{Ser323}$, creating a docking site for 14-3-3 that prevents access of substrates to the catalytic site. Dissociation of CHK1 from the centrosomes at the end of G_2 phase, together with positive regulatory phosphorylation of $CDC25B^{Ser353}$ by Aurora-A, enables CDC25B to activate the centrosomal cyclin B1-CDK1 and initiate mitosis.[66]

CDC25A is arguably the most important member of the CDC25 family due to its nonredundant role in mouse cells. CDC25A is targeted for rapid degradation by CHK1/CHK2 through a ubiquitin-mediated mechanism. CDC25A stability is controlled by APC/C^{CDH1} complexes during mitotic exit and early G_1 and by $SCF^{\beta\text{-}TrCP}$ complexes during interphase. Importantly, the $SCF^{\beta\text{-}TrCP}$-dependent turnover of CDC25A is enhanced in response to DNA damage. Phosphorylation of $CDC25A^{Ser76}$ by CHK1 is required for the phosphorylation of a phosphodegron centered at Ser82 (by an as-yet-unidentified kinase), creating a binding site for β-TrCP. Interestingly, β-TrCP also binds to a separate nonphosphorylated sequence in CDC25A (the DDG motif) and plays a role in CHK1-induced ubiquitination and degradation of CDC25A.[66]

There is also evidence that CHK1 can phosphorylate and activate WEE1 by promoting 14-3-3 binding.[67,68] Suppression of CDC25s or activation of WEE1 promotes $CDK1^{Thr14/Tyr15}$ phosphorylation, thus preventing damaged cells from entering mitosis. Other mechanisms are also known to play critical roles in the G_2 DNA damage checkpoint. For example, the p53

downstream target 14-3-3σ is involved in sequestering cyclin B1-CDK1 in the cytoplasm after DNA damage.[69]

Cells have also evolved checkpoints in S phase to prevent replication of damaged DNA. One of the better-understood S phase checkpoints is the intra-S DNA damage checkpoint, which is important for the responses to double strand breaks. A hallmark of the intra-S DNA damage checkpoint is that it slows down but does not stop DNA synthesis. In addition, there is no strong correlation between the sensitivity to DNA damage and the loss of the checkpoint.[70] For these reasons, it has been suggested that the intra-S DNA damage checkpoint may be involved in tolerating damage during replication rather than actually repairing the damage.[71] The checkpoint affects two distinct processes: origin firing and the rate of replication folk progression. As a global response, origins distant from the site of DNA damaged are prevented from firing through checkpoint activation. The mechanism involves ATM/ATR-dependent activation of CHK1/CHK2, which then phosphorylate CDC25A, leading to its rapid degradation. This prevents the dephosphorylation of $CDK2^{Thr14/Tyr15}$ and inhibits S phase progression by preventing the loading of replication initiation protein CDC45 onto the origin.[72,73,74]

Replication fork slowing may represent a more local response to DNA damage during S phase. The current paradigm states that two separate pathways downstream to ATM, namely CHK2 and MRN complexes, are required for folk slowing. Mutations in either pathway results in radioresistance DNA synthesis. However, activation of CHK2, degradation of CDC25A and inactivation of CDK2 occur normally in irradiated MRN complexes-defective cells.[74] Precisely how MRN complexes reduce the rate of replication folk progression is still unclear. One of the downstream effectors may be SMC1, which functions in the cohesion of sister chromatids following DNA replication and homologous recombination DNA repair.[75,76] It has been shown that SMC1 is phosphorylated by ATM upon ionizing radiation-induced DNA damage, but the details of the mechanism await further clarification.[77]

When cells suffer DNA damage during G_1 phase, it is critical for them to halt the entry into S phase until the DNA is repaired. It is well established that the G_1 DNA damage checkpoint involves the stabilization and activation of p53, which in turns transcriptionally activates the CDK inhibitor $p21^{CIP1/WAF1}$, leading to the inhibition of cyclin E-CDK2 complexes and G_1 arrest. The activity of p53 is highly regulated by posttranslational mechanisms including protein-protein interaction, acetylation, neddylation, phosphorylation, sumoylation and ubiquitination.[78] In unstressed cells, p53 is restrained by binding to MDM2, itself a transcriptional target of p53, in a negative feedback loop. MDM2 directly binds to the NH_2-terminal transactivation domain of p53 to inhibit its transcriptional activity and shuttles p53 out from the nucleus by the virtue of its nuclear exporting signal. In addition, MDM2 is also a ubiquitin ligase that targets p53 for ubiquitin-mediated proteolysis.

The crucial event in p53 activation and stabilization is the phosphorylation of the NH_2-terminal residues by checkpoint-stimulated protein kinases. Upon DNA damage, ATM and ATR are activated and phosphorylate $p53^{Ser15}$, which inhibits the interaction of p53 with MDM2, resulting in p53 stabilization.[79,80] Apart from directly phosphorylating p53, ATM also induces $p53^{Ser20}$ phosphorylation indirectly via CHK1 and CHK2.[81-83]

In addition to its well-known role in the G_1 DNA damage checkpoint, a growing body of evidence also indicates the importance of the p53-$p21^{CIP1/WAF1}$ axis in the G_2 DNA damage checkpoint.[84] In a recent study, $p21^{CIP1/WAF1}$ was found to downregulate EMI1.[85] Since EMI1 is an inhibitor of APC/C, it is possible that $p21^{CIP1/WAF1}$ contributes to the maintenance of G_2 arrest by stimulating the degradation of the mitotic cyclins.

Polyploidization Induced by DNA Damage

It is vital to prevent the precocious activation of cyclins-CDKs to provide sufficient time for DNA repair. It is well known that bypass of the classic DNA damage checkpoint pathways described above promotes premature entry into mitosis. Checkpoint-uncoupled cells then

undergo mitotic catastrophe, a special form of cell death during mitosis. For instance, cells lacking p53, $p21^{CIP1/WAF1}$, or 14-3-3σ fail to arrest in G_2 after DNA damage and undergo mitotic catastrophe.[69,84] However, a significant proportion of checkpoint-bypassed cells survive the aberrant mitosis. While these cells are able to enter and exit mitosis, they often fail to complete cytokinesis properly, giving rise to tetraploidy.[84,86-88] In support of this, a population of polyploid cells can frequently be detected in malignant tumors.[89] Polyploid cells are also observed in colonies that survive after treatments with DNA damaging agents.[90,91] The spindle-assembly checkpoint is required for mitotic catastrophe induced by abrogation of the DNA damage checkpoint,[40,92] suggesting a trap in mitosis is required for these types of cell death. Thus a weakened spindle-assembly checkpoint may potentiate with the bypass of the DNA damage checkpoint to induce polyploidization.

How DNA damage leads to polyploidization is still not completely understood. A likely explanation is that mitosis occuring in the presence of damaged DNA generates either chromosome fragments or entire lagging chromosomes, leading to cytokinesis failure or cell fusion.[87] Indeed, it has been demonstrated that lagging chromosomes are able to promote cleavage furrow regression and tetraploidization.[21] The frequent presence of micronucleus (which are formed from chromosome fragments and lagging chromosomes) in polyploid cells also reflects the role of chromosomal damage in polyploidization (reviewed in ref. 93). Furthermore, incorrect fusion of chromosomes during repair of double strand breaks can lead to formation of chromosome bridges.[94] Aurora B appears to be part of a sensor that responds to unsegregated chromatin at the cleavage site.[41]

Uncoupling of the DNA damage checkpoint is thus a key event in polyploidization. In fact, the G_2 DNA damage checkpoint is partially impaired in many cancer cells.[95] They are unable to maintain G_2 arrest and eventually undergo aberrant mitosis.[96] Uncoupling of the ATM/ATR-CHK1/CHK2 axis is well documented. Ablation of the G_2 DNA damage checkpoints induces unscheduled activation of cyclin B1-CDK1 and premature entry into mitosis.[69,97,98] Cells that contain defective ATM, such as those derived from ataxia-telangiectasia, often exhibit radio-resistant DNA synthesis.[99] Likewise, IR-induced G_1 arrest is impaired in $CHK2^{-/-}$ mouse embryonic fibroblasts.[100] Studies using conditional CHK1 knock-out mice also revealed that CHK1 deficiency causes inappropriate S phase entry, accumulation of DNA damage during replication and premature entry into mitosis.[101,102] Since the ultimate effect of the ATM/ATR-CHK1/CHK2 pathway is the inhibitory phosphorylation of CDK1, it is not surprising that expression of a nonphosphorylatable mutant of CDK1 can also trigger premature entry into mitosis.[102-104]

Chemical agents that inhibit the ATM/ATR-CHK1/CHK2 pathway can induce checkpoint bypass and many are potential chemotherapeutic agents. Caffeine is a classic inhibitor of ATM/ATR.[105-107] The checkpoints can also be uncoupled with CHK1 inhibitors such as UCN-01.[108-110] Inhibition of CHK1 with UCN-01 after DNA damage overcomes the DNA damage checkpoints, inducing premature activation of cyclin B1-CDK1 and mitotic catastrophe. However, UCN-01 is also a potent inhibitor of Protein Kinase C, CDKs, MK2, AKT (through inhibition of phosphoinositide-dependent kinase 1) and other kinases. This promiscuous nature of UCN-01 makes defining its precise role difficult. In fact, the two kinases that can phosphorylate $CDC25C^{Ser216}$—C-TAK1 and CHK1—can both be inhibited by UCN-01.[111] Likewise, inhibition of CHK2 promotes premature entry of mitosis after DNA damage.[112]

Another possible mechanism that can promote polyploidization after DNA damage is due to the inhibition of CDK1. As CDK1 is turned off by inhibitory phosphorylation after DNA damage, it is possible that prolonged inhibition of CDK1 may induce endoreduplication cycles, similar to those in cells such as megakaryocytes (see above). However, there is little experimental support of this hypothesis at this stage.

Similarly, defects of the MRN complexes can also ablate the intra-S-phase DNA damage checkpoint and induce polyploidization. Nijmegen breakage syndrome is a rare autosomal

recessive disorder characterized by microcephaly, immunodeficiency and predisposition to hematopoietic malignancy, sharing a wide range of clinical features with ataxia telangiectasia. The disorder is caused by mutation of the *NBS1* gene, which encodes a member of the MRN complex. Hypomorphic mutations have also been found in MRE11.[113] Complete inactivation of MRE11, RAD50, or NBS1 leads to early embryonic lethality in mice through accumulation of double strand breaks during development.[114-116] Furthermore, cells derived from mice that lacking functional NBS1 share similar phenotype with ATM-defective cells, showing an impairment of the intra-S DNA damage checkpoint after ionizing radiation.[117] Moreover, NBS1-deficient B lymphocytes show defective intra-S phase checkpoint, chromosomal translocation and tetraploid DNA content.[118] Finally, it has been well known that simian virus 40 induces host endoreduplication by large T antigen.[119] In fact, large T antigen interacts with NBS1 and disrupts the function of MRN complexes, leading to DNA rereplication and tetraploidization.[120]

The Sensitivity of Polyploid Cells to DNA Damage

As described above, a growing body of evidence indicates that tetraploidization promotes chromosome instability and transformation. Nevertheless, tetraploidy appears to be a relatively more stable state than other aneuploidy.[6] Artificially generated tetraploids can be maintained in culture for a long period without any obvious collapse of the ploidy.[121,122] However, there is also evidence that polyploids are under more stress and are less robust than diploids. In budding yeast, diploids take over tetraploids in long-term culture.[123] Tetraploid yeasts are notably genetically unstable, with high levels of both chromosome loss and interhomolog recombination.[13] Similarly, in chimeric mice produced from the combination of diploid and tetraploid cells, the tetraploid cells are out-competed and ultimately produces fetuses completely composed of diploid cells.[124] Mammalian tetraploid cells exhibit an increase in the basal expression of p53 and an enhanced rate of apoptosis.[121] This may reflect an elevated level of stress in tetraploid cells. In budding yeasts, several genes involved in DNA repair are essential for the viability of polyploid cells (but not in diploids), suggesting that polyploidization may elevate the levels of DNA damage.[13]

Whether ploidy influences the responses to genotoxic stress remains incompletely understood. It is likely that the increase amount of DNA per cell may raise the chance of receiving damage. Given that tetraploid mouse mammary epithelial cells were more prone to transformation after exposure to a carcinogen than diploid cells,[15] one possibility is that the increase in sensitivity to DNA-damaging agents in tetraploid cells may increase mutagenesis. This is supported by the findings that tetraploidization of Hep3B cells or human fibroblasts sensitizes cells to genotoxic stress inflicted by ionizing radiation and topoisomerase inhibitors.[122] Tetraploid cells contain higher number of γ-H2AX foci after ionizing radiation than their diploid counterparts. However, results described by Castedo et al indicate that tetraploid HCT116 and RKO cells are more resistant to DNA damaging agents (camptothecin, cisplatin, oxaliplatin, gamma- and UVC-irradiation) than their diploid counterparts.[121,125]

An additional factor that may affect the sensitivity of polyploid cells to genotoxic agents is the increase in cell volume. Ploidy is one of the key intrinsic factors that influence cell volume (reviewed in ref. 126,127). For instance, *Drosophila* polyploid salivary gland cells are more than 1,000 times larger than diploid cells and cells from tetraploid mice are about twice the size of those of diploid cells.[128] The increase in cell volume is believed to provide a metabolic growth advantage for polyploid cells.[129] It is possible that the increase in cell volume and surface area may allow polyploid cells to receive a higher dose of genotoxic agents.

Polyploidization and Cancer Therapies

Polyploidization can be a double-edged sword in cancer research. On the one hand, as we have discussed in detail above, aberrant polyploidization is believed to be a critical factor of tumorigenesis. Deciphering the mechanism of polyploidization will help us to understand the basis of tumorigenesis. On the other hand, polyploidization could be exploited as a strategy to induce cell death in cancer therapies.

For many types of potential therapeutic genotoxic agents, it is often not immediately obvious why they should selectively target cancer cells but spare normal cells.[130] Many effective anticancer agents are believed to take advantage of the severe imbalance of various cellular regulators and components in cancer cells. The obvious doubling of DNA and other cellular components in polyploid cells in relation to their diploid counterpart may offer an opportunity for designing novel therapeutic approaches.

If tetraploid cells are indeed more sensitive to DNA damage,[122] an implication for chemotherapeutic intervention is that some cancer cells can be sensitized to genotoxic agents by a preceding step that induces polyploidization. This will probably be especially apt for cells that contain a weakened or defective spindle-assembly checkpoint. Treatment of cancer cells with spindle poisons can induce mitotic arrest and apoptosis. However, cells can also undergo mitotic slippage and enter a tetraploid G_1 state. If additional checkpoint is lacking (such as being p53 defective), tetraploid cells can further undergo DNA replication and become polyploids. Thus it may be of advantage in cancer therapy to first induce polyploidization before treatments with DNA-damaging agents. In this scenario, sequential rather than simultaneous treatment with spindle inhibitors and DNA damaging agents will be critical, as cells are sensitized to DNA damaging agents only after mitotic slippage.

Mitotic slippage can also be promoted with inhibitors of mitotic kinases such as CDK1.[131] Mitotic slippage per se does not appear to be toxic, but a substantial portion of cells may be killed during the subsequent multipolar mitosis.[131] CDKs themselves are important targets for cancer therapies. Several small chemical inhibitors (purine analogs such as flavopiridol, BMS-387032, E7070 and roscovitine) have shown preclinical and clinical anticancer activity. In particular, roscovitine (Seliciclib, CYC202 or Cyclacel) is a potent chemotherapeutic agent and has been tested in clinical trials for a variety of cancers.[132] Hence it is possible that sequential treatment of a spindle poison followed by roscovitine and DNA damaging agents may prove effective against some cancers.

There are also other reports indicating that tetraploid cells are more resistant to DNA damaging agents.[121,125] The presence of polyploid giant cells in cancers may also account for resistant to cancer therapy. Following DNA damage (in particular with relatively low dose of DNA damaging agents), many polyploid cells appear after an initial phase of mitotic catastrophe and survive for weeks as mono- or multi-nucleated giant cells.[90,91] Whether these cells still retain proliferative potential is controversial. Although some studies indicate that giant cells have reduced or no proliferative potential,[133] other studies have shown that giant cells can undergo multipolar mitosis or de-polyploidization to return to near diploid state.[134] The latter studies suggest that the multistep process of escaping cell death through polyploidization and then depolyploidization may account for tumor relapse after initial efficient cancer therapy.

Conclusion

Several intricate DNA damage checkpoints ensure that cell cycle progression is delayed after DNA damage. Defects of the checkpoints in several disorders permits mitotic entry even in the presence of DNA damage. Likewise, aberrant entry into mitosis can be induced by chemicals that uncouple the checkpoints. Forcing cells with damaged DNA into mitosis causes severe chromosome segregation defects, including lagging chromosomes, chromosomal fragments and chromosomal bridges. The presence of these lesions in the cleavage plane is believed to abort cytokinesis. If this is coupled with defects of the p53-dependent postmitotic checkpoint pathway, cells can enter S phase and become polyploids. Several lines of evidence indicate that polyploidization triggers chromosomal instability and contributes to tumorigenesis (Fig. 2). Other mechanisms, including the prolonged inhibition of CDK1 activity and defects of the intra-S checkpoint, may also provide a link between DNA damage and polyploidization. These recent advances raise several important issues that require further investigation. Outstanding issues include the need of more compelling evidence of the linkage between DNA damage and polyploidization, as well as a direct demonstration of the importance of polyploidization in human cancers.

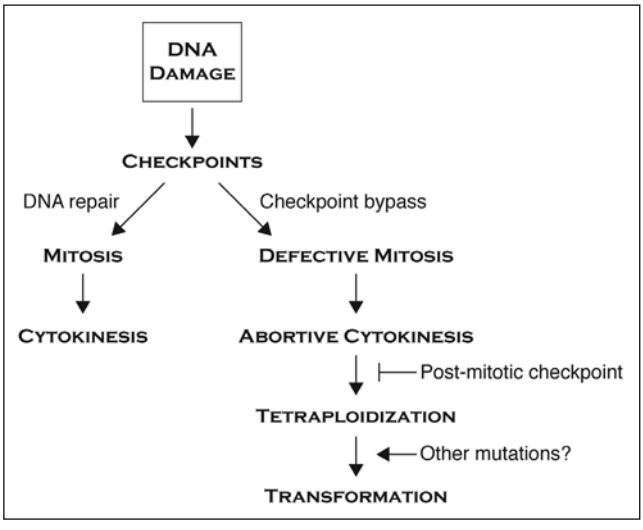

Figure 2. Promotion of polyploidization and transformation by DNA damage. The DNA damage checkpoints delay cell cycle progression. After the damaged DNA is repaired, the checkpoints are inactivated and the cell cycle can proceed. If the checkpoints are bypassed, however, cells can enter mitosis containing damaged DNA. This results in chromosome segregation defects that include lagging chromosomes, chromosomal fragments and chromosomal bridges, thereby preventing the success completion of cytokinesis. These cells can become tetraploids if the p53-dependent postmitotic checkpoint is defective. Tetraploidization (possibly coupled with other mutations) triggers chromosomal instability and contributes to tumorigenesis.

Polyploidization of cancer cells may offer an opportunity for drug intervention. Different strategies that trigger mitotic arrest, mitotic slippage and DNA damage should be explored to see if they sensitize various types of cancer cells. More vigorous studies are also required to provide a comprehensive picture of whether polyploidization sensitizes cancer cells to DNA damaging therapeutic agents.

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Chapter 5

Role of the p53 Family in Stabilizing the Genome and Preventing Polyploidization

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Abstract

Cellular defects resulting in chromosomal instability and aneuploidy are the most common features of human cancers. As a major tumor suppressor and intrinsic part of several cellular checkpoints, p53 contributes to maintenance of the stability of the genetic material, both in quality (ensures faithful replication) and quantity (preservation of diploidy). Although the exact trigger of p53 in case of numerical chromosomal aberrations is unknown, the absence of p53 allows polyploid cells to proliferate and generate unstable aneuploid progeny. A more recent addition to the p53 family, p73, emerged as an important contributor to genomic integrity when p53 is inactivated. p73 loss in p53-null background leads to a rapid increase in polyploidy and aneuploidy, markedly exceeding that caused by p53 loss alone. Constitutive deregulation of Cyclin-Cdk and p27/Kip1 activities and excess failure of the G2/M DNA damage checkpoint are important deficiencies associated with p73 loss.

p53—Tumor Suppressor

The p53 tumor suppressor gene encodes a multi-functional protein involved in the comprehensive control of cellular responses to genotoxic stress.[1,2] Its tumor suppressor effects are mediated by a variety of mechanisms including cell cycle arrest, apoptosis and cellular senescence that prevent cells with damaged DNA to pass on their genomes to progeny.[3] In unstressed cells, p53 is maintained at very low levels, but it becomes rapidly stabilized and activated in conditions of genotoxic stress. In the absence of p53, cells with damaged DNA fail to properly respond to DNA damage checkpoints but instead continue to proliferate, which results in random mutations, gene amplifications, chromosomal re-arrangements and aneuploidy. This is frequently associated with tumorigenesis. Consequently, p53 is functionally inactivated in more than half of human cancers ranging from carcinomas, sarcomas and lymphomas.[4-8] Moreover, alterations of the p53 gene occur not only as acquired somatic mutations in human cancers but also as germline mutations in patients with the cancer-prone Li-Fraumeni syndrome.[9-11]

The importance of p53 in tumor suppression was confirmed by animal models. p53 knock-out mice are highly prone to spontaneous tumor formation (T-cells lymphomas and fibrosarcomas).[12] Moreover, a single dose of 4 Gy γ-irradiation dramatically decreases the latency for tumor development in $p53^{+/-}$ heterozygous mice.[13] Cells derived from $p53^{-/-}$ null mice show signs of spontaneous genomic instability. For instance, mouse normal embryonic fibroblasts (MEFs) and pancreatic cells obtained from $p53^{-/-}$ mice exhibit a high degree of aneuploidy.[14-16] Murine models also proved

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that p53 loss synergizes with (proto)oncogenes (such as Myc, Ras, Wnt-1) in accelerating tumor formation.[17-21]

Analysis of human tumors revealed that the p53 gene is mainly targeted by missense mutations, which generate abnormally stabilized protein, while complete gene deletions are relatively rare.[22] Recent advances in clarifying the role of p53 hotspot mutants (i.e., which recur over and over in many patients) in a physiological context were offered by two knock-in mice that harbor the structural mutant p53R172H and the contact mutant p53R270H (corresponding to codons 175 and 273 in humans). p53R270H/+ and p53R172H/+ mice model the Li-Fraumeni syndrome. Of note, they developed allele-specific tumor spectra that were distinct from $p53^{-/+}$ mice. Moreover, p53R270H/– and p53R172H/– mice again developed a different tumor spectrum compared to $p53^{-/-}$ mice, including more frequent carcinomas and endothelial tumors. These results demonstrate that missense mutant p53 alleles expressed under physiological control have enhanced oncogenic potential (gain-of-function) that goes beyond the simple loss-of-function of p53 null alleles.[22] As the underlying mechanism it was suggested that p73 and p63 protective functions are concomitantly disabled by binding to the missense mutant p53 protein.[22,23]

p53 and Genomic Stability

p53 and Cell Cycle Checkpoints

p53 is one of the main effectors of cell cycle checkpoints. However, the precise mechanisms of its actions are still controversial. Clearly, p53 mediates G1 arrest in response to DNA damage, thus preventing DNA synthesis from damaged templates.[24] Apart from this, p53 is involved in regulating the cell cycle at transitions of G1/S and G2/M and within S-phase.[25-27] Evidence for a possible role of p53 in M-phase came from observations that p53 contributes to the control of centrosome duplication[28,29] and to the prevention of DNA rereplication when chromosome segregation is impaired by spindle inhibitors.[28,29] The various cell cycle checkpoints and a simplified view of p53 contribution to them are summarized below:

G1/S Checkpoint

Blocks replication of damaged DNA (e.g., if the nucleotide pool is inadequate for genome duplication[30,31] or under drugs that produce DNA damage).

p53 Model

DNA double-strand breaks activate ATM. ATM phosphorylates downstream effectors, either directly or through its immediate target Chk2. Phosphorylated histone H2AX (γH2AX) marks the chromatin site of the actual damage. p53 can be phosphorylated either by ATM or indirectly by Chk2. This contributes to p53's stabilization and activation. Through its transcriptional targets (such as cyclin-dependent kinase inhibitor p21) p53 delays cycle progression.[32-34]

Intra-S Phase Checkpoint

Stops DNA synthesis if the damage occurs in S-phase or cells with damaged DNA slipped through a G1/S block.

p53 Model

Intrinsic events or genotoxic stress (e.g., hydroxyurea and UV) during S-phase activate a checkpoint that prevents the progression of replication forks. The main player in this checkpoint is ATR. ATR phosphorylates and activates Chk1, γH2AX and BLM helicase. p53 becomes activated by ATR or Chk1 and transported by BLM to sites of stalled replication forks. Through direct interactions with components of the replication machinery and through activation of target genes, p53 is able to slow down replication.[34-37] p53-proficient cells show a lower level of double strand breaks (DSB), while in the absence of p53, ssDNA regions associated with stalled replication forks turn into DSB and generate major chromosomal abnormalities.[38]

G2/M Checkpoint

Employed when cells containing wrongly replicated (under- or overreplicated) DNA exit S-phase. The G2/M restriction point prevents mitosis of such cells. Loosening the G2/M checkpoint is one of the hallmarks of malignant transformation. This checkpoint is the "last barrier" prior to mitotic division that can block the perpetuation of mutations or an unstable genome.[39] The G2/M transition is under very strict control and integrates multiple pathways that are p53 dependent and independent.

p53 Model

It is thought that p53 is able to block cells in G2 via indirect inactivation of CDK1. Gadd45, p21 and 14-3-3σ, all p53 transcriptional targets, are able to inhibit CDK1. On the other hand, cyclin B, the regulatory subunit of CDK1, can be transcriptionally repressed by p53.[40] However, alternative pathways (ATM/ATR-dependent, caffeine and UCN-01 inhibitor sensitive) explain why p53-null cells are still able to arrest in G2/M.[41] Cells can stop the cell cycle in G2 by activating the ATM/ATR pathway and downregulate CDK1 via Chk1 and Chk2.[40] The Chk kinases inactivate Cdc25C, the phosphatase responsible for eliminating the inhibitory phosphorylations on CDK1.

Mitotic Checkpoint or Spindle Assembly Checkpoint

Does not allow anaphase to proceed until all chromosomes are properly attached to the spindle microtubule apparatus. Checkpoint proteins are components of the kinetochore, a macromolecular complex that resides at centromeres of chromosomes that establishes connections with spindle microtubules. Mitotic exit with abnormal chromosomes results in arrest.[41,42]

The p53 Model Is Controversial

p53-deficient fibroblasts, but not their wild-type counterparts, fail to arrest in response to spindle inhibitors and undergo another round of replication without mitosis to become polyploidy.[28,43] However, more careful studies revealed that p53 acts, in fact, at the subsequent G1 step to induce arrest. In response to spindle inhibitors, both wild-type and p53-null MEFs moved equally well from M into G1. The difference was that wild-type cells remained arrested, while p53 null cells were able to restart DNA synthesis and thus became polyploid. Eventually, some of these cells would escape the postmitotic block and divide, generating aneuploidy.[44]

However, p53 might still play some role in the mitotic checkpoint proper through its reported colocalization with centrosomes and a direct involvement in preventing multipolar mitotic spindles and centrosome amplification.[29] Loss of p53 was reported to be associated with accumulation of centrosome abnormalities, multiple spindle poles and missegregation of chromosomes into daughter cells in a fraction of mouse fibroblasts.[29]

p53 in DNA Repair

In addition to DNA damage-induced transcription-dependent p53 functions, evidence has accumulated for a direct role of p53 in DNA repair, DNA replication and DNA repair associated with active replication.[45] Genetic studies using KO mice for different genes involved in nonhomologous end joining (NHEJ) showed that p53 is a negative regulator of error prone NHEJ.[42,46-48] Additionally, p53 has been reported to have intrinsic Mg^{2+}-dependent exonuclease function.[49] p53 colocalizes with PCNA, DNA polymerase α, DNA ligase and RPA in the nuclei of Herpes virus—infected cells[50] and binds recombinases (such as Rad51[51] and Rad54[52]) in human cells. Thus, p53 is likely to negatively modulate homologous recombination.[53] Moreover, p53 null mice show an increased frequency of homologous recombination at different stages of development.[54] Recent studies of human cells revealed a requirement for p53 in global modulation of chromatin structure upon localized subcellular UV irradiation.[55] Thus, p53 has a role in increasing global chromatin accessibility, potentially through histone acetylation.

Deficiencies in cell cycle checkpoints or in the system that detects and repairs DNA damage have a deep impact on genomic stability and increase the probability of tumor formation.[24] Thus, p53 may help maintain genomic stability by preventing DNA replication of damaged DNA,

preventing replication in conditions that could harm the DNA and preventing rereplication of DNA that could lead to aneuploidy.[24]

Although its precise role in tumorigenesis is still controversial as to being cause or consequence, aneuploidy is a hallmark of cancer development. While aneuploidy can arise directly from defects in duplication, maturation or segregation of centrosomes,[56] it is generally thought that the precursor of aneuploidy is a polyploid state. One proposed route to aneuploid cancer cells is through an unstable tetraploid intermediate.[57,58] Supporting this idea, recent studies demonstrate that tetraploidy promotes chromosomal aberrations and tumorigenesis in vivo.[59-61]

Mechanisms of Polyploidization

Without excluding the possibility of other ways, it is considered that a diploid organism can acquire polyploid cells through several general mechanism: cell fusion, endoreplication (also called endomitosis) and a variety of defects that result in a nondividing cell cycle (nonmitotic, abortive cell cycle).[62]

DNA endoreplication is widely observed in the plant kingdom and selectively occurs in many animals as a response to developmental needs.[63] In humans, endoreplication can occur during differentiation (e.g., in megakaryocytes, hepatocytes and trophoblasts) or as a physiological response to metabolic stress (muscle cells). Megakaryocytes are bone marrow precursors that generate platelets. Specifically, megakaryocytes traverse the initial stages of mitosis (anaphase A) including centrosome duplication, but skip anaphase B and cytokinesis, resulting in polyploid cells.[62] Metabolic stress also facilitates polyploidy in several tissues. An increase in the percentage of polyploid hepatocytes is seen in ischemic lesions of the liver, regrowth of the liver after partial hepatectomy and in advanced age.[64,65] Hypertensive humans or rats show polyploid heart muscle and vascular smooth-muscle cells.[66] Another example are tetraploid fibroblasts frequently observed during wound healing in biopsies.[67] Thus, an increase in the amount of DNA and, consequently, in cell volume is regarded as beneficial for cells that have high metabolic rates like liver or muscle cells. Of note, this tetraploid state does not trigger any p53-depedent checkpoint. This led many authors to conclude that although mechanistically very similar, endoreduplication and polyloidization are not the same phenomenon.[63] Along the same lines, it is unclear if polyploid cells resulting from physiologic endoreduplication are more prone to genomic instability than their diploid parental tissue and if they associate with future organ pathologies.

From an evolutionary point of view, polyploidy could be advantageous for the entire organism due to better use of heterozygosity, the buffering effect of gene redundancy on mutations and, in certain cases the facilitation of reproduction through self-fertilization or by asexual means.[63]

Polyploidization resulting from an abnormal cell cycle, however, puts cells at risk for aberrant mitotic divisions and for subsequent mitosis with multipolar spindles, which eventually lead to aneuploidy. In conditions of abnormal DNA replication, sister-chromatid nondisjunction, mitotic spindle dysfunction or defective cytokinesis, cells are not able to proceed through a proper mitotic division. Regularly, many of these defects that result in abortive cell cycles trigger checkpoint responses that block cell-cycle progression or, in some cases, trigger apoptosis. However, checkpoint activation often produces only transient delays in cell-cycle progression. So, even if the initial insult persists, the possibility exists where some cells 'slip' past the arrest, exiting as a tetraploid from the defective cell division.[62] One of the important checkpoint responses to abnormal passage through mitosis is the activation of p53 in the next G1 phase.[68]

Disadvantages of Polyploidy

The obvious consequences of increasing the DNA content of a cell or organism include the disrupting effects of nuclear and cellular enlargement, the propensity of polyploid mitosis and meiosis to produce aneuploid cells and the epigenetic instability that affects gene regulation.[63] Increasing the genomic content of an organism usually increases cell volume, with a subsequent change in the spatial relationships between various components of the cell. Recent studies suggest that any imbalance of the ratio between the internal and the surface components of the nucleus has regulatory repercussions.[63] For instance, the volume of budding yeast cells increases linearly with

each extra chromosome pair.[62,69] In the nucleus, the precise localization of telomeric and centromeric heterochromatin ensures proper organization of chromosomes in the nucleus.[70] Lamins, which form a fibrous network that lines the inside of the nuclear envelope, interact with heterochromatin and have a function that is vital to the cell. This is demonstrated by the phenotypic effects of laminar abnormalities on human health.[71]

Chromosomes in polyploid cells of Arabidopsis thaliana have a greater mobility within the interphase nucleus relative to chromosomes in diploids, due to loss of the nuclear substructure that normally restricts chromosome movement.[70]

Most interestingly, when compared with haploids or diploids, tetraploid budding yeast cells have significantly increased rates of chromosome loss and recombination.[72] They also show increased sensitivity to gamma-irradiation and to other DNA-damaging agents.[62] Likewise, polyploid fission yeast undergoes chromosome missegregation at a high frequency.[73] Also, p53 null tetraploid cells are highly competent to induce tumors in nude mice. Polyploidization sensitized cells to genotoxic stress imposed by ionizing radiation and topoisomerase inhibitors.[74] These findings raise the possibility that an increase in ploidy generally impairs genomic stability.[61]

Tetraploidy Checkpoint Theory

Several views exist on the possible fate of polyploid cells. In the best case scenarios, multipolar mitosis of tetraploid cells can lead to the formation of diploid cells through a poorly understood process, known as 'reduction mitosis'. Alternatively, some cells 'adapt' to multiple centrosomes by clustering them at the spindle poles. This allows a bipolar mitosis to occur, which seems to progress normally. This mechanism is employed by many cancer cells as a way to avoid mitotic catastrophe.[75,76]

Another view suggests that tetraploid cells undergo cell cycle arrest via the so-called p53-mediated 'G1 tetraploidy checkpoint', which can then trigger apoptosis.[77,78] However, recent doubts about the existence of this checkpoint came from studies of cancer cell lines treated with mitotic spindle inhibitors. A proportion of these cell populations escape the mitotic arrest and enter into a "G1-like state" with a 4N set of chromosomes. This phenomenon is known as 'mitotic slippage'. If these tetraploid cells have functional p53, they arrest in this G1 phase.[44] However, p53-deficient cells progress through the next S-phase, undergo an abnormal mitosis and become aneuploidy.[79] It was initially considered that p53 might directly monitor the ploidy status of cells, perhaps via DNA content or centrosome number.[77] However, it was later shown that the high concentrations of spindle inhibitors (e.g., cytochalasin B) that were used in these experiments also caused DNA damage and that was the actual cause for inducing a p53 response. When repeated with lower but still effective doses, tetraploid cells formed, but did not arrest in G1 and instead showed normal cell cycle progression despite the p53 presence.[80,81] In sum, these studies concluded that mammalian cells do not possess a "tetraploidy" checkpoint, meaning that an abnormal chromosome number is not the direct trigger of the checkpoint response.[80,81]

The p53-dependent cell-cycle arrest of tetraploid cells shares features with the p53-dependent G1 arrest upon DNA damage. For instance, in both cases cell-cycle arrest coincides with induction of the CDK inhibitor p21 and hypophosphorylated retinoblastoma protein (Rb).[75,82] Furthermore, tetraploid cells that lack either p21, Rb or p53 all fail to arrest in G1 and proceed into an aberrant cell division.[62] Not without significance, p53 and Rb are the most frequent tumor suppressors functionally inactivated in human cancers. Disruption of the cytoskeleton by failed cytokinesis, abnormal spindle geometry in tetraploids,[80] or redistribution of nuclear proportions and lamins by increased DNA content[71] were also proposed as potential p53 activators. Thus, although its precise trigger is unknown, p53 prevents abnormal polyploid cells to occur in vitro as well as in vivo. The polyploid/aneuploid cells that arise from multipolar mitosis are rapidly eliminated in cells that contain p53.[61] Moreover, p53 null mice have 23% 4N cells in the pancreas compared with 7% in wild type mice.[28] Also, disabling p53 by a pancreas-specific SV40 T-antigen produced >45% polyploid pancreatic cells.[28] Thus, it will be important to establish the degree to which the antipolyploidization effect of p53 contributes to cancer suppression.

Agonists and Antagonists of p53 Function in Genome Stability

a. Mice deficient in genes important for telomere function, DNA damage checkpoint activation and DNA repair (both nonhomologous end joining (NHEJ) and homologous recombination) allow proliferation of cells with damaged DNA and a high degree of genomic instability.[83] The majority of these mice present with developmental problems or even embryonic lethality, all attributable to the activation of p53 in response to DNA damage signals, followed by induction of apoptosis. The developmental abnormalities are rescued by codeleting p53, but with a price: mice predisposed to genomic instability and lacking p53 are highly tumor-prone. For instance, the lethality of mutations in genes for DNA ligase IV or XRCC4 can be relieved by a mutation in p53 or ATM, but the double-knock-out mice develop T-cell lymphomas at a very early age.[47,84,85] These latter mouse models prove that p53-mediated apoptosis is an essential tumor suppressor mechanism to eliminate cells that are genomically unstable.[83]
b. Msh2 and its heterodimeric binding partner Msh3 are necessary for removal of nonhomologous tails during recombination.[86] In vivo, combined loss of Msh2 and p53 leads to embryonic lethality of female mice and to synergistically increased tumorigenesis in males on a C57BL6J background.[87] Drug-induced polyploidization studies in MEFs revealed that, while $Msh2^{-/-}$ MEFs showed no increase in the 8N population compared with WT MEFs, the $p53^{-/-}$ $Msh2^{-/-}$ MEFs showed a clear increase in cells with an 8N DNA content, over and above that seen in MEFs deficient in p53 alone. On a larger scale of DNA repair, it is possible that p53 monitors unresolved or aberrant recombination structures and allows cells to mend such DNA structures. However, when the signal from mismatch repair proteins is missing, these cells are not able to complete mitosis. Moreover, when p53 is also missing, cells may aberrantly re-enter S-phase. Thus, polyploid cells might appear from a combined defect in DNA repair and checkpoints.[88]
c. Lats2: The tumour suppressor Lats2, which is localized at the centrosome during a normal cell cycle, interacts with and inhibits the Mdm2 E3 ligase activity and thereby promotes p53 activation in cells with mitotic spindle defects. The Lats2-Mdm2 interaction occurs specifically when centrosome function is disrupted. Moreover, RNAi knockdown of Lats2 in cells that lack p53 function leads to accumulation of polyploid cells after exposure to nocodazole. However, if p53 is activated, proliferation of these cells is prevented. Thus, p53 and Lats2 cooperate via Mdm2 in preventing tetraploidization upon spindle defects.[89]
d. Wnt-1 is a member of a family of cysteine-rich, glycosylated signaling proteins that intervene in diverse developmental processes ranging from the control of cell proliferation, adhesion, cell polarity, to the establishment of cell fates. Alterations of Wnts are associated with carcinogenesis. Wnt-1 transgenic mice crossed into $p53^{-/-}$ nullizygosity develop mammary tumors with increased genomic instability, aneuploidy, amplifications and deletions.[18]
e. p73 is another member of the p53 family that contributes to the maintenance of genomic integrity. Mouse cells that harbor deletions in both p53 and p73 are marked by a higher degree of polyploidy and aneuploidy than the one observed in p53 null cells. Its role is detailed in the next part.

Introduction to p73

20 years after p53 was discovered, two structurally similar genes—p63 and p73—were discovered and placed into the same family. Initially thought to be tumor suppressors like p53, these two proteins proved to have a more complicated and intriguing behavior. Structurally, p63 and p73 have in common with p53 an amino-terminal transactivation domain, a highly conserved central DNA binding domain and a carboxy-terminal tetramerization domain. In overexpression studies, p63 and p73 can function as sequence-specific transcription factors that activate expression of genes containing p53-binding sites (like Bax and p21).[90,91] Moreover, tumor-associated stress signals (i.e., deregulated oncogenes and DNA damage) that activate p53 also induce p73.[91]

However, p53 and p73 are not functionally equivalent in vivo. While p53 plays a clear role in tumor suppression, p73 has long eluded efforts to place it into a defined category of cancer biology. Indeed, p53 null mice are highly tumor-susceptible but show no major developmental defects. In contrast, p73-deficient mice show defects in neuronal development and immune function, with the majority of animals dying within 2 months after birth due to chronic infection.[92] p63-deficient mice show epithelial defects with absence of skin, hair, truncated or absent limbs, craniofacial malformations and perinatal death due to desiccation.[93] Although p73 null mice were originally reported as not tumor prone,[92] a more recent re-analysis of ageing $p53^{+/-}p73^{+/-}$ mice (as well as a small number of $p53^{+/+}p73^{-/-}$ and $p53^{+/+}p73^{+/-}$ mice) did reveal an elevated incidence of microscopic carcinomas, T-lymphomas and sarcomas and increased metastatic ability, compared to $p53^{+/-}$ mice. Moreover, these tumors underwent loss of heterozygosity and loss of the remaining wtp73 allele. Thus, heterozygous loss of p73 increases the p53-dependent tumor phenotype in severity, frequency and breadth of tumor spectrum.[94] The latter result suggests that p73 acts as a tumor suppressor in certain tissues.[94] In the same study, an increase in tumor burden and spectrum was noticed in ageing $p63^{+/-}$ or $p63^{+/-}$ $p53^{+/-}$ mice.

On the other hand, p63 and p73 do not contribute to gamma irradiation-induced p53-mediated T-cell lymphoma suppression in vivo.[95] Moreover, in a different p63 KO mouse strain, Mills et al obtained a different result: no signs of malignancy, but accelerated aging instead.[96]

The story gets even more complicated in the analysis of human tumors. Unlike the clear picture that p53 offers with mostly inactivating mutations or rarely deletions in more than 50% of human cancers, p63 or p73 inactivating mutations are rarely found in human tumors.[97,98] One major reason for the lack of clear interpretability lies in the complex gene loci of p63 and p73, which produces two classes of isoforms with opposing activities. Thus, splice variants of p63/p73 exist that lack the N-terminal transactivation domains (ΔN p63/p73) and may function to interfere with the activity of their full-length counterparts (TA p63/p73).[91,99] Splice variations in the C-terminus adds another layer of isoforms (named α, β, γ, etc.) in the already extensive family.[100] The longest α variant of p63 and p73 contains a sterile-α motif (SAM), a known protein-protein interaction domain.[101]

Thus, multiple primary tumor types and tumor cell lines overexpress these genes and often concomitantly the TA as well as the reportedly oncogenic ΔNp isoforms (see below).[100,102] Numerous studies highlight the oncogenic potential of ΔNp63 in skin, the main isoform grossly overexpressed in human squamous cell carcinoma, including its clinical correlation to poor prognosis.[103] However, other tumor types (mainly some lymphomas and leukemias) show loss or reduced levels of p63 and/or p73.[98]

Further complicating the picture is a potential interference with p73 activity by mutant forms of p53, which might contribute to cancer development in vivo.[22,23,104]

p73 Functions

Although cancer cells deficient for p53 are more resistant to chemotherapy, they are still responsive to drugs, suggesting that other pro-apoptotic pathways are also involved. One of these rescue pathways might be mediated by the activation of p73. Thus, the majority of studies on p73 focused on its pro-apoptotic role. Based mostly on overexpression studies, it is thought that TAp73 has p53-like functions, while ΔNp73 isoforms have an opposing inhibitory role. When ectopically overexpressed, TAp73 can replace p53 in various cancer cell lines and induce apoptosis, cell cycle arrest and DNA repair by activating effectors like Bax, p53AIP1,[105] p21, GADD45, 14-3-3σ and p53R2.[106] However, while common promoters for p53 and p73 are numerous, differentially sensitive genes also exist.[107]

TAp73 participates in apoptosis and growth suppression in p53 null cells in response to DNA damage (chemotherapeutic drugs or γ-irradiation) or oncogenic stress (E2F1, cMyc, E1A).[107] In response to cisplatin, the apoptosis-inducing function of p73 is regulated by the c-Abl kinase and the mismatch-repair system.[108] Moreover, as part of normal T-cell development and selection, E2F1-p73 pathway induces cell death in response to T-cell receptor activation.[109] Consistently, in

radiation-induced mouse T-cell lymphomas, the p73 locus undergoes LOH in 33% of the cases.[110] Flores et al showed transcriptional cooperation between p53 and either p63 or p73 in inducing apoptotic effector genes in E1A-expressing MEFs and primary neuronal cells.[111] In their experiments, adriamycin-induced death was dependent on the copresence of at least two of the family members. While the expression of p21 (cell cycle arrest related protein) was not changed, the expression of Bax, Noxa and PERP was suppressed in $p63^{-/-}p73^{-/-}$ MEFs that were still $p53^{+/+}$.

The Role of p73 in Genomic Stability

Using genetically defined primary MEFs, we recently showed that p73 indeed plays an intriguing and unique role in genomic integrity that is manifested when p53 is lost. Isolated p73 loss does not induce genomic instability but instead results in impaired proliferation, transformation and premature senescence due to compensatory constitutive activation of p53. Combined loss of p73 and p53 completely rescues these defects, but at the expense of markedly exacerbating genomic instability. This leads to a rapid increase in polyploidy and aneuploidy, markedly exceeding that of p53 loss alone. Constitutive deregulation of Cyclin-Cdk and p27/Kip1 activities and excess failure of the G2/M DNA damage checkpoint fuel the increased ploidy abnormalities, while primary mitotic defects do not play a causal role. Thus, interference with p73 function—in the absence of protective mechanisms provided by p53—markedly exacerbates polyploidy and random loss or gain of chromosomes.[112] Of note, in human tumors concomitant inactivation of p53 and p73 often co-exist.[102] Below are highlights of our results:

Combined Loss of p53 and p73 Leads to Excess Polyploidy and Aneuploidy

When assayed on the 3T3 protocol, WT and $p73^{-/-}$ MEFs retained diploid status throughout their lifespan until they senesced at passage 7 and 4, respectively (Fig. 1). On the other hand, $p53^{-/-}$ MEFs (SKO) show a gradual increase in hyperdiploid cells with passaging, while also preserving significant diploidy (Fig. 1).[28,68] In contrast, the majority of freshly isolated $p53^{-/-}p73^{-/-}$ (DKO) MEFs were already hyperdiploid and after only 5 passages, virtually all cells were tetraploid and octaploid (Fig. 1). By FACS quantitation at p5, DKOs showed 88% aneuploidy and only 12% diploidy, in contrast to SKOs with 41% aneuploidy and 59% diploidy. Since each passage corresponds to 3 population doublings, we calculated that at least 5% of DKO cells, but only 2.5% of SKO cells lost their diploid status with each round of cell division. Thus, DKO cells have twice the rate of polyploidization than SKO cells.

By SKY (Spectral Karyotype Analysis) analysis at p7, DKO cells had the lowest diploid populations (only 2% with 2n) (n = 20 chrom. in mouse cells), but the highest polyploidy and aneuploidy (98% with 4n + >4n and 36% with >4n) (Fig. 2A). In contrast, SKOs had an intermediate phenotype, while WT cells were diploid. Most strikingly, extreme cases of DKOs were readily detectable with cells containing >300-400 chromosomes, a phenotype never seen in SKOs of the same passage (Fig. 2B). Notably, freshly isolated thymoctes from a young, healthy DKO mouse already contained a small (6%) but definite subpopulation of triploid normal T-cells, in contrast to its age-matched $p53^{-/-}$ control that had none, supporting polyploidization in vivo and in another tissue (not shown).

Thus, loss pf p73 in normal young thymocytes in vivo may have an impact on genomic stability. Human lymphomas can be triploid or near triploid, see reference 114.[113] Most DKO MEFs also contained unequal chromosome numbers by SKY (aneuploidy), reflected by the broad spread obtained when metaphases were quantitated for individual chromosomes (Fig. 2C). However, chromosomal translocations were rare in SKO and DKO. Thus, while p73 is not sufficient to completely prevent polyploidy upon p53 loss, it clearly acts to prevent further genomic destabilization.

The Ploidy Defect Is Not Due to a Mitotic Defect but a Failure of Premitotic Mechanisms

Polyploidy can be caused by several means that uncouple DNA replication from mitotic completion.[40] We therefore scrutinized the mitotic competence of DKOs, but found no intrinsic mitotic defects. First, concerning centrosome hyperamplification (2 being normal), DKOs had a

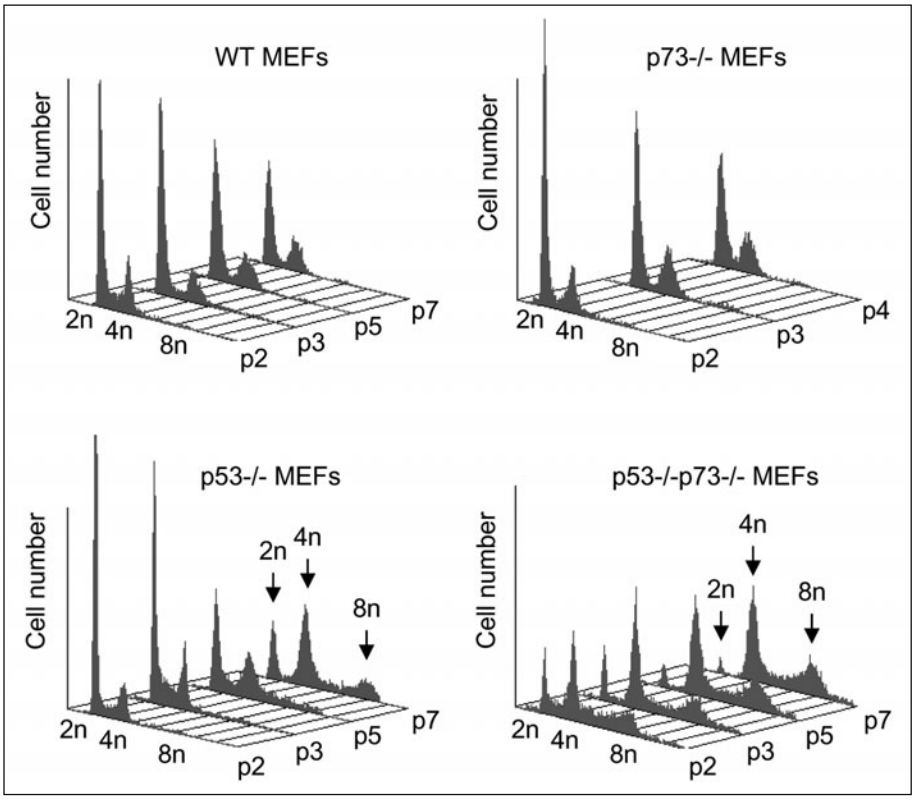

Figure 1. DNA histograms of freshly isolated WT, $p73^{-/-}$, $p53^{-/-}$ and DKO MEFs passaged on the 3T3 protocol. Number of passages in culture is indicated on the right. Ploidy (2n, 4n, 8n) is indicated.

better, rather than a worse phenotype compared to SKOs (Fig. 3A). Second, p7 DKOs were mainly mononucleated and had the lowest number of cells with two or more nuclei (Fig. 3A). Third, DKOs, when forced to undergo chromosomal segregation, showed no mitotic defects. To this end, cells were synchronized by mitotic spindle inhibitor nocodazole (which induced mainly tetraploidy in SKOs and tetraploidy and octaploidy in DKOs) and then released into media containing a G1/S roadblock (imposed by L-mimosine, hydroxyurea (HU) or aphidicolin) (see Fig. 3B). Both genotypes—since they could not go forward—went straight back through a proper mitosis within 4 hrs after release and regained their original ploidy. This was confirmed by direct visualization of chromosome condensation that follows real-time mitotic progression via GFP-tagged histone H2B (Fig. 3B). Thus, p73 loss does not cause defects in the centrosome duplication cycle, mitotic spindle checkpoint, karyokinesis and cytokinesis.

Excess Failure of the G2/M DNA Damage Checkpoint and Constitutive Deregulation of Cyclin-Cdk and p27/Kip1 Fuel Aberrant Ploidy upon p73 Loss

CDK-Cyclins are the driving force of the cell cycle. To determine if and where in the cycle DKOs are defective, we looked for CDK deregulation in cycling DKOs only subject to endogenous DNA damage (endogenous ROS modifies ~ 20,000 bases/day/cell).[114] Compared to SKOs, DKOs indeed have a higher and longer peak of Cyclin E-Cdk2 activity in early S and an elevated

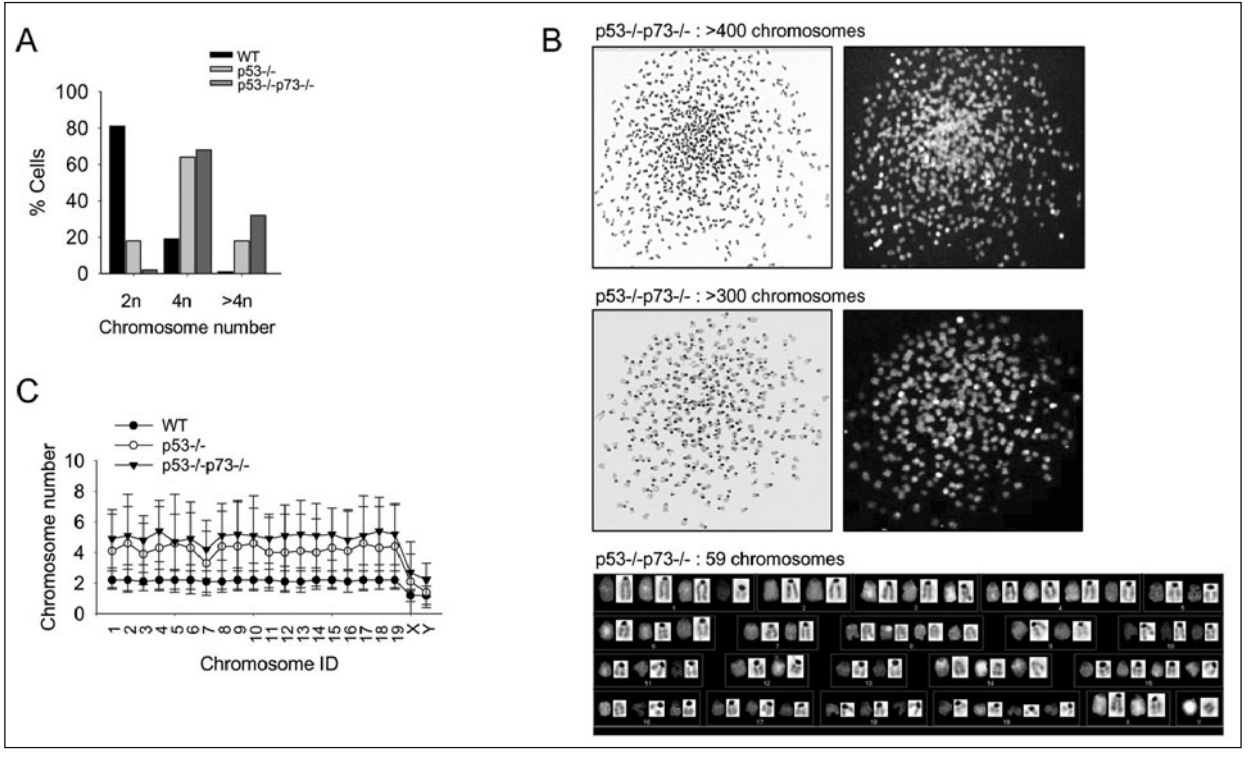

Figure 2. A) Karyotype analysis of >100 random metaphases each from MEFs of the indicated genotypes at passage 7. Note lack of diploidy (2n) and highest degree of polyploidy and aneuploidy (4n plus >4n) in DKO MEFs. B) Polyploidization of DKO cells at passage 7, as shown by Spectral Karyotyping (top). Most cells contain unequal chromosome numbers, indicating aneuploidy (bottom). C) Individual chromosome contents of WT, p53$^{-/-}$ and DKO MEFs at passage 7. Over 100 moderately polyploid metaphases were analyzed for each genotype. Error bars represent the standard deviation.

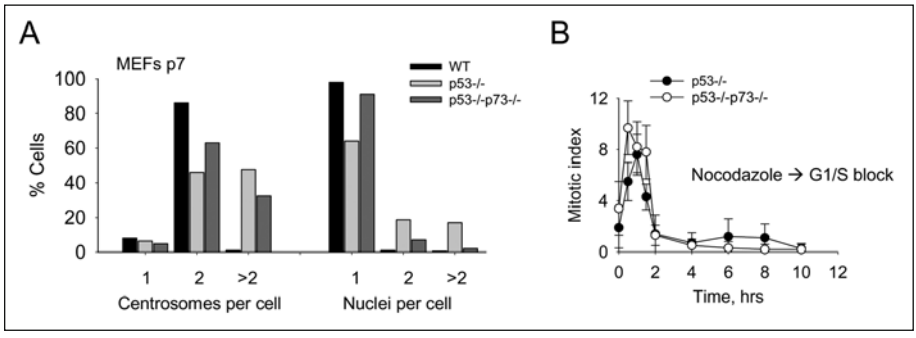

Figure 3. A) Total number of centrosomes (determined by γ-tubulin staining) and nuclei (determined by Hoechst staining) per cell in WT, $p53^{-/-}$ and DKO cells at passage 7. For each genotype, >500 random cells were counted. DKO cells have an improved centrosomal phenotype and are predominantly mononucleated. B) DNA histograms of $p53^{-/-}$ and DKO MEFs at passage 7 treated with nocodazole for 12 hrs, washed out and then released into media containing the G1/S blocker L-mimosine. Both genotypes recover their original ploidy within 4 hours (i.e., 2n for $p5^{-/-}$ MEFs and 4n for DKO cells), indicating that they had passed through a proper mitosis.

Cyclin A-Cdk2 activity in late S (Fig. 4A). Most importantly, DKOs show uncoordinated and premature collapse of Cyclin B-Cdk1 activity, leaving insufficient levels to go through mitosis. We identified that the source of CDK deregulation are not Cdks or Cyclins themselves but constitutively elevated *p27/Kip1* (Fig. 4B). p27 acts as a positive regulator of early phase Cyclin E-Cdk2[115,116] but a negative regulator of late phase Cyclin B1-Cdk1.[117] DKOs have constitutively elevated p27 levels in all phases of the cycle (Fig. 4B) and more p27 bound to Cyclin B-Cdk1 in G2M (not shown). As a consequence, since entry into mitosis depends on sufficient Cyclin B-Cdk1 activity,[118] more DKOs than SKOs are blocked from entering mitosis. Thus, many more DKOs skip mitosis and are reset from G2 back to G1, enabling another round of replication.[40,118] Together with the deregulated S phase, these events contribute to twice the polyploidization rate of p73-deficient DKOs. Of note, deregulated p27Kip1 and Cyclin E drive polyploidization of normal tissues in vivo. E.g., constitutively elevated p27 levels in Skp2 null mice, a component of the SCF ubiquitin-proteolysis system which degrades p27, causes excess polyploidization in many tissues and this is exclusively due to their p27 abnormality, since $p27^{-/-}$ $Skp2^{-/-}$ double knock-out mice rescue this phenotype completely.[119,120] Also, Cyclin $E^{-/-}$ embryos lack normally polyploid megakaryocytes and trophoblasts. Conversely, overexpressed Cyclin E drives non-endoreplicating megakaryoblasts into endomitosis.[121,122] Of note, in our system, retroviral overexpression of Cyclin E and its corresponding Cdk2 in $p53^{-/-}$ cells altered (impaired) the G2 checkpoint to resemble the one of DKO cells (not shown).

The G2M checkpoint is critical because it is the last barrier before mitotic division for cells with wrongly replicated or damaged DNA. Also, polyploid cells are prevented from re-entering mitosis through engagement of the G2M checkpoint.[44,123] Conversely, a defective G2 checkpoint in itself can cause polyploidy and aneuploidy after DNA damage, because cells with inadequate DNA repair in G2 proceed into a catastrophic mitosis, where massive bridging prevents chromosome segregation from which they will exit as polyploid/aneuploid G1 progeny.[44,123] p73-proficient SKOs respond to the DNA damaging G2 inhibitors adriamycin and VM26 with a robust S/G2 block. In contrast, DKOs fail to mount an effective G2M checkpoint and instead continue cell cycle progression, accumulating a large proportion of octaploid cells that enter mitosis (Fig. 5A,B). Conversely, retroviral re-introduction of TAp73α, the major TAp73 isoform in MEFs, re-establishes an efficient S/G2 arrest in VM26- and adriamycin-treated DKOs (Fig. 5C).

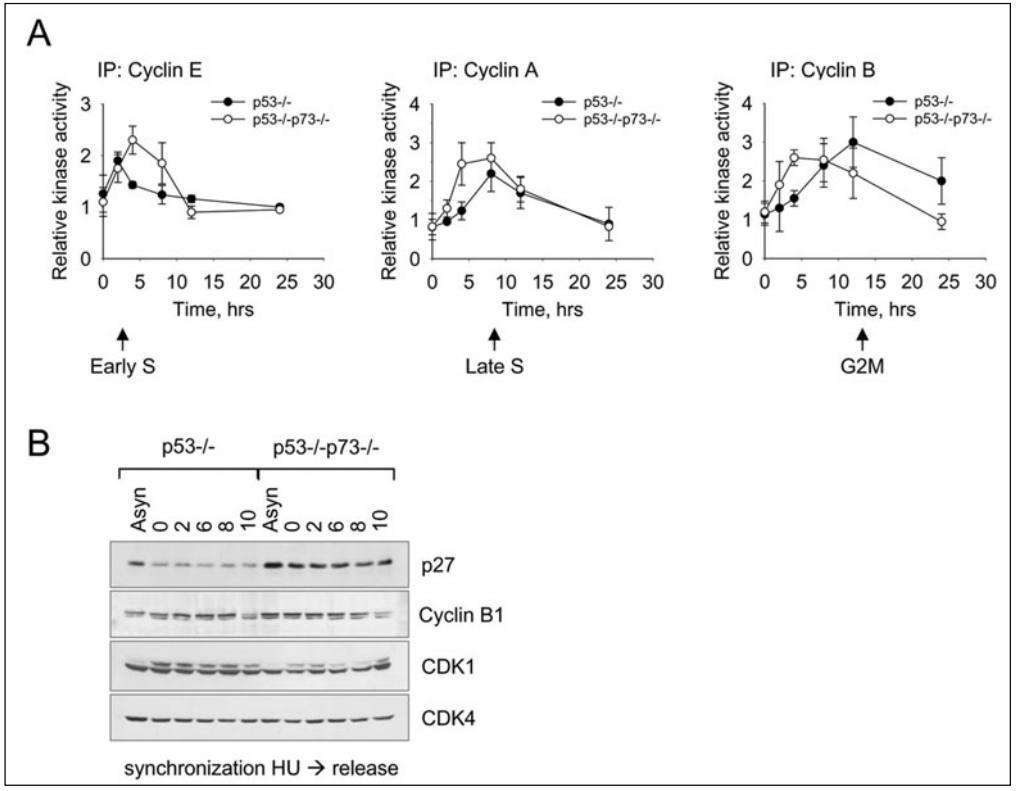

Figure 4. A) In vitro kinase assays of immunoprecipitated Cyclin E-, Cyclin A- and Cyclin B-associated Cdk2 and Cdk1 activity in synchronized $p53^{-/-}$ and DKO MEFs. Cells were analyzed at the indicated times after release from G1/S-phase block with hydroxyurea. Three experiments each, error bars represent standard deviation. B) $p53^{-/-}$ and DKO MEFs were synchronized at the G1/S border by hydroxyurea, Protein lysates prepared at the indicated hours were immunoblotted. Continuously growing (Asyn) MEFs are also shown. DKO cells exhibit constitutively elevated p27 levels.

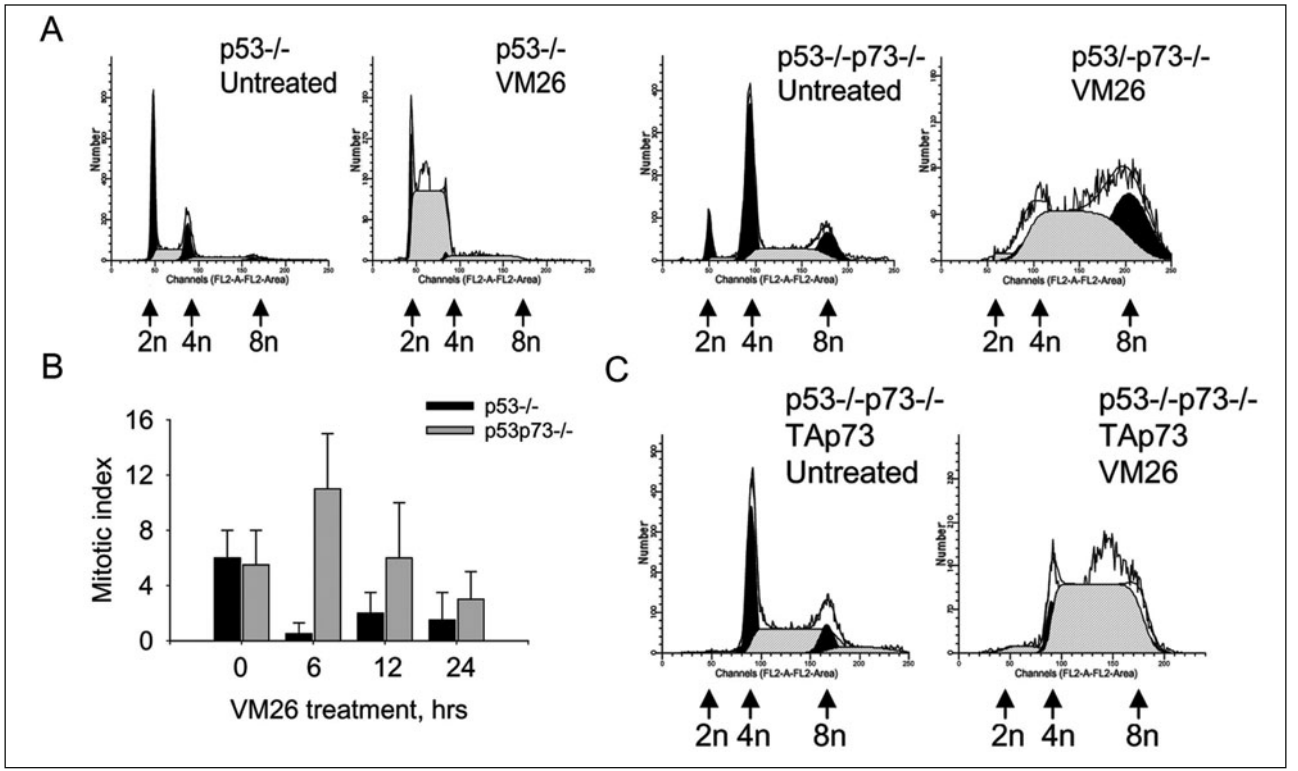

Figure 5. A) DNA contents of $p53^{-/-}$ and DKO MEFs incubated for 20 hrs in the absence or presence of VM26. Note the baseline difference in ploidy between untreated $p53^{-/-}$ and DKO MEFs. B) More DKO than $p53^{-/-}$ cells enter mitosis despite DNA damage. Mitotic indices were determined by real-time quantitative chromosome evaluation of H2B-GFP-expressing $p53^{-/-}$ and DKO cells, treated with VM26 for up to 24 hrs. At least 500 random cells were scored for each time point. Error bars represent standard deviation. C) TAp73 re-establishes an efficient S/G2 arrest and rescues the G2M checkpoint defect of DKO cells. Cell cycle profiles of DKO MEFs expressing ectopic TAp73α in the absence or presence of DNA damage.

The G2M checkpoint is controlled through the ATM/Chk/Cdc25 pathway that blocks Cdk activity.[124] Indeed, while SKO dropped their Cdk1 activity upon VM26, DKOs increased and maintained high levels of Cdk1, enabling DKOs with misreplicated and damaged DNA to exit G2M and enter mitosis (not shown). In contrast, dominant negative mutants of Cdk2 (D145N), Cdk1 (D146N) or Cdc25A (C430S), or pharmacologic Cdk1/2 and Cdc25 inhibitors significantly blocked further polyploidization of DKOs upon DNA damage (data not shown). In sum, this indicates that p73 activates a G2M DNA damage checkpoint.

Conclusion

The exact mechanism by which p73 influences the G2M checkpoint, including its effects on Chk1/Chk2 activity, requires further elucidation. Recent studies identified CDK inhibitors p21Cip1 and p57Kip2 as targets of p73 regulation.[125,126] Although p73 affects expression of these cell cycle regulatory proteins, it is unlikely that variations in the expression levels of a limited number of genes could account for the observed dramatic phenotype resulting from p73 loss. Being a chromatin accessibility factor, p53 contributes to the DNA repair processes by both transcription-dependent and transcription-independent mechanisms.[42,53] Likewise, it is conceivable that transcription-dependent and transcription-independent mechanisms also contribute to the p73-loss-induced phenotype.

Indeed, comparing cell cycles of primary cells, p73-deficient DKO MEFs exhibit a significant increase of DNA replication over $p53^{-/-}$ MEFs, associated with a constitutively deregulated S-phase Cdk2 activity. Recent studies underscore a critical role of the Rb tumor suppressor in maintaining chromatin structure and in DNA-damage checkpoint signaling in S-phase.[127-129] Notably, Rb-null rodent cells are polyploid even in the presence of wild-type p53.[127,129] The mechanism of this Rb activity remains unknown, although it appears to be E2F-independent and therefore transcription-independent.[129] Because a constitutively elevated Cdk2 activity would result in rapid Rb inactivation with dire consequences for the genomic stability of cells, a potential role of Rb in generating the p73-deficient phenotype needs to be addressed by future genetic and biochemical studies. In our study, reintroduction of TAp73α—but not of ΔNp73α—re-established an efficient G2M arrest upon adriamycin and VM26 in DKO MEFs. However, both TA and ΔN isoforms in isolation had minimal effects on polyploidization of DKO cells when passaged on the 3T3 protocol (data not shown), suggesting that a combination of isoforms in the right proportion might be necessary to completely rescue the DKO phenotype. The possible involvement of each of the p73 isoforms in maintenance of genomic stability and tumor suppression requires further investigation. The importance of p73 for genomic stability in the context of human tumors can only be addressed in correlational studies between expression of different p73 isoforms in human tumors and their degree of polyploidy/aneuploidy. Finally, generation of isoform-specific p73 knock-in mice should give insights to the most stringent question: what is the contribution of each isoform to tumor suppression/development? Answering this question will also contribute to the ultimate goal of studying p73: modulation of p73 levels in human tumors in order to activate p73-dependent apoptosis and cell cycle checkpoints.

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Chapter 6

Centrosomes, Polyploidy and Cancer

Anette Duensing and Stefan Duensing*

Abstract

Cancer cells are frequently characterized by ploidy changes including tetra-, poly- or aneuploidy. At the same time, malignant cells often contain supernumerary centrosomes. Aneuploidy and centrosome alterations are both hallmarks of tumor aggressiveness and increase with malignant progression. It has been proposed that aneuploidy results from a sequence of events in which failed mitoses produce tetra-/polyploid cells that enter a subsequent cell division with an increased number of centrosomes and hence with an increased risk for multipolar spindle formation and chromosome missegregation. Although this model attempts to integrate several common findings in cancer cells, it has been difficult to prove. Findings that centrosome aberrations can arise in diploid cells and the uncertain proliferative potential of polyploid cells suggest that alternative routes to chromosomal instability may exist. We discuss here recent results on centrosome biogenesis and the possible link between ploidy changes, centrosome aberrations and cancer.

Introduction

Genomic instability is a hallmark of cancer and tumor cells frequently contain grossly altered genomes with a tetra-, polyploid or aneuploid chromosomal content.[1] In addition to ploidy changes, the vast majority of cancers harbor tumor cells with abnormal centrosome numbers.[2,3] Centrosomes function as major microtubule organizing centers in animal and human cells and contribute to the organization of the mitotic spindle.[4] Aberrant multipolar mitoses have been long recognized as hallmarks of cancer. Whether centrosome aberrations are a cause or consequence of genomic instability, however, is still under debate; it is very likely that both are correct.[5-7]

The incidence of centrosome aberrations and aneuploidy in many advanced stage malignancies has led to the general belief that abnormal centrosome numbers are a consequence of tetra-/polyploidy after a failed mitosis. Aneuploidy would be a result of a two-step process in which the accumulated centrosomes in tetraploid cells increase the risk of chromosome missegregation when cells re-enter the cell division cycle.[6,8] This model attempts to integrate several key findings in tumor cells; however, the question whether cells that have failed cell division once can re-enter mitosis and produce viable and genomically unstable daughter cells has been difficult to prove. This is exemplified by the finding that a fusion of two diploid cells to induce tetraploidy does not necessarily provoke aneuploidy[9-11] and that inactivation of p53 is needed to prompt such outcome.[12] These results leave the possibility that polyploidy and aneuploidy may develop independently, at least at early stages of neoplastic progression when p53 function is often normal. Moreover, tumors can contain numerous cells with aberrant centrosome numbers without signs of ongoing genomic instability[13] whereas, on the contrary, genomically unstable tumors can contain normal centrosome numbers.[14] Centrosome accumulation after failed mitosis is not the only pathway that

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can lead to aberrant centrosome numbers and oncogenic stimuli that rapidly disrupt the centrosome duplication cycle in otherwise normal diploid cells have been identified, for example the human papillomavirus Type 16 (HPV-16) E7 oncoprotein.[15] In addition, there are reports showing that the frequency of centrosome-induced spindle abnormalities in metaphase cells may not be mirrored by a similar increase of such alterations in ana- or telophase, suggesting that many cells undergoing multipolar mitosis are unlikely to ultimately produce daughter cells.[16]

This chapter describes distinct mechanisms leading to centrosome amplification and discusses their potential impact on genome integrity. Mechanisms leading to ploidy alterations and possible consequences with respect to centrosome-mediated chromosomal instability are highlighted.

The Centrosome Duplication Cycle

Centrosomes function as major microtubule-organizing centers in most animal and human cells. During mitosis, centrosomes contribute to the organization of the mitotic spindle. Centrosomes have been implicated in various other cellular processes, many of which involve cell polarization.[17] It is noteworthy that centrioles, the core forming units of centrosomes, have important functions in the formation of sensory and motile cilia by forming basal bodies.[18]

Centrosomes typically contain two centrioles, short microtubule cylinders that are embedded in pericentriolar material (PCM). Nondividing cells contain a single centrosome which duplicates prior to mitosis in synchrony with the cell division cycle.[19] The morphological changes that occur during this process are well characterized; the molecular basis of centrosome duplication, however, is much less well understood. One molecular player that has recently been identified is separase. This protein is involved in the earliest steps of centrosome duplication, the movement of the two centrioles from a perpendicular arrangement to a near parallel position during G1 phase of the cell division cycle (centriole disengagement).[20] During the subsequent S phase, single daughter centrioles form in close proximity to the pre-existing centrioles (mother centrioles). This process involves regulation by Polo-like kinase 4 (PLK4) and HsSAS-6.[21,22] In addition, cyclin-dependent kinase 2 (CDK2) has been implicated in the regulation of centriole duplication. However, CDK2-deficient cells have no apparent centrosome anomalies[23] but it is possible that compensatory CDKs maintain normal centrosome duplication. Nonetheless, CDK2 is indispensable for oncogene-induced centrosome overduplication.[23]The precise role of CDKs and whether they function directly at the centrosome awaits further clarification. At the end of G2 phase, each maternal centriole has nucleated a single daughter centriole and the two centriole pairs separate in late G2. The two centriole pairs now start to move to the opposite spindle poles, which is accompanied by a massive increase of their microtubule-nucleating capacity. The centrosomes then participate in mitotic spindle formation and its three-dimensional orientation. Although mitotic spindles can form without centrosomes,[24] there is evidence that the presence of centrosomes is important for proper completion of cell division and the generation of viable progeny.[25]

In the nematode *Caenorhabditis elegans*, a cascade of events that controls centrosome duplication has been characterized.[26-28] The process is initiated by CDK2-mediated recruitment of the SPD-2 protein to centrioles. SPD-2 is then involved in the recruitment of the ZYG-1 kinase, which in turn recruits two additional proteins, SAS-5 and SAS-6. The latter two have been implicated in the formation of the central tube, the first step of pro-centriole formation.[29] Next, the SAS-4 protein mediates the assembly of microtubules onto the central tube (*C. elegans* centrioles consist of singlet microtubules and not of triplets as in mammalian cells). Although it is conceivable that higher organisms have a more complex network of proteins that regulate centriole biogenesis, recent results suggest a surprisingly high level of conservation.[21-22]

Aberrant Centrosome Numbers in Cancer Cells

Numerical and/or structural centrosome abnormalities have been detected in virtually all human cancers.[30] Many cancer cells contain more than the normal one or two centrosomes and such aberrations are easily detected using antibodies against the pericentriolar material such as γ-tubulin.[31] Structural centrosomal aberrations are typically recognized by an increase in centrosome size and

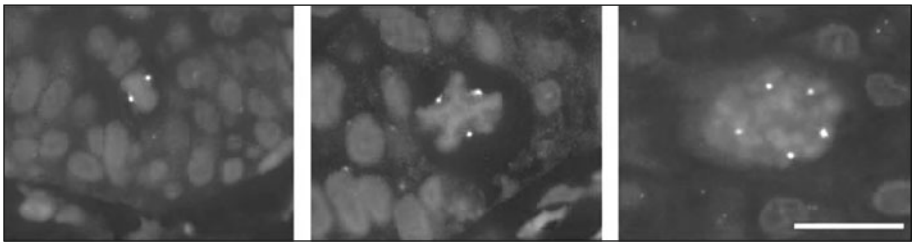

Figure 1. Multipolar mitoses are hallmarks of cancer. Bipolar mitotic spindle (left) in comparision to multipolar metaphases (middle, right) detected by immunofluorescence microscopic analysis of control tissue (left) or high-risk HPV-associated neoplasms (middle, right) for the PCM marker γ-tubulin (green). Note the increase of chromosomal material associated with both multipolar mitoses, a finding that commonly indicates a polyploidy/aneuploid chromosomal content. Chromosomes stained with DAPI. Scale bar indicates 50 μm.

an irregular shape and/or fragmentation of centrioles.[32] Together with centrosomal aberrations, abnormal multipolar mitoses are considered hallmarks of malignant tumors (Fig. 1) and were suggested as a potential source of genomically unstable tumor cells more than 100 years ago.[5] Several studies suggest that the frequency of centrosome aberrations in tumors correlates with increased aneuploidy and certain clinical characteristics that reflect tumor aggressiveness.[33-35]

Multiple Pathways Can Lead to Aberrant Centrosome Numbers: Studies Using Human Papillomavirus (HPV) Oncoproteins

Tumor viruses are elegant tools to explore basic mechanisms of cellular transformation and chromosomal instability because of the limited number of oncogenic proteins that they encode. Human papillomaviruses (HPVs) are the cause of cervical cancer and have also been implicated in the pathogenesis of squamous cell carcinomas of other anatomic locations such anal or oral carcinomas.[36] High-risk HPV types such as HPV-16 encode two major transforming oncogenes, E6 and E7. These oncoproteins function during the viral life cycle to promote efficient replication of the viral genomes.[37] Remarkably, they do so by subverting host cell tumor suppressor pathways that normally restrict DNA replication and that are also altered in the vast majority of nonvirus-associated malignancies.[38] Whereas a major function of the high-risk HPV E6 oncoprotein is to target the p53 tumor suppressor, the high-risk HPV E7 oncoprotein binds and inactivates the pRB tumor suppressor as well as the pRB family members p107 and p130 and interacts with a number of other host cell proteins.[39]

Both HPV-16 E6 and E7 can stimulate abnormal centrosome numbers in primary human cells when expressed under stable conditions.[15] In striking contrast, transient overexpression of HPV-16 E6 or E7 revealed that only the HPV-16 E7 oncoprotein can rapidly induce abnormal centrosome numbers when expressed for approximately 48 h. In contrast, HPV-16 E6 had no effect on centrosome numbers under transient conditions. Further analyses showed that the unique property of HPV-16 E7 to stimulate aberrant centrosome numbers within a short period of time was associated with an excessive formation of daughter centrioles.[40] Moreover, HPV-16 E7 was able to provoke numerical centrosome aberrations in morphologically normal, diploid cells and hence as a potential cause of cell division errors.[40] Recently it was found that HPV-16 E7 stimulates centriole overduplication through a pathway that involves the concurrent formation of more than one daughter centriole at a single maternal centriole (Fig. 2).[41] Interestingly, such phenotype is normally limited to multiciliated epithelial cells that produce numerous basal bodies during ciliogenesis.[42]

The HPV-16 E6 oncoprotein was found to stimulate abnormal centrosome numbers mostly in parallel with significant nuclear atypia including multinucleation and micronuclei.[40] There was a correlation between the degree of nuclear alterations and centrosome aberrations, indicating

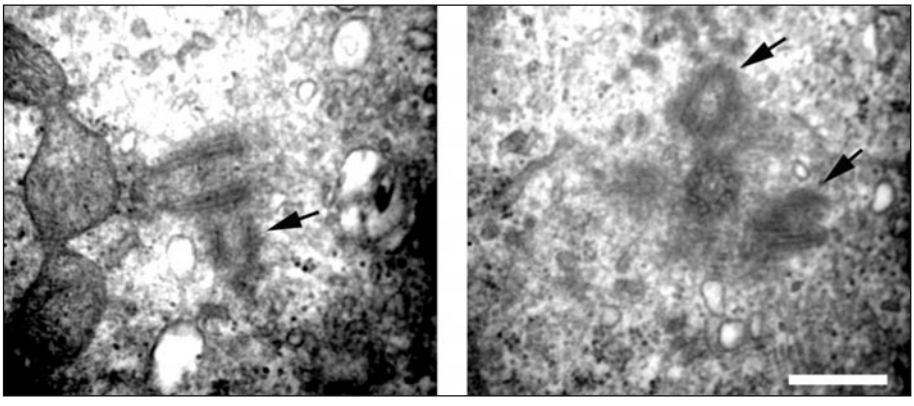

Figure 2. Centriole overduplication through formation of more than one daughter per maternal centriole. Electron micrograph of a normal mother-daughter centriole pair (left) and a mother centriole that nucleates the concurrent formation of two daughter centrioles (arrows). Such phenotype has been identified, for example, in cells expressing the HPV-16 E7 oncoprotein. Scale bar indicates 500 nm.

that these changes developed in parallel. Many of these cells were terminally growth arrested and therefore unable to produce viable progeny (see also below). It is hence likely that centrosome aberrations in HPV-16 E6-expressing cells develop as a consequence of genomic instability reflected by the gross nuclear changes and not as a potential cause as in HPV-16 E7-expressing cells. Moreover, the finding that nuclei of HPV-16 E6 expressing cells were frequently enlarged suggested that these cells were also polyploid. Based on these findings, we have proposed that distinct mechanisms can lead to aberrant centrosome numbers in tumor cells as outlined below. Importantly, the impact on genome integrity may vary depending on the mechanism and the cellular background of abnormal centrosome formation.[43]

Mechanisms of Centrosome Amplification in Tumor Cells

Primary Centrosome Overduplication

Primary centrosome overduplication should be considered when excessive numbers of immature centrioles are induced within a single cell division cycle. The discovery of proteins that specifically label mature centrioles such as ninein[44] or Cep170[45] has been instrumental in proving the existence of this mechanism. In addition, at least a fraction of such changes should occur in morphologically normal diploid cells. Whether oncogenic stimuli that rapidly induce supernumerary centrioles always trigger a concurrent formation of more than one daughter centriole in the presence of a single maternal centriole as recently reported for HPV-16 E7[41] remains to be determined. A similar phenotype has been reported in cells overexpressing Polo-like-kinase 4 (PLK4) and HsSAS-6.[21,22]Given the rapid induction of abnormal centrosome numbers in cells that have not yet acquired a highly abnormal phenotype, it is likely that primary centrosome overduplication increases the risk for cell division errors in subsequent mitoses.

Recently, loss of the CDK inhibitor $p21^{Cip1}$ was found to stimulate centriole overduplication in murine myeloblasts.[46] This finding, together with the results obtained with HPV-16 E7, raise the possibility that an impaired $p21^{Cip1}$-cyclin-CDK2-pRB signaling axis is a frequent stimulus for centriole overduplication.

In breast cancer, centrosome amplification in the absence of genomic instability has been reported. Intriguingly, aberrant centrosome numbers were detected independently of p53 inactivation and it is thus possible that certain oncogenic insults involved in breast carcinogenesis function as a trigger for primary centrosome overduplication.[47]

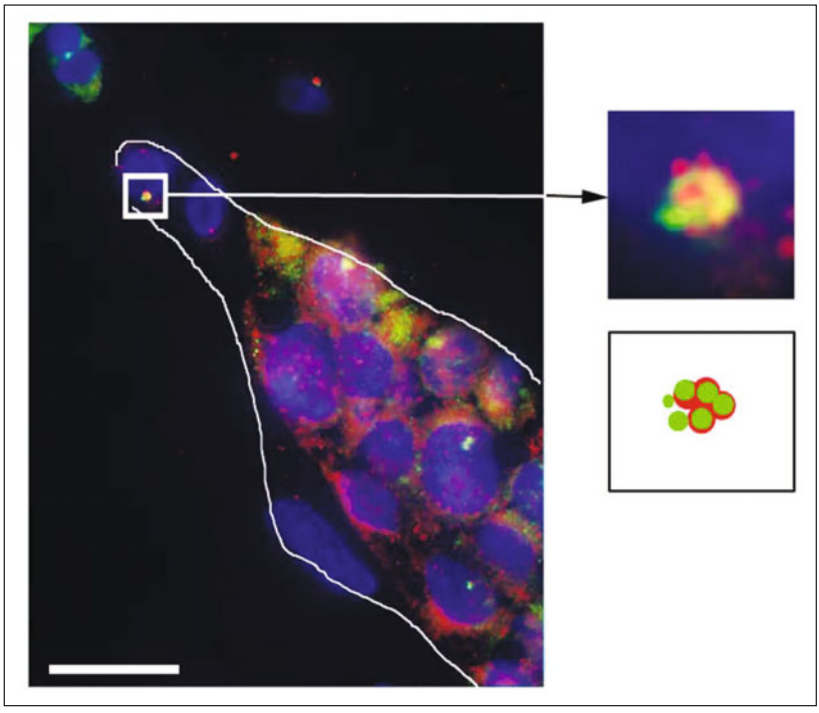

Figure 3. Centrosome accumulation in a malignant tumor. Immunofluorescence analysis of a high-risk HPV-associated squamous cell carcinoma for the PCM marker γ-tubulin (green) and a marker for mature maternal centrioles, Cep170 (red). Note the presence of multiple centrosomes that colocalize with Cep170, indicating an accumulation of mature mother centrioles (in the case of primary centrosome overduplication, only a single centrosome would be Cep170 positive since the supernumerary centrioles would be immature). Nuclei stained with DAPI. Scale bar indicates 25 μm.

Permanent Centrosome Accumulation

In contrast to a primary centrosome duplication error, centrosome accumulation is characterized by the generation of supernumerary centrosomes that does not involve uncontrolled centrosome synthesis but cellular insults that lead to an impaired segregation of centrosomes into daughter cells (for example, cytokinesis defects). The very nature of this mechanism makes it unlikely that centrosomes contribute to cell division errors. Although only long-term live cell imaging can provide an ultimate proof, there is circumstantial evidence that cells with centrosome accumulation have lost the ability to generate daughter cells. Human keratinocytes stably expressing the HPV-16 E6 oncoprotein were found to contain abnormal numbers of centrosomes together with a highly altered nuclear morphology, in particular multinucleation and micronuclei. Approximately one-third of these cells were positive when tested for senescence-associated β-galactosidase activity, indicating a permanent cell cycle arrest.[40] Interestingly, more than 40% of cells were still expressing the proliferation marker Ki67, indicating active DNA replication despite the presence of multiple nuclei.[40] It is likely that such cells undergo repeated rounds of DNA replication without producing daughter cells. HPV-16 E6 inactivates p53[48] and it is noteworthy that p53-deficient cells can also become multinucleated, in particular at later passage numbers.[49] Despite the fact that p53 can also more directly interfere with centrosome homeostasis,[50] the high frequency of p53 inactivation in human cancers suggests that centrosome accumulation may be the prevailing mechanism for supernumerary centrosomes in tumors (Fig. 3).

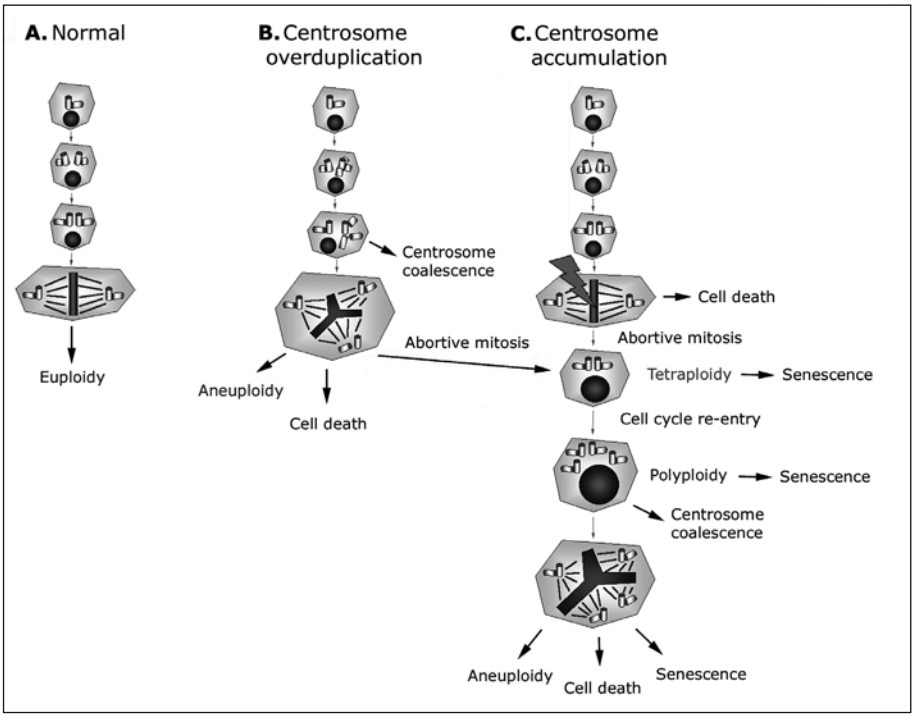

Figure 4. Ploidy changes and centrosome aberrations are in tumor cells. Normal centrosome duplication and bipolar mitotic spindle formation (A) in comparison to centrosome overduplication (B) and centrosome accumulation (C). A defect of the centriole duplication cycle itself (for example induced by the HPV-16 E7 oncoprotein) can lead to an overduplication of centrioles in otherwise normal diploid cells (B). In a subsequent mitosis, a multipolar mitotic spindle may form from which various outcomes can be envisioned. If viable daughter cells are produced, they may become aneuploid. However, the chromosomal alterations may also be detrimental, forcing cells into apoptosis. Another possibility would be that the spindle defects cause cells to adapt and re-enter a G1-like state with a tetraploid chromosomal content. It needs to be pointed out that centrosome aberrations per se have not been implicated in mitotic spindle checkpoint activation. However, prolonged activation of the mitotic spindle checkpoint (bolt of lightening) may lead to abortive mitosis and cells with a tetraploid chromosomal content and accumulation of supernumerary centrosomes (C). If such cells are able to re-enter the cell division cycle (promoted, for example, by p53 deficiency), they would not only replicate their DNA and become polyploid but also duplicate their centrosomes leading to an increased risk for multipolar mitoses. The outcome, again, would be aneuploidy (if daughter cells are viable) or cell death. Another possibility would be a permanent growth arrest and senescence. Note that centrosome coalescence can potentially alleviate centrosome-mediated cell division errors by formation of an essentially bipolar spindle despite the presence of multiple centrosomes.

More direct evidence for centrosome accumulation in cells unable to complete cell division stems from experiments in which $p21^{Cip1}$- or p53-deficient cells were exposed to DNA damage and then followed through mitosis by time-lapse videomicroscopy. Although such cells did enter mitosis, they were found to be unable to complete cytokinesis. Such cells were frequently binucleated and/or contained chromatin bridges,[51] similar to cells expressing the HPV-16 E6 oncoprotein.[52] Furthermore, these results highlight that identical oncogenic insults i.e., loss of $p21^{Cip1}$, may stimulate distinct pathways leading to aberrant centrosome numbers suggesting that

primary centrosome overduplication and centrosome accumulation may co-exist in cell populations and maybe even in the same cell.

Transient Centrosome Accumulation

The idea that cells with a diploid karyotype can become genomically unstable through an abnormal multipolar mitosis has raised concerns regarding cell viability since major chromosomal gains and/or losses in diploid cells may be detrimental. It has hence been proposed that cell division errors as a cause of genomic instability are more likely to occur in cells that enter mitosis with a tetraploid chromosome content. Tetraploid cells can arise through multiple mechanisms including endoreduplication of DNA, abortive mitosis, rereplication of DNA (i.e., re-initiation of DNA replication before completion of S phase) or cell fusion (see also below). It is conceivable that all these conditions lead to cells that contain more than the normal number of centrosomes (Fig. 4). One can imagine that re-entry into the cell division cycle of such cells is very likely to be associated with increased numbers of spindle poles. Whether cells are able to re-enter the cell division cycle may depend mainly on the p53 status but it is probable that other checkpoint proteins are also involved.[53] Possible consequences include multi- or tetrapolar spindle formation or spindles where two or more centrosomes function together as one spindle pole (coalescence).[54] It is noteworthy that centrosome coalescence could lead to more subtle changes of spindle function resulting in chromosome segregation defects that cells are able to cope with even when they are diploid. Examples of oncogenic stimuli that may lead to a transient centrosome accumulation, in particular in the absence of p53, are overexpression of Aurora-A or Polo-like kinase 1 (PLK1).[8] In addition, various other oncogenic insults such as inactivation of BRCA1, BRCA2 or SKP2[55-57]have been shown to cause centrosome amplification together with increased ploidy.

Aberrant Centrosome Numbers as a Consequence of Polyploidy—Implications for Genomic Instability in Cancer

Since ploidy alterations and numerical centrosome anomalies frequently coincide in tumor cells, the following paragraphs discuss different pathways to ploidy alterations, how they may affect centrosome numbers and the potential outcome regarding chromosomal instability.

Endoreduplication

Polyploidy per se is not pathological and several cell types in humans, including megakaryocytes or trophoblast cells, normally contain a polyploid chromosomal content. In addition, regenerative processes can increase the proportion of polyploid cells in certain tissues, for example in the liver. It is believed that nonmalignant polyploid cells have undergone rounds of endoreduplication of their genomes. During this process, DNA replication is not followed by a productive cell division. Megakaryocytes enter mitosis and form a mitotic spindle but do not complete cytokinesis.[58] Such cells can contain dozens of centrosomes and centrioles,[59] which underscores the notion that polyploidization can cause accumulation of multiple centrosomes. Daughter cells are usually not generated and it is, therefore, unlikely that endoreduplication increases the risk for chromosomal instability.

Abortive Mitoses

Cell cycle checkpoints are commonly disrupted in cancer cells, which may permit progression of cells beyond the G2/M checkpoint and into mitosis despite the presence of altered DNA or other cellular abnormalities. The mitotic spindle checkpoint is the major checkpoint that is active during mitosis and it monitors proper attachment of spindle microtubules to the kinetochores of mitotic chromosomes. The checkpoint becomes activated when spindle microtubules are not properly attached to kinetochores and delay anaphase onset. There is increasing evidence that other cellular insults including genotoxic stress can also delay or block anaphase entry.[60] The meta- to anaphase transition may hence function as a last line of defense to prevent the propagation of altered chromosomes. It is important to consider, however, what the fate of cells that have been arrested during mitosis may be. Besides undergoing apoptosis directly from mitosis (mitotic

catastrophe), it is possible that cells undergo adaptation during which they enter a G1-like state and decondense their chromosomes with a tetraploid chromosomal content.[61] It has been suggested that in this situation the cell fate critically depends on the p53 status. Based on the fact that cells with impaired p53 function can re-enter the cell cycle after prolonged spindle disruption, a tetraploidy checkpoint has been proposed.[62] Whether this p53-dependent postmitotic arrest is in fact triggered by increased ploidy or by other changes associated with spindle disruption and/or cytoskeletal aberrations remains controversial.[10,11]

Regardless of whether a ploidy checkpoint exists, there is solid evidence that p53-deficient cells are prone to initiate multiple rounds of DNA replication.[63] It has recently been reported that p53-deficient tetraploid cells become aneuploid and such cells were found to be able to form tumors in vivo.[12] The precise role of tetraploidy is difficult to ascertain from these experiments but it has been proposed that altered ploidy in yeast profoundly affects cell homeostasis and genome integrity.[64] As expected, the majority of tumor cells derived from tetraploid p53-deficient tumor cells contained supernumerary centrosomes.[12] It is conceivable that abortive mitoses generally lead to centrosome accumulation and that the genetic background of a cell determines whether it arrests permanently or re-enters mitosis. In the latter case, centrosome aberrations would increase the risk for multipolar spindles and chromosome missegregation. A detailed analysis of centrosome-mediated chromosomal instability after tetraploidization, however, has not yet been performed.

Cell Fusion

It has been proposed that cells can fuse under various conditions and that cell fusion may be a particularly common event in malignant tumors.[65] Such a mechanism would lead to tetraploid or polyploid cells and such cells would likely contain supernumerary centrosomes. Fusion events between differentiated normal cells have been shown to result in multinucleated cells that are unable to proliferate. Fusion events between malignant cells have been proposed to result in cells that are capable of producing viable progeny, a prerequisite for centrosome-mediated cell division errors and chromosomal instability. However, the survival rate of cell fusion products (hybrids) is low, suggesting that cell fusion is followed by massive cell death or permanent growth arrest in the vast majority of cells.[65] Whether cell fusion exists in primary human tumors and the contribution of fused tumor cells to chromosomal instability remains elusive. If such a mechanism can be substantiated and cells are capable of producing viable daughter cells, it is likely that supernumerary centrosomes may contribute to cell division errors and chromosomal instability.

DNA Rereplication

Another mechanism that can lead to tetra- or polyploidy is DNA rereplication i.e., an initiation of DNA replication before the previous S phase is completed.[66] It is conceivable that this can lead to aberrant DNA structures and it was found that rereplication of DNA causes an activation of DNA damage checkpoints. A prolonged DNA damage checkpoint activation, however, may allow centrosome overduplication in G2 phase.[67] In addition, overexpression of cyclin E, which is detected in many cancers, may trigger rereplication of DNA by inactivation of geminin, a major inhibitor of licensing of DNA replication.[66] At the same time, cyclin E has been implicated in centrosome overduplication.[68] Hence, cells undergoing DNA rereplication may be prone to centrosome-mediated cell division errors, in particular when loss of p53 impedes antiproliferative responses.

Aberrant Centrosome Numbers as a Cause of Polyploidy

The question whether centrosome aberrations can be a cause of polyploidy has not been addressed in detail. Previous studies suggest that a "centrosome checkpoint" which would block the progression of multipolar metaphases[69,70] into anaphase does not exist. Nonetheless, there are reports showing a discrepancy between the frequency of multipolarity in metaphase cells in comparison to anaphase cells,[40,71] which raises the question whether other yet to be identified mechanisms can hinder progression from multipolar metaphase into later stages of mitosis. A failure of such cells to complete cytokinesis would result in increased ploidy and centrosome accumulation (Fig. 4).

As discussed above, the further cell fate would likely be determined by the genetic background, specifically the p53 status.

Conclusion

Altered ploidy and centrosome aberrations are hallmarks of malignant growth and frequently arise concomitantly in tumor cells. This coincidence has led to the idea that tetra- or polyploid cells represent "seeds" of chromosomal instability. However, there are clearly examples where the formation of supernumerary centrosomes does not require an altered ploidy and that tetraploid cells do not necessarily become aneuploid. A key task for the future will be to ascertain the frequency at which cells that have failed mitosis once will re-enter mitosis and give rise to viable progeny. Moreover, the role of centrosome-mediated cell division errors in chromosomal instability requires further investigation since many studies have shown significant underrepresentation of multipolar ana- or telophase cells when compared to metaphases. In future studies, it will be important to distinguish between centrosome aberrations that have the potential to trigger cell division errors and those that merely are a side effect of cellular insults. Such studies will be critical for the use of centrosome aberrations as surrogate biomarkers of chromosomal instability and tumor progression.

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Chapter 7

Polyploidy:
Mechanisms and Cancer Promotion in Hematopoietic and Other Cells

Hao G. Nguyen and Katya Ravid*

Abstract

Polyploidy, the state of having greater than a diploid content of DNA (e.g., tetraploid, octaploid, etc) has been recognized in a large variety of both, plant and animal cells. Human and murine megakaryocytes, hepatocytes, arterial smooth muscle cells and cardiac myocytes, all develop a certain degree of polyploidy during their normal lifespan. In addition, polyploid cells may be found in some tissues under conditions of stress, including uterine smooth muscle during pregnancy, aortic vascular smooth muscle cells during aging and hypertension, beta-cells in diabetic human or mouse thyroid cells in hyperthyroidism and cells in seminal vesicles with aging. Polyploid cells are also found in malignant tissues in which they are believed to contribute to the development of cells with intermediate DNA content values (e.g., 3n, 4.5n, etc.) (reviewed in refs. 1,2). With the use of micro-array, researchers have demonstrated that genetically identical yeast strains (*Saccharomyces cerevisiae)* with differences only in ploidy status (from haploid to tetraploid) display a substantial difference in gene expression, including of the G1 cyclins.[3] This finding has suggested that DNA content per se might affect cellular functions.

Overview: Characteristics of Polyploidy and Its Induction Under Different Conditions

Currently, the relationships between polyploidy and aneuploidy has not been studied extensively considering the prominent role of genetic instability in tumorigenesis.[4] An understanding of the biochemical, gene expression and signaling pathways that drive normal and abnormal polyploidization could lead to useful insights with respect to novel anticancer therapeutic approaches. The occurrence of polyploidy in normal and transformed cells poses a number of questions. Is polyploidy a protective mechanism upon stress, as suggested,[2,5,6] or rather a maladaptive response? What mechanisms or signaling pathways are employed by normal developing polyploid cells (e.g., megakaryocytes) to safeguard them from becoming aneuploid?

In megakaryocytes, polyploidization up to 128N can be attained, if the cells undergo repeated endomitotic cell cycles, characterized by a well coordinated entry of cells into a normal early mitotic phase, which includes prophase, metaphase and early anaphase. However, these cells skip late anaphase and cytokinesis (this truncated mitosis is referred to as *polyploidy* via *endomitosis*, reviewed in ref. 2). In contrast, polyploidy may result from another type of truncated mitosis, referred to as *polyploidy* via *abortive mitosis* to describe the generally uncoordinated events that are driven by spindle checkpoint defects or by chemical treatments. These events are often associated with pathological

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conditions (reviewed in refs. 1,2,4 and see Illustration 1). It has been shown, in both tissue culture and in transgenic mice, that polyploidy via endomitosis in megakaryocytes is tightly regulated by a series of signaling pathways and gene expressions, including signaling through thrombopoietin (TPO), binding to its receptors c-Mpl[1,2] and is associated with elevated cyclin D3 expression and a rapid reentry into S-phase.[7-9] There is also evidence that these cells possess a gene expression profile that is different from their diploid counterparts, including low expression of the tumor suppressor gene p53[10] in conjunction with high expression of the cell cycle inhibitor p21 to allow a short-lived progression through G1 phase.[11,12]

Numerous studies have shown that normal diploid cells of other lineages can be induced to undergo polyploidization via endomitosis as a consequence of stress (e.g., hypertension and senescence (reviewed in ref. 2). In addition, polyploid hepatocytes have been shown to increase in number dramatically upon oxidative stress or after partial hepatoectomy.[13-15] Endothelial cells and fibroblasts have been shown in tissue biopsies and in cell culture to become polyploid upon aging and during tissue repair.[6,16] Hypertension can induce vascular smooth muscle cells and cardiac myocytes to become polyploid.[17,18] In these cases, polyploidy is believe to be a protective mechanism, which acts to prevent cellular proliferation in the vasculature or to increase DNA content in order to compensate for mutations introduced by genotoxic agents.[2,19] On the other hand, tetraploidy (cells with a double diploid DNA content) may reflect tissue damage as in Barret's esophagus,[20] in which there is dysplasia of the

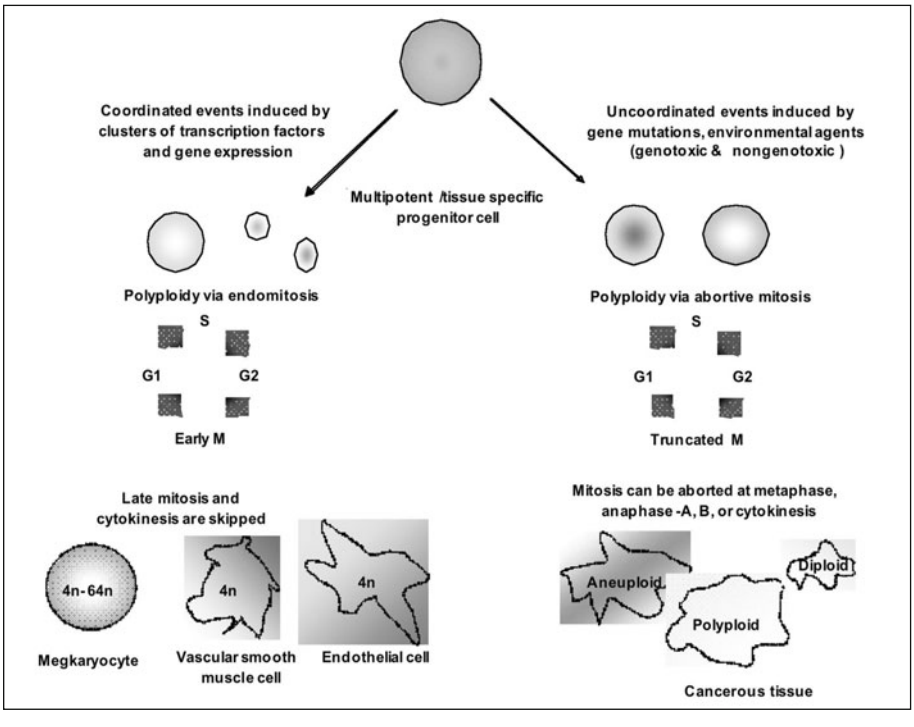

Figure 1. Pathways to polyploidy. Left panel) Polyploidy via Endomitosis—a shortened mitosis without anaphase-B and cytokinesis, followed by reentry into G1 phase of cell-cycle. This well-controlled truncated mitosis is a part of megakaryocytes development.[2,4] Right panel) Polyploidy via Abortive Mitosis—an abrupt termination of mitosis at metaphase, anaphase A, anaphase B or cytokinesis, followed by reentry into the cell-cycle with a tetraploid DNA content. These cells can have a single or multiple nuclei, depending on the timing of the defective events. This phenomenon is often associated with pathological conditions, including cancer (reviewed in refs. 2,4).

esophageal epithelium following repeated exposure to acid reflux. The most pronounced dysplastic changes include the appearance of tetraploid cells and predict for esophageal cancer.[20]

Tetraploidy can be induced in a variety of ways, including aberrant expression of proteins regulating the G2/M phase (Cyclin-B1, Aurora-A, Forkhead transcription factor M3),[21,22] mitotic spindle checkpoint proteins (BUBR1, Mad2 Aurora-B, Survivin)[23,24] leading to abortive cytokinesis. Tetraploidy can also be induced by chemical agents and/or irradiation and be associated with tumorigenesis[25-27] (see Fig. 1). This latter type of polyploidy is thought to be a by-product of uncoordinated events during mitosis in which a defect in mitotic spindle checkpoint arrest allows for a "mitosis slippage", resulting in cells with truncated mitosis, sometime at anaphase A, other times at anaphase B, or at cytokinesis.[28] The resulting tetraploid cells can be cell cycle arrested, undergo apoptosis or continue to the next division, to produce aneuploid daughter cells.[29-32]

Prevalence of Polyploidy/Aneuploidy in Different Cancers

Polyploidy often precedes aneuploidy during the events of tumorigenesis that are associated with high incidence of malignancy and poor prognosis.[33-35] It is generally accepted that aneuploidy in cancer cells is the rule and not the exception. Most heterogeneous tumor tissues (colorectal cancer, lung, breast, prostate, renal cell carcinoma, bladder cancer, thyroid cancer, some types of leukemia, glioblastoma and melanoma and rare childhood tumors) contain large populations of aneuploid cells in conjunction with a relatively smaller percentage of polyploid cells.[36-45] Among hematological malignancies, a shift in ploidy is often observed in acute lymphoblastic leukemia (ALL). In addition to a high frequency of translocations, deletions and fusion of chromosomes (70% of adults and 80% of children), a common cytogenetic abnormality in childhood ALL is the occurrence of massive hyper-diploid (defined as having greater than 50-65 chromosomes, a condition observed in 20-30% of the cases). Polyploidy has also been described in choroid plexus carcinoma, a rare form of childhood brain tumor, in which freshly isolated tumor cells were found to have up to 200-400 chromosomes.[46] Moreover, there is a recent report that primary keratinocytes infected with human papillomavirus (HPV) Type 16 E6, E7 become polyploid, possibly by abortive

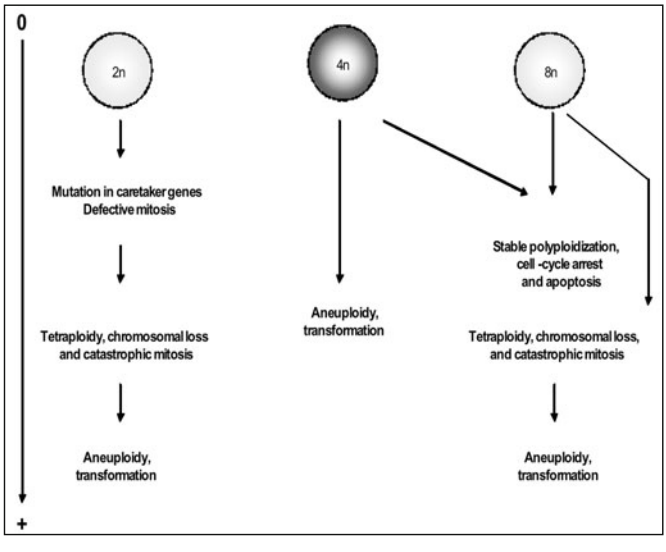

Figure 2. Possible models for polyploidy acquisition and tumorigenesis. Shown are the potential events that a cell with diploid, tetraploid or octaploid DNA content may acquire to reach malignant transformation as a function of time. This model predicts that cells with a tetraploid content are more prone to cancer development with a shortest latency period when compared to their counterparts. This model is based on previous works as reviewed in reference 4.

mitosis.[47] In most solid cancers, the modal chromosome number is near triploid, or near tetraploid. In some instances, the appearance of polyploid cells from a normal diploid cell background may be mediated by the tetraploidy/polyploidy checkpoints. This checkpoint ensures that cells with greater than 2N DNA content do not progress past G1 after exiting from mitosis.[29] There are several mechanisms that can be envisioned as causes of a ploidy shift, including doubling of a hyper-haploid cell (defined as having a total of 30-40 chromosomes), a single event of aberrant mitosis, or normal polyploidization with subsequent loss and/or gain of chromosomes.[48] Because of the high rate of chromosome loss in cycling polyploid cells,[31,49,50] it is very possible that aneuploidy develops from a tetraploid/polyploid population of cells during tumorigenesis (see Fig. 2).

Cancer Theories: Potential Involvement of Aneuploidy in Cancer Promotion

Malignant cancer cells are generally defined as cells that: (1) escape programmed cell death; (2) enter a proliferative state without mitogenic signals; (3) are unresponsive to antiproliferative signals; (4) escape programmed senescence; 5) metastasize and thrive in different tissues, including recruitment of new blood vessel formation; and (6) can eventually kill the host organism.[51] However, the transforming events that allow cancer cells to develop are not fully understood. Nonetheless, a number of theories focused on multi-step gene mutation,[52] genomic instability[53] and aneuploidy,[54] offer at least partial explanations for tumorigenesis and the development of cancers.

The Somatic Gene Mutation Theory

Cancer cells are characterized by a variety of genomic defects, such as inactivation of DNA repair genes, over-expression of growth promoting oncogenes, possession of extra or missing chromosomes, an abnormal number of centrosomes and aberrant mitosis and cytokinesis.[23] The Somatic Gene Mutation theory (SMT) has emerged as the basis for much of current cancer research and rests upon three principal assumptions. (1) Cancer is a genetic disease caused by mutations in cancer related genes, such as p53, Rb and Ras. (2) Mutations of tumor suppressor genes and oncogenes allow cells to disregard inhibitory growth signals and permit them to grow uncontrollably. (3) For a cell to become malignant, several damaging gene mutations are required, or both alleles of those genes must be affected (two hits hypothesis).[55,56] First, this theory implies that cancers are derived from individual cell clones that have accumulated mutations sequentially over time (i.e., tumors are monoclonal in nature). Second, this theory suggests that normal cells destined to become cancerous must have faster than normal rates of mutation to acquire these genetic changes (i.e., a fast rate of 10^{-3} mutations as oppose to a normal rate of 10^{-7} to 10^{-8} mutation per nucleotide per cell division). Numerous tumor suppressor genes and oncogenes have been identified and mutations of these genes have been shown to lead to neoplastic transformation in transgenic mice.[51] Yet, the somatic gene mutation theory fails to explain why cells within the same invasive tumor do not uniformly share the same mutations of relatively important genes, i.e., Ras and p53. Such cells also may share substantial differences in chromosome numbers, although they are thought to originate from clonal expansion.[57] The heterogeneous nature of tumor cells (both in the rate and type of gene mutations and ploidy status) has prompted scientists to look for additional or alternative unifying principles to explain tumorigenesis.

The Mutator Phenotype Hypothesis

Loeb and colleagues proposed the *Mutator Phenotype Hypothesis* to explain why cancer cells have a much faster rate of random mutations and how this phenotype may account for the genetic changes observed in cancer.[58,59] This theory postulates that once normal cells acquire mutations of genes that control the fidelity of DNA replication and repair, they develop an explosive increase in random mutations (Mutator Phenotype). Some of these mutations may permit cells to have selective advantages to expand and achieve clonal dominance.[60,61] This theory implies that genetic instability/aneuploidy is a consequence of these random mutations.

The Genomic Instability Theory

Lengauer and Vogelstein observed that a very high degree of genomic instability, characterized by the gain or loss of portions of chromosomes or entire chromosomes is present in the early stages (preneoplastic) of colon cancer development. Based on this finding, they proposed the *Genomic Instability Theory* of cancer in 1997. This theory argued that, at least in colon cancer, chromosomal losses or gains are the early events that lead to the loss of tumor suppressor genes and/or gain of oncogenes, which are widely believed to drive malignant transformation.[34,53,62-64] One of the main assertions of this theory is that as cells acquire mutations in master genes (or genes required for cell division and segregation of chromosomes), subsequent divisions are prone to result in more mistakes, leading to an instability in chromosome number, a critical early event in tumorigenesis. While emphasizing the importance of genetic instability as early events, this theory still holds that mutations in cancer related genes are a prerequisite for transformation. This theory explains a number of characteristic of tumor cells, including aneuploidy and fast rates of mutation. Compelling evidence in support of this theory was recently reported by Hanks et al (2004),[65] in relation to individuals with a rare genetic disorder, mosaic variegated aneuploidy, in which more than 25% of the cells in the body may be found to be aneuploid. This phenotype is characterized by mutation in both alleles of the chromosome segregation gene, BUB1B. Affected individuals frequently develop childhood cancers such as rhabdomyosarcoma and leukemia. This report is the first to suggest that aneuploidy may have a direct causal role in the development of cancer in human.

The Aneuploidy Theory of Cancer

The Mutator Phenotype and Early Genetic Instability Theories cannot explain malignancies caused by nongenotoxic carcinogens, which are not mutagens but can act as aneugens (chemical agents that disrupt the mitotic spindle and cause chromosome mis-segregation) and are associated with tumorigenesis. For instance, asbestos, a nonmutagenic carcinogen, has been shown to bind to the mitotic spindle, causing chromosome mis-segregation and genetic instability.[49] Asbestos has not been reported in the literature to cause specific cancer related gene mutations. In light of this, Duesberg and colleagues proposed the Aneuploidy Theory in 1999.[54,66] The first assumption of this theory is that cancer is not a disease of gene mutations per se but a disease of gene dosage (i.e., having 3, 5, or zero copy/copies of a normal set of genes via random aneuploidization). The second assumption is that carcinogens or spontaneous cell-cycle accidents are more effective inducers of aneuploidy than specific mutations. Hence, according to this theory cancer development does not necessarily require mutations in cancer related genes at the DNA level but an imbalance in the dosage of thousands of normal genes caused by chromosomal gains or losses. Therefore, cells may become transformed before mutations of tumor suppressor genes and/or oncogenes occur.

Regardless of which theory of cancer evolution best explains individual types of cancer, they each identify aneuploidy and genetic instability as having a causal role in tumorigenesis.

Regulators of Mitosis and Mechanisms Leading to Aneuploidy

There are a variety of ways in which cells may become aneuploid,[34] including: (1) Telomere dysfunction, which has been linked to aneuploidy in cancer. Studies have shown that telomere shortening in telomerase knockout mice after succeeding generations is associated with tumorigenesis. Cells with truncated telomeres are more prone to chromosome translocation and fusion, (reviewed in ref. 67). (2) Defective mitotic spindle checkpoint. During the transition from metaphase into anaphase, cells evolve surveillance mechanisms to ensure proper attachment of mitotic spindles to kinetochore/centromere before the segregation of chromosomes begins. Important protein components of this spindle checkpoint include: BUB1, 2, 3, Mad-1,2,3 and the chromosome passenger protein Aurora-B. Seminal studies in diverse species, ranging from yeasts to humans, have concluded that defects in this checkpoint allow the cell to progress through metaphase/anaphase with unequal attachment of the spindle/kinetochore, giving rise to aneuploid daughter cells (reviewed in refs. 68,69). (3) Defective Mitotic spindle assembly. The aberrant duplication of centrosomes at early mitosis (often due to mutation of genes involved in centrosome matura-

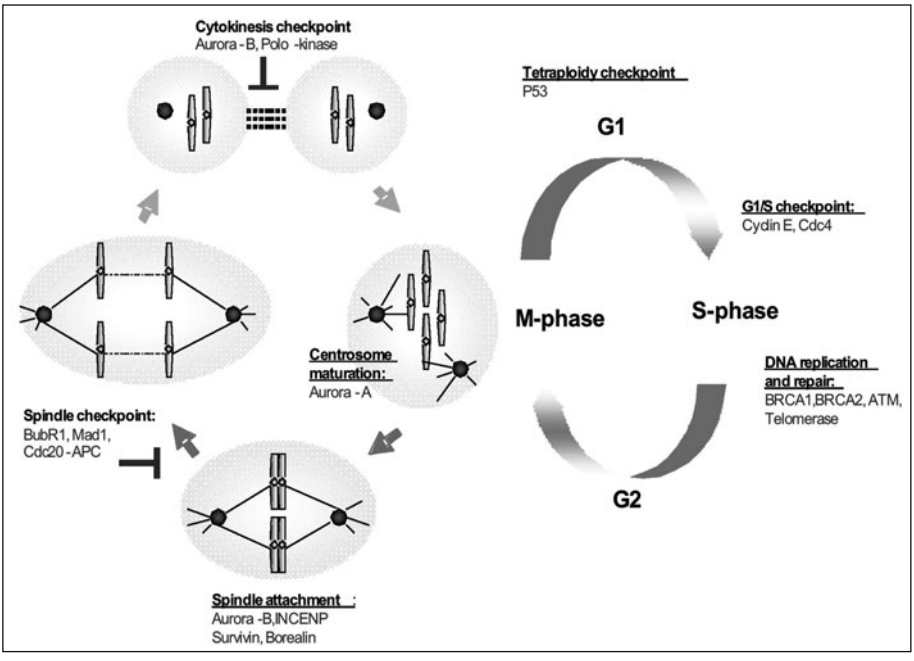

Figure 3. Pathways to aneuploidy. Shown are crucial events (underlined text) of the cell cycle that have been implicated in the generation of aneuploidy. The deregulated genes (blue text) have been shown to underlie the mechanism leading to aneuploidy. Defective regulation of mitotic genes seems to be the main routes to aneuploidy. This illustration was adapted from two review articles.[34,72] A color version of this figure is available at www.landesbioscience.com/curie.

tion and duplication, including Aurora-A, or via chemical agents) has been demonstrated to cause polyploid or aneuploid daughter cells. The resulting functional defects of mitotic spindle assembly lead to lagging chromosomes as they segregate during a precise time frame at the transition into anaphase.[31] (4) Abnormal chromosomal rearrangement, breakage and fusion, has been demonstrated to be a source of aneuploidy.[70] (5) Abortive cytokinesis, if occurred in diploid cells can lead to the formation of polyploid cells and has been suggested to cause aneuploidy via tetraploid intermediates (see Fig. 3).[71]

Stem Cells and Cancer Development

With the findings of stem cells in breast cancers, Wilm's tumors, hematological malignancy and neuroblastomas[73,74] (referred as tumor stem cell, TSC), there has been increased interest in understanding the role of tumor stem cells in tumorigenesis. Given the scarcity of stem cells in tumors, their existence in the heterogeneous tumor tissue has been demonstrated experimentally only recently, although hypothesized decades ago.[73] Tumor stem cells are phenotypically similar to normal stem cells in their abilities to self-renew and to differentiate into multiple tissues or cell lineages within the same tissue. However, they differ from normal stem cells with respect to the balance of self-renewal and differentiation. Normal stem cells generally give rise to progenitors cells, which commit into a specific cell type with a limited life span. Under normal physiological conditions, the self-renewal capability of normal stem cells is inhibited by cell cycle inhibitors, such as p21 and p18 and is tightly and reversibly regulated by the need for differentiation or tissue regeneration (reviewed in ref. 73). In p21 $^{-/-}$ mice, hematopoeitic stem cells (HSC) tend to cycle faster than wild-type cells, while the proliferation of marrow progenitor cells is repressed,

resulting in a larger pool of HSC and a smaller pool of lineage committed progenitors.[12] Hence, as observed in other systems, the proliferation of normal stem cells requires a balance between differentiation and self-renewal, depending on their stage of development.[73] In contrast, tumor stem cells are believed to have irreversible defects in cyclin inhibitors, coupled with disruptions in feedback mechanisms to control differentiation or apoptosis.[67,75] Tumor stem cells in leukemia are thought to originate directly from hematopoeitic stem cells or marrow progenitors, depending on the developmental stage at which genetic changes occur.[43] It is reasonable to hypothesize that some cancers may evolve from tissue specific progenitor stem cells because of their self-renewal, tissue evasion and ineffective senescent properties. The propensity for environmental agents, reactive oxygen species and hormones to cause genetic and epigenetic changes in stem cell is greater than that in their short-lived, differentiated counterparts. Although not yet proven, studies[74,76,77] have suggested that loss of heterozygosity of cancer related genes in mammary stem cells may contribute to genetic instability in progeny cells and subsequent breast cancer development. With the exception of polyploidy resulting from stem cell fusion,[78] a role of polyploidy and aneuploidy in the development of tumor stem cells has not been reported. However, given the important role of polyploidy and aneuploidy in tumorigenesis, an analysis of the degree of changes in ploidy in tumor stem cells would be worthwhile.

Chromosome Passenger Proteins and Their Role in Ploidy Promotion

Accurate segregation of chromosomes following each cell division requires a perfect synchrony of regulated protein proteolysis, phosphorylation and dephosphorylation, the localization and recruitment of a chromosome passenger complex and the physical interaction between the centromere and the mitotic spindles at the metaphase to anaphase transition. At the same time, the surveillance mechanism orchestrated by Mad1 and BubR1 ensures that the separation of chromosomes does not progress if these processes become asynchronous. In mammalian cells, the protein complex consisting of Aurora B kinase, Survivin, INCENP and Borealin, (also referred as the Chromosome Passenger Complex (CPC)) displays a distinct localization pattern throughout mitosis, suggesting that it has an important function in regulating mitosis. During prophase, this complex associates with condensed chromosomes and then concentrates at the inner centromere during prometaphase. At the onset of anaphase, the complex relocates to the central spindle. As the central spindle elongates at cytokinesis, the chromosome passenger proteins coalesce at the midbody, the site of the cleavage furrow. It is hypothesized that during telophase, this complex must be degraded for cells to exit mitosis normally. Various studies have demonstrated that altered subcellular localization patterns are associated with mitotic arrest, mis-segregation of chromosomes, abortive cytokinesis and polyploidy (reviewed in ref. 79). Moreover, the progeny of cells with such defects have been shown to be tumorigenic in xenograft mouse models.[72,80]

INCENP (Properties and Effects of Its Deregulated Expression on the Cell Cycle)

Inner Centromeric Protein (INCENP) was the first protein identified in the chromosome passenger protein complex. The C-terminus (IN-Box) of this protein is conserved from yeast to humans. INCENP binds to Aurora-B through the IN-Box sequences and stimulates its kinase activity during mitosis.[81] Deletion analysis of this protein has revealed that an N-terminal region (amino acid 1-68) is important for targeting INCENP to the centromere/kinetochore and midzone at anaphase.[82] INCENP is an essential gene, given that its targeted deletion in mice leads to polyploidization of embryonic cells and induces early embryonic lethality (32-64 cell stage).[83] RNAi mediated down regulation of endogenous INCENP has been shown to produce severe mitotic mis-segregation of chromosomes in *C. elegans* and Drosophila.[84,85] Overexpression of the dominant negative form of INCENP in mammalian cells showed similar defects in addition to the appearance of abnormal number of centrosomes. Thus, tight regulation of INCENP is clearly essential for cell division.[86] In vitro studies have shown that aberrant levels of INCENP disrupt the chromosome passenger complex and cause Aurora-B and Survivin to mislocalize in prometaphase.[84,87,88] Aberrant expression of INCENP also induces chromosome mis-segregation

and abortive cytokinesis in yeast, fruit flies and mammalian cells.[88,89] It has been shown in yeast that dephosphorylation of INCENP by Cdc14 is required for the transfer of the chromosome passenger complex to the central spindle at anaphase. Point mutations that generate a nonphosphorylated INCENP resulted in daughter cells with chromosomal loss, likely due to lagging chromosomes.[90] Interestingly, chromosomal alignment remained intact while the nonphosphorylated INCENP localized prematurely at the centromere prior to anaphase onset.[91] This study implies that the function of Aurora-B as a guardian of spindle attachment and alignment does not depend solely on the localization of INCENP. Hence, the protein level, kinase activities and sub-cellular localization of the Chromosome Passenger Complex proteins appear to be equally important in preventing polyploid and aneuploid phenotypes.

Borealin (Properties and Effects of Its Deregulated Expression on the Cell Cycle)

Borealin (alternatively called Dasra) was recently cloned and characterized as a new member of the chromosome passenger complex in vertebrate.[92,93] Borealin displays a typical pattern of subcellular localization to the centromere, central spindle and midbody during mitosis. Depletion of Survivin or INCENP by RNAi has been shown to disrupt this specific localization of Borealin. Similar to other chromosome passenger proteins, RNAi mediated knock down of endogenous Borealin also causes spindle defects, chromosome mis-segregation and pronounced disruption of spindle assembly.[93] Interestingly, Borealin appears to act prior to the onset of anaphase. Borealin is a direct substrate of Aurora-B and is required to target the CPPs to the centromere but not to the midzone during anaphase.[92] Given the similarity in the expression pattern and functions of Borealin to those of other CPPs, it will be important to elucidate the functional links between Borealin, Survivin, INCENP and Aurora-B in normal and cancerous cells.

Survivin (Properties and Effects of Its Deregulated Expression on the Cell Cycle)

Survivin, a 16 KDa protein as a monomer and 32 KDa as a dimer, is the smallest member in the Inhibitor of Apoptosis Protein (IAP) family and contains a BIR domain, which is characteristic of this family of proteins. Unlike other members of the IAP family, Survivin does not have ubiquitin ligase activity (E3) and is the only member protein that forms a homodimer in solution.[94-96] Interestingly, it is also a component of the chromosome passenger complex that associates with Aurora-B and it follows a similar pattern of expression and localization during mitosis. Its expression has been found to peak at G2/M and its degradation occurs in a cell-cycle dependent manner.[97] In differentiated tissue, Survivin expression is virtually absent, in contrast to its high expression in actively proliferating lineages, including CD34+ hematopoietic stem and progenitor cells (when stimulated by the combination of Thrombopoietin (TPO), Stem Cell Factor (SCF) and Flt3 ligand (FL)),[98] vascular endothelial cells,[99] vascular smooth muscle cells,[100] thymus T- and B-cells[101] and particularly in tumor cells (reviewed in ref. 102). Survivin can be found in three splice variants that differ in size (Survivin 2B, Delta Ex3 and 3B) as a result from translation of an alternate exon 2B, skipping of exon 3 and/or a frameshift with premature stop codon.[103] However, these splice variants still retain two features in common: the dimer interphase and the BIR domain at the N-terminus.[102] Published studies have suggested that survivin can form homodimers or heterodimers with its splice variants.[102,104] These homodimers/heterodimers are hypothesized to have distinct functions in regulating apoptosis or cellular proliferation, depending on the type of dimer and its subcellular localization.[104] The Survivin 2B variant is cytosolic, while the Delta Ex3 variant is localized mainly in the nucleus. The Delta Ex3 variant contains a nuclear localization sequence (NLS, R/K-rich region ^{81}RRKNLRKLRRK^{91}).[102] Survivin 2B expression is lost at later stages of malignancy, while normal Survivin and its Delta Ex3 variant maintain a high expression profile, suggesting a differential role in tumor development.[105,106] In vitro studies have demonstrated that survivin's localization to the central spindle and midbody at telophase is dependent on phosphorylation at Thr^{117} by Aurora-B and mutation of this site leads to disruption of its association with INCENP,[107] suggesting that phosphorylation of Thr^{117} is important for Survivin's role as a chromosome passenger protein. Homozygous deletion of Survivin in mice results in embryonic lethality at day 4.5, characterized by the presence of catastrophic mitosis (cell

death during mitosis), giant multinucleate cells, in addition to a large population of polyploid cells.[108] Forced overexpression of Survivin has been shown to inhibit IL-3 induced apoptosis in B-lymphocytes[109] and in UV-induced apoptosis in primary keratinocytes.[110] In addition, published studies have suggested that overexpression of Survivin shortens G1 phase arrest and accelerates S phase, potentially through activation of Cdk2/Cyclin-E complex.[111,112] The important role of Survivin in regulating endomitosis in polyploidizing megakaryocytes and vascular smooth muscle cells has been implicated in work reported from our laboratory.[113] In addition, it has been shown that during the endomitotic cell cycle of vascular smooth muscle cells, Survivin does not colocalize with Aurora-B or INCENP, as typically observed at the centromere and at the central spindle/midbody during cytokinesis of normally dividing cells. Interestingly, defects in sister chromatid separation and reversal of cytokinesis has also been observed in this generally normal and well coordinated endomitotic events.[113] Overexpression of Survivin has also been shown to reduce polyploidization in cultured primary vascular smooth cells.[113] Hence, the atypical localization pattern of Survivin appears to account for polyploidization in this lineage. Based on these studies, the function of Survivin in regulating endomitosis appears to be important. In accordance with our earlier report, survivin was not detected in endomitotic murine megakaryocytes, although these authors questioned the quality of the antibody used. In low ploidy human megakaryocytes, survivin was described as being properly localized in endomitotic megakaryocytes.[114] Several possibilities could account for the discrepancy in the reports on Survivin expression/localization in human and mouse megakaryocytes: a. According to Baccini et al, a group that has extensively studied megakaryocytes biology (e.g., refs. 115,116), human megakaryocytes grown in vitro present a defect in their polyploidization and hence, the authors caution others from using them as a model system for the study of endomitosis.[115] In vivo, most polyploid human megakaryocytes are 16N and 32N, as is the case in mice and rats. However, while the mouse cultures mimic the in vivo profile, the human cultures present less than 10% of the cells as polyploid, with the vast majority having a ploidy level not greater than 8N.[115,116] Hence, in the recent study that used this culture system,[114] survivin localization might have been primarily followed in proliferating or very low-ploidy megakaryocytes (as also pointed out by the authors); b. In the literature there is a recognized controversy about the specificity of available antibodies to survivin. For instance, W. Earnshaw's lab described conflicting survivin localization in mitosis with two published antibodies and noted that researchers need to also confirm data on protein localization with ectopically expressed GFP-labeled protein.[88] c. Although there is one survivin gene, there are three splice forms of mRNA yielding three different proteins, of which only the longer one (142 amino acids) displays typical properties attributed to survivin.[103] Some of the available antibodies might be detecting one of the nonfunctional splice variants.

With regard to its antiapoptotic properties, Survivin has been shown to bind to Smac/Diablo, a caspase activator and/or to procaspase 9 via the hepatitis B X-interacting protein (HPXIP) complex to mediate this effect.[117,118] A study by Song et al (2004)[104] demonstrated that a single amino acid change (Asp53→Ala53) converts Survivin from an antiapoptotic to proapoptotic regulator, suggesting that it has a dual role in controlling cell death at mitosis. Studies of Survivin function as both a Chromosome Passenger Protein and as an anti/pro apoptotic factor has been a subject of much interest. Recent work has described a new type of cell death, termed "mitosis catastrophe", often observed in cells with defective mitosis spindle assembly checkpoint, chromosome mis-segregation and abortive cytokinesis (reviewed in ref. 119). Although "mitosis catastrophe" is believed to be triggered by aberrant events during mitosis and not signals originating in G1 or S-phase, this type of programmed cell death still converges on the action of caspases, as suggested by several studies.[110,120,121] It is tempting to hypothesize that Survivin is a critical regulatory protein that determines the life and death of a cell undergoing division. Survivin may ensure the survival of cells with correct chromosome segregation by directly inhibiting caspases through its anti-apoptosis and/or chromosome checkpoint properties. On the other hand, Survivin, through its pro-apoptotic properties, may also ensure that cells undergo apoptosis if mitotic events are defective.

Aurora-B (Properties and Effects of Its Deregulated Expression on the Cell Cycle)

The Aurora/Ipl1 (Increase-in-Ploidy protein-1) protein kinases have been shown to orchestrate vital mitotic events, including G2/M transition, centrosome duplication, chromosome condensation, bi-polar spindle-kinetochore attachment, chromosome segregation and cytokinesis. Their roles are evolutionarily conserved in yeast, nematodes and mammalian cells (reviewed in refs. 72,122). While lower organisms have only one form of Aurora kinase (Ipl-1), mammalian cells have three types, Aurora-A, Aurora-B and Aurora-C, whose function and localization are distinct in space and time during cell division. The function of Aurora-C in mammalian cells has not been studied extensively. Aurora-A localizes to the centrosomes during early anaphase and is required for mitotic entry.[123] Aurora-B, (also called AIM-1, Stk-5) regulates the formation of a stable bi-polar spindle-kinetochore attachment in mitosis. It colocalizes with Survivin, Inner Centromere Protein (INCENP) and a recently discovered protein named Borealin or hDasra B to form the chromosome passenger complex, needed for chromosome segregation and cytokinesis.[21,92,93] Aurora-B is regulated at the mRNA level, at the protein level and at the level of its kinase activities (reviewed in refs. 72,122,124,125). INCENP has been shown to stimulate the kinase activity of Aurora-B[84,126,127] and there are conflicting reports on the regulation of Aurora-B by Survivin.[126] In a cell-free system, Survivin seems to enhance the kinase activity of Aurora-B (via Histone-H3-Ser10 phosphorylation),[128] provided that its kinase activity is first reduced in cells with siRNA-mediated Survivin knock down. How Aurora-B activity/function is terminated at the end of mitosis is an additional intriguing question.[23] Studies pursued in this thesis demonstrate that Aurora-B is regulated by protein degradation through the A-box and KEN box sequences.[129] Most importantly, overexpression of a nondegradable A-Box mutant leads to aneuploid/polyploidy, suggesting that Aurora-B's proteolysis plays an important role in the regulation of Aurora-B and chromosome stability at each cell division.[129] A recent study[130] identified a very short sequence in the C-terminus of Aurora-B (326-331) as responsible for its function and subcellular localization. Taken together, these studies demonstrate that Aurora-B's stability is regulated through its N-terminus, whereas the C-terminus contains the sequences required for its function and subcellular localization.

The most extensively studied function of Aurora-B is its involvement in mitotic spindle attachment. In order for chromosomes to separate equally, a synchronized alignment of sister chromatids at metaphase coupled with stable bi-polar attachments between the mitotic spindle and the kinetochore must take place. During this dynamic process, there are various ways in which the kinetochore-microtubule can form unstable attachments. This includes the case of kinetochore attaching to the spindle from both poles (merotellic) or when both sister kinetochores are attached to the same spindle pole (syntellic). If these unstable attachments are not corrected in time as the cell enters anaphase, lagging chromosomes and unequal separation of chromosomes occur in the daughter cells. Reduction of endogenous Aurora-B by genetic (iRNA) or pharmacological agents (ZM447439 and Hesperadin) results in merotellic and/or syntellic attachment and subsequent disruption of chromosome segregation.[21] Experiments using microinjection of anti-Aurora-B antibodies reveals that inhibition of Aurora-B in mitotic Xenopus tissue culture cells abrogates the spindle checkpoint and causes an early exit from mitosis with no evidence of anaphase or cytokinesis, concomitantly with the appearance of chromosome misalignment and polyploid cells.[131] How Aurora-B promotes stable bi-polar attachment and prevents unstable merotellic and/or syntellic attachment continues to be under investigation. The current model, derived from various studies with both yeast and mammalian cells, proposes that Aurora-B, through its kinase activity and interaction with various proteins (such as the Mitotic Centromere Associated Kinesin, MCAK), actively facilitates the depolymerization of microtubules associated with unstable attachments. Evidence for this model in mammalian cells includes the finding that Aurora-B directly interacts with MCAK to promote microtubule depolymerization.[132,133] In addition, its interaction with protein phosphatase I (PPI) keeps depolymerization in check, once stable attachments are achieved.[134,135] In budding yeast, Aurora-B is believed to function as a sensor for the pulling force and tension generated by the spindle-kinetochore complex. Yeast mutants, unable to generate spindle tensions accumulate merotellic and/or syntellic spindle attachments.[136-139] Hence, as a sensor of

the pulling force, Aurora-B may sense unequal forces and promote their elimination.[139] Recently, the yeast Aurora-B homolog, Ipl1 has been shown to interact with the Damp1 complexes, which interact directly with the kinetochore and microtubule to regulate bipolar attachment of mitotic spindle.[140] Specific mutation (S to A) of all four Ipl1 phosphorylation sites in the Dam1p protein causes cell death, suggesting an essential role for Ipl1/Dam1p phosphorylation.[140] Because of its vital role in correcting chromosome-spindle attachment, deregulated expression of Aurora-B/Ipl1 can be expected to impair chromosome segregation and mitotic progression.

Another function of Aurora-B, that has been described, concerns its role in the spindle checkpoint. To ensure viable and functional progeny after each cell division, cells have evolved several cell cycle checkpoints to allow adequate time for repair prior to progression to subsequent stages of cell division. One of the most important and final checkpoints of the cell cycle is the spindle assembly checkpoint during the transition into anaphase. Defective mitotic spindle assembly or detachment of the kinetochore directly triggers Bub1, Mad1 and other spindle checkpoint proteins to bind and inhibit the activity of the Cdc20-APC/c E3 ligase (a component of proteasome mediated degradation, as described below and also regarded as the effector of the spindle checkpoint), leading to a transient arrest of the cells at metaphase. Several studies have demonstrated that Aurora-B participates in the recruitment/association of Mps1, Bub1, CENP-E, Bub3, Mad1 and Mad2 to kinetochores.[141,142] Studies described in this thesis show that Aurora-B directly associates with the Cdc20-APC/c complex.[129] Moreover, other studies indicate that depletion of endogenous Aurora-B impairs the cells' ability to localize Cdc20, Cdc27 and Cdc23 (subcomponents of the APC/c) to unattached kinetochores such that cells fail to activate the spindle checkpoint in response to microtubule destabilization.[139,142-144] These functional studies have demonstrated that Aurora-B is an indispensable member of the spindle assembly checkpoint, acting upstream of Bub1 and Mad1 and indicate that deregulation of Aurora-B disrupts this protein composition to prevent the spindle checkpoint.[145]

During telophase, Aurora-B also has a role in ensuring the completion of cytokinesis.[146-149] Drosophila cells lacking Aurora-B protein do not undergo cytokinesis and undergo polyploidization.[150] Drug-mediated inhibition of this kinase in proliferating mammalian cells can also induce polyploidy[21] and/or cell death by "catastrophic mitosis".[151] In bone marrow megakaryocytes (platelet precursors), which undergo endomitotic cell cycles and polyploidization during normal development, Aurora-B has been shown to be absent from the midzone.[152] Of note, a very recent study reported results similar to ours with respect to proper Aurora-B expression/localization during early mitosis and lack of it at late anaphase in murine megakaryocytes.[114] In few human megakaryocytes examined,[114] Aurora-B was detected at late anaphase. In this study, Aurora-B kinase activity was measured in polyploid, nonsynchronized megakaryocytes and it was concluded that Aurora-B is not limiting for polyploidy in this lineage. Similar to our conclusion, this study also suggested that further study of midzone organization and composition is needed. Aurora kinases have been found to be overexpressed in a variety of malignant cancers (as listed in: http://cgap.nci.nih.gov) and this overexpression is suspected to contribute to chromosome instability.[153] Studies by our group[152] have shown that in vivo overexpression of Aurora-B transgene in megakaryocytes increases the proliferative potential of these cells, but does not by itself induce malignant transformation.[152]

Chromosome Passenger Proteins and Cancer (Emphasis on the Role of Aurora-B)

The link between overexpression of the Aurora kinases in mammalian cells and carcinogenesis is believed to be causal and to be dependent on perhaps, the disruption of normal centrosome or centromere function, spindle checkpoint regulation and cytokinesis.[30,80,153-155] Overexpression of the Chromosome Passenger Proteins (CPP), including Aurora-A, Aurora-B, Survivin and INCENP has been observed in ovarian, breast and prostate cancers and shown to correlate with aneuploidy.[72,81,156-159] In addition, chromosomes containing the CPP are often affected in aneuploid cells.[160,161] The mechanisms that explain how overexpression of CPP proteins, individually or together, promotes aneuploidy remain an important, unanswered question. Only recently,

have studies been carried out to determine whether ectopic expression of the CPP drives cellular transformation by means of increasing proliferation, by centrosome amplification, or by inducing chromosome instability. Overexpression of Aurora-A has been shown to potentiate HRAS (Harvey sarcoma virus oncogenes) induced transformation in-vitro, whereas reduced endogenous Aurora-A expression by short hair-pin RNA (shRNA) decreased transformation.[162] It has been suggested that overexpression of Aurora-A results in cells with increased number of centrosomes (3-4) and consequently impairs their ability to segregate chromosomes equally.[50] Similarly, correlative data showing overexpression of Aurora-B kinase in solid tumors and tumor cell lines has been reported.[80,157,160,163-165] However, given the tight regulation of Aurora-B at the protein level,[152] only a handful of studies have been able to demonstrate overexpression of Aurora-B induces oncogenic transformation. These studies include those using xenograft models of localized tumor formation in mice injected with cells overexpressing Aurora-B (80 and Nguyen Hao and Katya Ravid unpublished data). In these studies, oncogenic transformation appears to be mediated by aneuploidy and to be a consequence of Aurora-B overexpression. Inhibition of the Aurora kinases, in general, blocks progression of the cell cycle and induces cell death by "catastrophic mitosis". Several studies have exploited this type of cell death using Aurora kinase inhibitors (VX-680 for Aurora-A, Hesperadin and ZM447439 for Aurora-B (refs. 21,166-168 and reviewed in ref. 151)) to suppress tumor growth in vivo. Reduction of endogenous Aurora-B expression by such means has been shown to diminish the growth of thyroid anaplastic carcinoma tumor cells.[163] However, it is possible that inhibition of Aurora kinases is unable to completely prevent tumor growth, since reduced expression of these kinases also leads to aneuploidy (as reviewed in refs. 88,119,122,124,125,155,158,169,170) and such inhibition would be expected to prevent the activation of the spindle checkpoint, causing cells to exit mitosis prematurely.

Overexpression of Survivin in a wide range of tumor tissues, including leukemia (ALL, AML), colo-rectal cancers, astrocytic tumors and breast cancer has been consistently reported in the literature.[102,159,171] Moreover, overexpression of Survivin in cancer tissues is closely correlated with poor prognosis.[172-176] The role of Survivin in cancer promotion has been studied using a transgenic mouse model.[110] Grossman et al[110] have shown that exogenous expression of Survivin (driven by the keratinocyte specific promoter (K14)) inhibits UVB induced apoptosis and thus inhibition is more pronounced when the expression of p53 is reduced. Hence, this study suggests that Survivin functions as an inhibitor of apoptosis and thereby contributes to transformation. However, given Survivin's role as a chromosome passenger protein, the consequences of its overexpression on chromosome stability have not been fully explored.

Conclusion

It is not clear yet if polyploidy is a protective mechanism upon stress, or rather a maladaptive response. Progress has been made, however, on mechanisms and signaling pathways that are employed by normal developing polyploid cells (e.g., megakaryocytes) to safeguard them from becoming aneuploid, as well as on the mechanisms leading to aneuploidy and its relation to cancer development.

Acknowledegments

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Chapter 8

Polyploidization of Liver Cells

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Abstract

Eukaryotic organisms usually contain a diploid complement of chromosomes. However, there are a number of exceptions. Organisms containing an increase in DNA content by whole number multiples of the entire set of chromosomes are defined as polyploid. Cells that contain more than two sets of chromosomes were first observed in plants about a century ago and it is now recognized that polyploidy cells form in many eukaryotes under a wide variety of circumstance. Although it is less common in mammals, some tissues, including the liver, show a high percentage of polyploid cells. Thus, during postnatal growth, the liver parenchyma undergoes dramatic changes characterized by gradual polyploidization during which hepatocytes of several ploidy classes emerge as a result of modified cell-division cycles. This process generates the successive appearance of tetraploid and octoploid cell classes with one or two nuclei (mononucleated or binucleated). Liver cells polyploidy is generally considered to indicate terminal differentiation and senescence and to lead both to the progressive loss of cell pluripotency and a markedly decreased replication capacity. In adults, liver polyploidization is differentially regulated upon loss of liver mass and liver damage. Interestingly, partial hepatectomy induces marked cell proliferation followed by an increase in liver ploidy. In contrast, during hepatocarcinoma (HCC), growth shifts to a nonpolyploidizing pattern and expansion of the diploid hepatocytes population is observed in neoplastic nodules. Here we review the current state of understanding about how polyploidization is regulated during normal and pathological liver growth and detail by which mechanisms hepatocytes become polyploid.

Introduction

The liver is an essential organ with a high regenerative capacity and complex functions.[1-3] This organ has a central role in metabolic homeostasis, as it is responsible for the metabolism, synthesis, storage and redistribution of nutrients, carbohydrates, fats and vitamins. Nutrients entering the liver are transformed into secreted proteins (albumin, most coagulation factors and several plasma carrier proteins), carbohydrates stored in the liver as glycogen (the main glucose reserve used for stabilization of glucose levels in the blood) and lipids sent as lipoproteins into the other tissues. Importantly, the liver is also the main detoxifying organ of the body, which removes wastes and xenobiotics by metabolic conversion and biliary excretion. The main cell type of the liver that carries out most of these functions is the parenchymal cells, or hepatocytes, which constitute approximately 60% of all cells in the liver and 90% of liver cell mass. The other 40% comprise the nonparenchymal cells, which include endothelial cells, kupffer cells, lymphocytes and stellate cells. Hepatocytes within the liver lobule differ in their enzyme content and subcellular structure according to their location (for reviews see ref. 4). In fact, based on the blood vessels location and the blood flow direction, the individual liver lobule can be subdivided into an upstream "periportal" and a downstream "perivenous"

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(pericentral) region. Amino-acid metabolism, gluconeogenesis, lipid oxidation, energy metabolism and glycogen synthesis from lactate take place in the upstream, periportal hepatocytes. On the other hand, glycolysis, lipogenesis, cytochrome P450-dependent detoxification and glycogen synthesis from glucose are located in the downstream, pericentral hepatocytes.

Polyploidy is a characteristic feature of mammalian hepatocytes.[5-8] During postnatal growth, the liver parenchyma undergoes dramatic changes characterized by gradual polyploidization during which hepatocytes of several ploidy classes emerge as a result of modified cell-division cycles. This process generates the successive appearance of tetraploid and octoploid cell classes with one or two nuclei.[8-10] The hepatocyte ploidy level practically reaches the plateau at maturity. Interestingly, a second wave of ploidy elevation has been also observed at senescence in different species.[11,12] The biological significance of hepatic polyploidy is not clear, but the presence of advanced polyploidy is generally considered to indicate terminal differentiation,[13,14] with decreased proliferative capacity.[15,16] In this chapter, we discuss how polyploidization is regulated during normal and pathological liver growth and try to understand by which mechanisms hepatocytes become polyploid.

Polyploidization During Normal (Developmental) Liver Growth

Hepatic development is an extended process that continues thought early postnatal life. Through E14, most hepatoblasts are bipotent with the ability to differentiate into hepatocytes as well as biliary cells but by E15 most hepatoblasts are committed to the hepatocyte lineage.[17,18] During the remaining period of gestation and the first four postnatal weeks, hepatoblasts acquire functions of the differentiated hepatocytes and metabolic zonation.[4,17,18] In parallel with this process of hepatocyte differentiation, there is a progressive decline in cellular proliferation. The DNA synthesis rate is elevated in rats two hours after birth with 18% of the hepatocytes incorporating ^{3}H-Thymidine.[19] Three weeks after birth, ~9% of the hepatocytes show evidence for DNA synthesis; within six weeks DNA synthesis is detected in only few hepatocytes, ~0.05% which is similar to a normal adult liver.

The onset of polyploidy is clearly correlated with the end of the proliferative state in the liver. Several techniques have been reported for ploidy determination using isolated hepatocytes (ex vivo studies). Hepatocyte ploidy has been investigated in the past essentially through karyometry[20] and cytophotometry.[21,22] More recently, several groups have used flow cytometry (FACS).[9,14,23] However, FACS cannot resolve nuclearity (counting binucleated cells) and a second step is required to count the number of nuclei per cell (microscopy approach).[24,25] Recently, reports have taken advantage of fluorescence imaging to directly assess in vivo nuclearity and to measure DNA content in order to determine the liver ploidy/binuclearity profile[26,27] (Fig. 1A,B). In fact, the results of ex vivo and in vivo studies are in line with each other. For example, in the newborn rat liver, all hepatocytes are diploid (Fig. 1C). From the first three weeks postnatal, the proportion of diploid cells starts to fall significantly, with the successive appearance of binucleated 2 × 2n and mononucleated 4n hepatocytes (Fig. 1C). Binucleated 2 × 2n cells are first detected after weaning (day 21) and then their proportion rise rapidly to reach ~30 % of the total hepatocytes population at day 30. The mononucleated 4n hepatocytes population is only present at significant levels 25 days after birth. At day 30, a sharp increase is observed in the number of mononucleated 4n hepatocytes and this hepatocytes contingent is in the majority at day 40, reaching ~45% of the total population (Fig. 1C). Octoploid (binucleated 2 × 4n and mononucleated 8n) hepatocytes accumulate in significant numbers during the second and third months, in parallel with a decline in the relative number of tetraploid hepatocytes.

The degree of polyploidization varies in different mammals (Table 1).[28,29] In humans, the number of polyploid cells averages 30% to 40% in the adult liver.[11-27] A negative correlation exists between the mitotic index in the liver and the level of hepatocyte polyploidization found in different species.[30,31] For example, a mouse liver has a much lower mitotic index than a rat liver and accordingly the higher level of hepatocyte polyploidization was found in the mouse liver. Polyploidization is minimal in a guinea pig liver which has the highest mitotic index of hepatocytes amongst rodents.[29] Increased cell size is the most obvious and consistent consequence of an increase in ploidy. Different studies have demonstrated in both human and mouse liver cells that the volume of hepatocytes is approximately twice with doubling DNA content.[8,25,32,33] Moreover, there is no significant difference in the volume

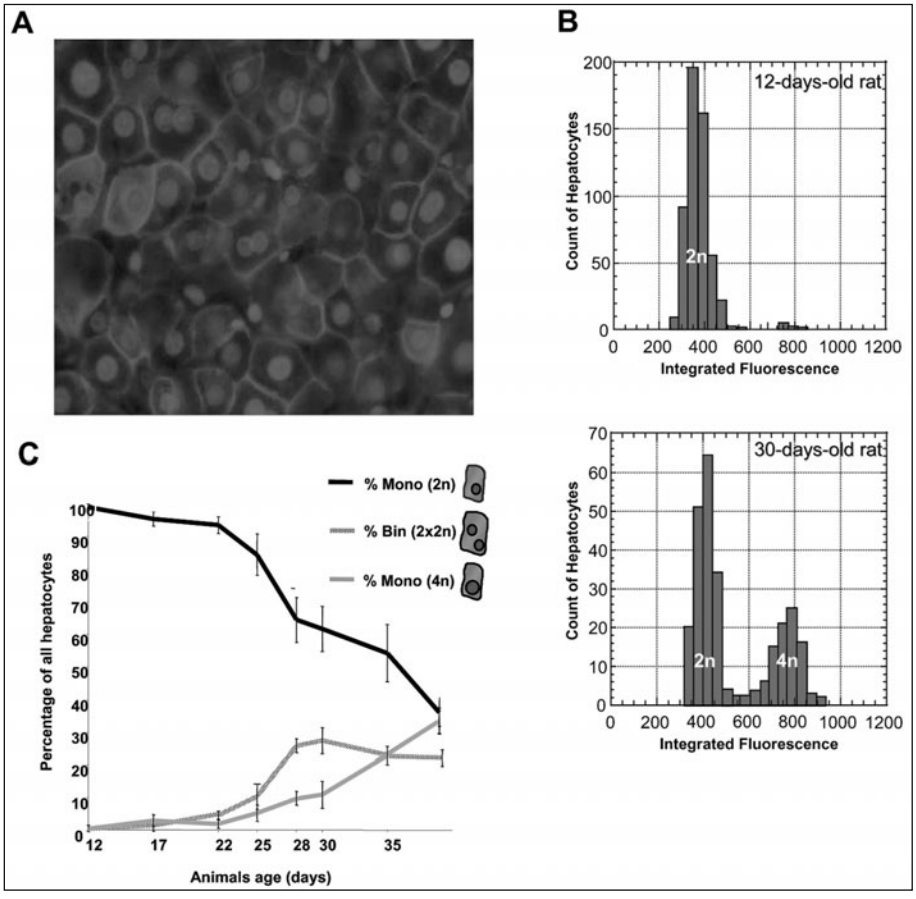

Figure 1. In situ analysis of hepatocyte polyploidy during liver growth. A) Imaging of a liver section after double staining with Hoechst (nuclear labelling) and β-catenin (plasma membrane labelling) enable a distinction between mononucleated 2n, 4n and binucleated 2 × 2n hepatocytes. B) Representative histogram of the DNA content distribution of mononucleated hepatocytes from 12-days-old and 30-days-old rats. DNA content is evaluated by recording the Hoechst integrated fluorescence in each nucleus. Integrated fluorescence is expressed in arbitrary units. The first peak is representative of hepatocytes with 2n DNA content. The second peak is positioned at twice the value of the first peak and is representative of hepatocytes with 4n DNA content. C) Polyploidization during postnatal liver growth: percentages of mononucleated 2n (black line), binucleated 2 × 2n (dark line) and mononucleated 4n (grey line) hepatocytes. The average percentage of each population is shown on the curve.

of binucleated 2 × 2n and mononucleated 4n hepatocytes and between binucleated 2 × 4n and mononucleated 8n hepatocytes.[25] The relationship between DNA content and cell volume is in fact conserved in evolutionarily distant eukaryotes. For example, the volume of budding yeast cells increases linearly with each extra complement of chromosomes.[34] How are polyploid hepatocytes distributed in adult hepatic lobules? Different studies have suggested the existence of ploidy zonation within the hepatic lobules, periportal hepatocytes exhibiting less ploidy and perivenous hepatocytes greater ploidy.[13,35,36] However, recent discrepant results have been reported suggesting that similar proportions of binucleated hepatocytes are present in both periportal and perivenous areas.[37]

Table 1. Distribution (%) of the different hepatocytes ploidy classes

	Hepatocyte Ploidy Classes					
	2n	**2 × 2n**	**4n**	**2 × 4n**	**8n**	**2 × 8n**
Bos taurus	97,3	2,0	0,7			
Equus caballus	96,7	2,3	1,0			
Ovis aries	92,7	4,3	3,0			
Homo sapiens	75,8	14.3	9,6	0,3		
Canis lupus	87,3	6,0	6,7			
Gorilla gorilla	81,3	12,21	5,6	0,6	0,3	
Cavia porcellus	68,7	28,0	3,3			
Talpa europaea	63,7	31,0	5,3			
Sorex araneus	15,0	84,7	0,3			
Rattus rattus	4,0	16,3	67,7	8,7	3,0	0,3
Rattus norvegicus	5,3	2,7	76,0	10,0	6,0	
Mus musculus	5,7	7,3	60,0	17,0	7,7	2,3

The molecular events that cause polyploidy remain elusive. The onset of polyploidy is clearly associated with weaning and independent feeding.[29] Liver polyploidy would be influenced by dietary restriction (DR). The effect of the DR on liver polyploidy has been analyzed by measuring the volume of hepatic nuclei. When dietary restriction is imposed on one group by reducing their food intake to 60% of ad libitum food intake, onset of polyploidization is delayed in food-deprived rats.[38] The same results are obtained if mice are fed with a low protein diet.[39] The endocrine regulation of the growth and polyploidization of liver nuclei has been extensively studied in the past. Forty years ago, morphometric studies revealed that hormones alter mitotic activity and hepatocyte ploidy, the regulation being predominantly carried out by growth and thyroid hormones with modulators effects of sex steroids hormones.[40-44] When summarizing the role for pituitary-mediated hormones, it was suggested that the action of the thyroid hormone is largely mediated through an effect on the release of the anterior pituitary growth hormone (GH).[30] However, more recently two studies have investigated the effect of T_3 in thyroidectomized rats on the ploidy of liver nuclei. A complete cessation of 4C nuclei formation was reported in these rats; treatment of thyroidectomized rats with a single dose of T_3 was sufficient to abruptly increase the percentage of 4C nuclei.[45,46] By contrast, hGH injection has no effect on the 4C nuclei fraction in the hypothyroid rats.[46] These results suggest that the processes of hepatocyte polyploidization are under endocrine control, with thyroid hormones playing the essential regulatory role.

Mechanism of Binucleation and Polyploidization

One fascinating question is how a diploid organism can give rise to polyploid cells in some tissues. In the liver, the cellular mechanisms that govern the passage from mononucleated 2n to binucleated 2 × 2n and/or mononucleated 4n hepatocytes have long been unknown. One explanation for liver polyploidization is that a mononucleated 2n hepatocyte gives rise directly to mononucleated 4n cells through endoreplication. During this physiological process, DNA replication is uncoupled from cell division: the cell undergoes several rounds of DNA replication without mitosis, leading to the genesis of terminally differentiated nondividing autopolyploids cells.[47] This process has already been described in plants, *Drosophila* and mammals, notably in megakaryocyte and trophoblast cells.[47-50] In the liver, such a mechanism has also been described but only in pathological murine models exhibiting an absence or deregulated expression of genes such as p21, S-phase kinase associated protein 2 (Skp2) and excision repair cross-complementing protein 1 (ERCC1). In these mice, endoreplication takes place and induces premature liver poly-

ploidization with an increase in the number of mononucleated polyploid fractions.[51-53] However, this process doesn't take into account the formation of binucleated cells.

An alternative mechanism could be cell fusion, where two mononucleated 2n cells can fuse together and produce one binucleated 2 × 2n hepatocyte. Hereafter, binucleated 2 × 2n hepatocytes may divide, leading to the genesis of two daughter mononucleated 4n hepatocytes. A mononucleated 4n hepatocyte then embarks upon a new round of binucleation/polyploidization with the formation of 2 × 4n and 8n cells. In some cell types (skeletal muscle cells and osteoclasts), cell fusion is a normal developmental programmed step, which leads to the production of terminally differentiated cells.[54,55] In the liver, there is no strong evidences that such phenomenon can occur between two hepatocytes, even if an old study suggests that cultured primary hepatocytes with one or two nuclei can fuse themselves spontaneously and that the frequency of this phenomenon increases with culture time.[56] However the relevance of this phenomenon is controversial and could be attributed to the cell culture conditions, as it has been described for other kind of cell types.[57]

Finally, it was suggested a long time ago that a process of abortive cell cycle could produce binucleated hepatocytes.[20] Generally correlated with pathological proliferation, this particular event is due to a wide variety of defects in different aspects of cell division such as DNA replication, dissolving sister-chromatid cohesion, mitotic spindle function and cytokinesis. Recently, two studies have clearly demonstrated that binuclated hepatocytes have a pivotal role for the establishment of liver cell polyploidization during postnatal development (Fig. 2). In the first study, Guidotti and collabora-

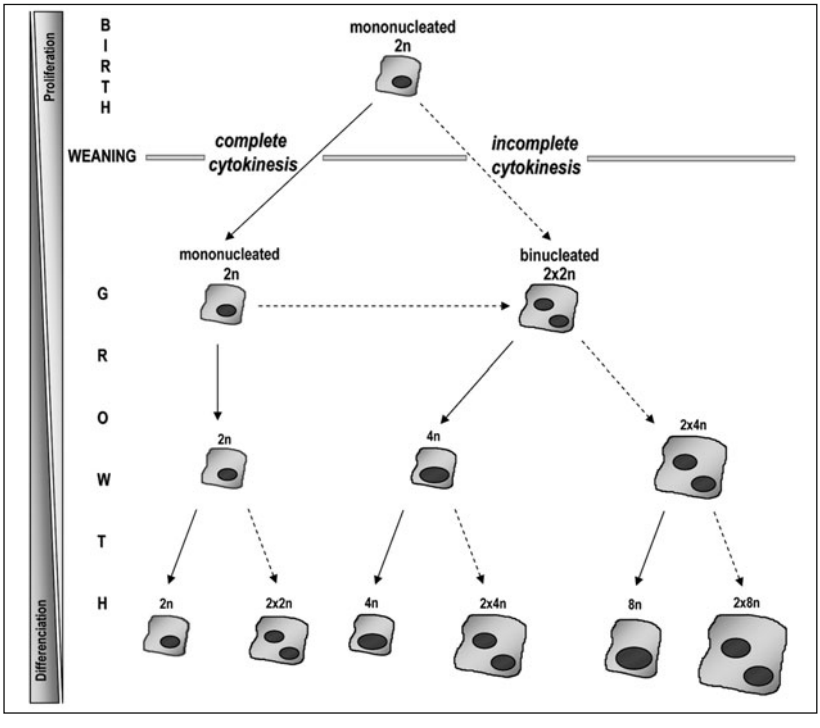

Figure 2. Lineage of hepatocytes of different ploidy during postnatal liver growth. Hepatocytes of newborns are exclusively diploid (mononucleated 2n). At the weaning period, the mononucleated 2n hepatocyte can engage either into a normal cell division cycle (whole arrow) and gives rise to two mononucleated 2n hepatocytes, or follow an adaptive cell cycle with incomplete cytokinesis (arrow in dotted line) and give rise to one binucleated 2 × 2n hepatocyte. During liver growth, progressive polyploidization appears and tetraploid and octoploid cells classes with one or two nuclei are formed.

tors showed that the formation of binucleated hepatocytes was the consequence of a modified cell cycle.[26] Indeed, using a live-cell microscopy approach, they clearly demonstrated that binucleated 2 × 2n hepatocytes derive from mononucleated 2n hepatocytes that have not completed cytokinesis. In another study, the molecular process of the incomplete cytokinesis was deciphered.[37] This specific division program is triggered by weaning. Indeed, in suckling rats, all late telophase hepatocytes

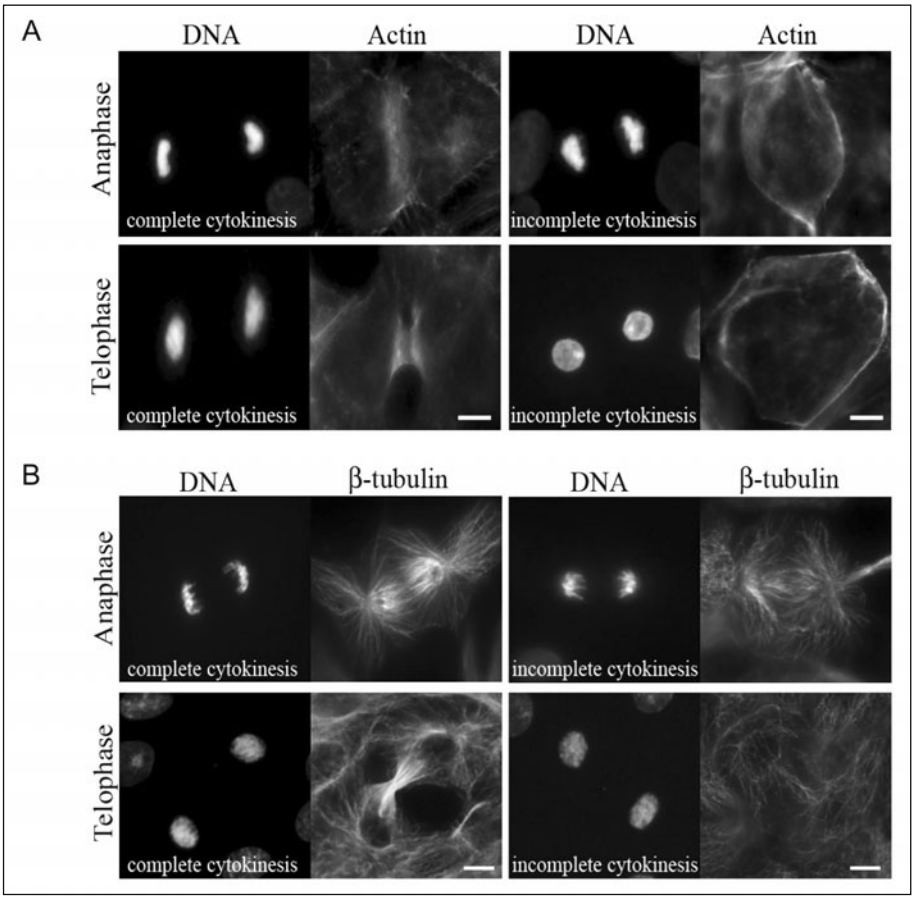

Figure 3. Physiological incomplete mode of cytokinesis in the liver. (Adapted from Margall-Ducos G et al. J Cell Sci 2007; 120:3633-3639)[37]. A) Actin cytoskeleton rearrangement doesn't occur during an incomplete cytokinesis process. When hepatocytes complete cytokinesis, the presence of an actin belt parallel to the cleavage plane is observed in anaphase with its ingression during telophase (left panels). By contrast, during incomplete cytokinesis, the actin belt is always absent, actin cytoskeleton not being able to reorganize at the cleavage plane (right panels). Consequently, during telophase, there is no ingression. Hepatocytes are stained with Alexa Fluor488 phalloidin (actin) and nuclei with Hoechst (DNA). Scale bars represent 5 μm. B) Organization of the microtubules network during incomplete cytokinesis. Staining for b tubulin reveals that the microtubules network is correctly organized in anaphase when hepatocytes complete cytokinesis, microtubules being compressed in the midzone during telophase as consequence of furrow ingression (left panels). When hepatocytes do not complete cytokinesis, the cells present disrupted microtubules network due to the absence of anchorage to the equatorial cortex (right panels). Scale bars represent 5 μm. Reproduced with permission of the Company of Biologists.

present a cell shape characteristic of cleavage furrow ingression.[58] In fed rats, although some late telophase hepatocytes are engaged in a normal cytokinesis process (60%), others present a round shape, indicating an absence of ingression (40%). Following living cells divisions after weaning, the authors established that anaphase cell elongation, a crucial step in the cytokinesis process, is clearly impaired in hepatocytes presenting an incomplete cytokinesis. In fact, the actin cytoskeleton is not reorganized to the cleavage plane during anaphase-telophase transition (Fig. 3A). Moreover, during an incomplete cytokinesis process there is an absence of astral microtubules anchorage to the equatorial cortex inducing a total destabilization of microtubules network (Fig. 3B). Signals transmitted by astral microtubules are not delivered to the equatorial cortex. In this condition, the RhoA pathway, the orchestrator of cytokinesis, is not activated. These findings reveal a new developmental cell division program in the liver which prevents cleavage-plane specification leading to the genesis of binucleated hepatocytes.

In contrast to a transformed cell that does not complete cytokinesis,[59] a binucleated 2 × 2n hepatocyte is able to proceed through a new cell cycle.[26] This cell progresses through the S phase (Fig. 4A) and during mitosis cytokinesis may or may not be completed, leading to the genesis of two

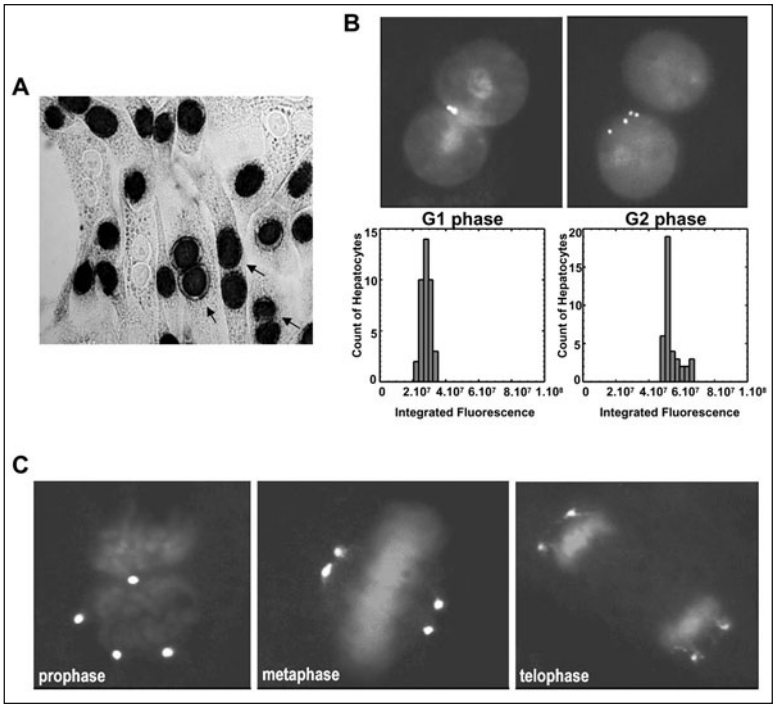

Figure 4. Centrosomes traffic during the division of binucleated 2 × 2n hepatocytes. (Adapted from Guidotti JE et al. J Biol Chem 2003 23; 278(21):19095-101)[26]. A) Binucleated hepatocytes progress through the S phase. Analysis of BrdU incorporation on primary cultures of hepatocytes reveals that DNA replication occurs in binucleated as well as mononucleated hepatocytes. B) A binucleated hepatocyte duplicates its centrosomes. A binucleated hepatocyte displays two centrosomes during G1 phase (γ-tubulin labelling) and four centrosomes during G2 phase. DNA content is evaluated by recording Hoechst fluorescence in each nucleus. The peak DNA content of hepatocyte (2 × 4n) with four centrosomes is positioned at twice the value of one of the hepatocyte (2 × 2n) with two centrosomes. C) Specific migration of centrosomes during mitosis of binucleated hepatocytes. During prophase, the four centrosomes move apart and at metaphase cluster in pairs at opposite poles of the cell. At telophase, centrosome clustering is maintained. Reproduced with permission of the Company of Biologists.

mononucleated 4n hepatocytes or one binucleated 2 × 4n hepatocyte (Fig. 2). Note that animal cells face a specific problem when they become tetraploid: they acquire an extra centrosome that could potentially compromise the assembly of a bipolar spindle during metaphase, contributing to the accuracy of chromosome segregation. Hyperamplification of a centrosome has been observed in many tumor tissues and cell lines and is linked with both aneuploidy and tumorigenesis.[60-65] In order to prevent proliferation or survival of tetraploids cells, the cell has evolved several mechanisms: G1 cell cycle arrest, apoptosis, adaptive silencing of extra centrosomes and specific clustering of these extra centrosomes.[59] Interestingly, binucleated hepatocytes during the S phase correctly duplicate their centrosomes (Fig. 4B). During mitosis these cells formed a unique bipolar spindle, leading to the alignment of all chromosomes on one metaphase plate. This event is driven by a specific clustering of supernumerary centrosomes, two by two at the cellular pole (Fig. 4C). This centrosome clustering is essential to give rise solely to viable polyploid progeny and prevent the genesis of aneuploid cells.[59] In conclusion, the hepatocyte constitutes a particularly interesting model of a ploidy process leading first to binucleated 2 × 2n cells, which then evolve into mononucleated 4n or binucleated 2 × 4n cell. Furthermore, it is quiet fascinating that hepatocytes can adapt to the presence of extra centrosomes. Understanding the mechanism that controls centrosome clustering would be important for cancer biology in order to prevent aberrant mitosis.

Polyploidy in Regenerating Liver and During Pathological States

The adult liver retains a high proliferative capacity. It responds to tissue injuries such as partial hepatectomy, toxin and drug-induced liver disease as well as the administration of a specific growth factor by priming quiescent hepatocytes.[66] During liver regeneration after partial hepatectomy, quiescent hepatocytes undergo one or two rounds of replication to restore the liver mass by a process of compensatory hyperplasia. Many studies have shown that during this process hepatic polyploidy is modified.[6,9,14,20,29] Regenerative liver growth differs markedly from developmental liver growth with the most striking difference being the rapid disappearance of binucleated hepatocytes (Fig. 5). The hepatocellular growth pattern is thus switched to a nonbinucleating mode of growth; nuclei with diploid or tetraploid DNA is converted to tetraploid and octoploid ones respectively.[14] Interestingly, the diploid hepatocytes seem to have a higher tendency that the polyploid ones to undergo several rounds of division.[14] Moreover, after partial hepatectomy, polyploid hepatocytes exhibit senescence-type changes with increased lipofuscin accumulation, β-galactosidase activity (a marker of cell senescence[67]) and accumulation of p21.[14] Different studies have also shown, in adult rodents, that induction of DNA synthesis by different chemicals is associated with disparate changes in liver ploidy and nuclearity profile. Thus, liver growth induced by the mitogen lead nitrate, unlike liver regeneration induced by PH, is associated with cellular polyploidy mainly resulting from an increase in binuclearity.[68] By contrast, an increase in mononucleated octoploid hepatocytes has been described following administration of hepatic mitogens as 1,4-dichlorobenzene[24] and sodium phenobarbitone (PB)[69] and peroxisome proliferators as WY-14 643 and methylclofenapate.[70] All these results suggest that different chemicals may selectively induce DNA synthesis in hepatocytes of one particular ploidy or nuclearity class.

Hepatic polyploidy can also be modified by metabolic overload that induces liver lesions (Fig. 5). It has been reported that Long-Evans Cinnamon (LEC) rats spontaneously develop a necrotizing hepatic injury.[71,72] These rats are deficient in the P-type copper ATPase gene (atp7b), the gene responsible for human Wilson's disease (WD). LEC rats accumulate excess copper in the liver but have decreased levels of serum ceruloplasmin activities, a clinical presentation similar to human WD.[73] Moreover, LEC rats also accumulate as much iron as copper.[74] In this animal's model, hepatocytes present large polyploid nuclei and a delay in mitotic progression has been also observed.[75,76] Similarly, in normal mice the injection of iron-dextran induces liver polyploidization; this effect is inhibited by the oral intake of iron chelator.[77] Oxidative damage to the liver is also associated with a pronounced increase in the population of polyploid hepatocytes. Gorla and collaborators demonstrated that subsequent to radiation, hepatocytes exhibit evidence for oxidative injury with the deletion of intracellular antioxidants (as glutathione and catalase) and for an

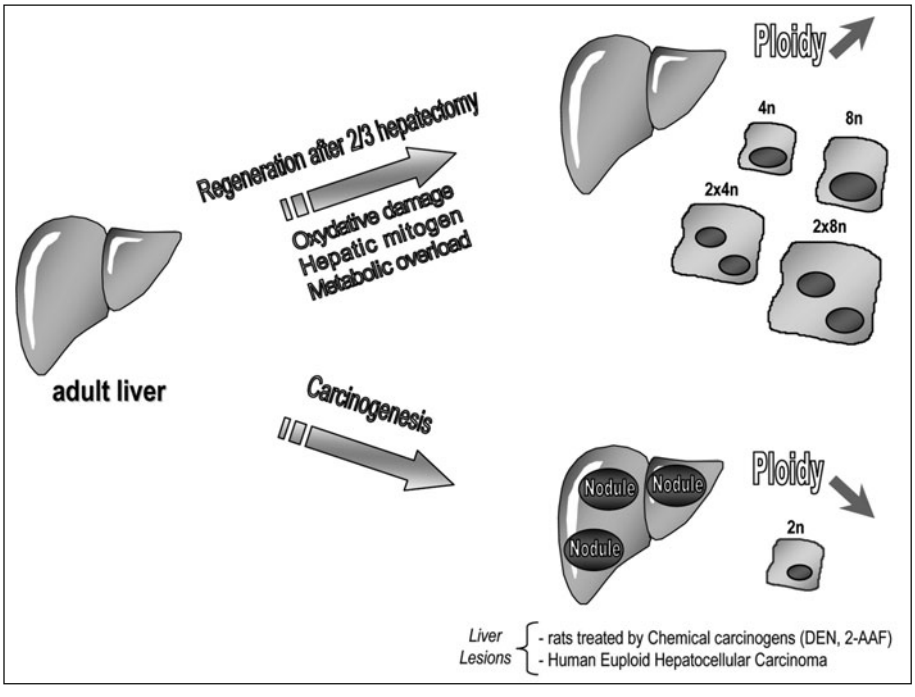

Figure 5. Ploidy modification during regenerative and pathological proliferation. In adults, liver polyploidization is regulated differently upon loss of liver mass and liver damage. Liver regeneration induced by partial hepatectomy leads to the disappearance of binucleated hepatocytes and the formation of mononucleated tetraploid and octoploid hepatocytes. Induction of DNA synthesis by chemicals, oxidative damage or metabolic overload is selectively associated with a pronounced increase in the population of polyploid hepatocytes of one particular ploidy or nuclearity class. Liver lesions induced in the rat by chemical carcinogens (diethylnitrosamine (DEN) and 2-acetyl-aminofluorene (2-AAF)) lead to an overall reduction in liver ploidy and an expansion of the diploid cell population. Increases in diploid mononucleated hepatocytes have been reported in human euploid hepatocellular carcinoma (HCC).

increase of polyploidy.[78,79] In the same line, another study demonstrated that the overexpression of antioxidant enzymes (glutathione peroxidase, Cu, Zn-superoxide dismutase) in transgenic mice decreases hepatocyte ploidy during liver regeneration.[80] All these results argue with the fact that an extensive correlation exists between the generation of polyploid hepatocytes and a variety of cellular stress as it has been demonstrated in other tissues.[59]

Finally and in contrast of what we described above, hepatocellular carcinoma has a lower polyploid fraction compared to an age-matched normal liver(Fig. 5).[81] Thus, liver lesions induced in the rat by chemical carcinogens (diethylnitrosamine (DEN) and 2-acetyl-aminofluorene (2-AAF)) lead to an overall reduction in liver ploidy and an expansion of the diploid cell population which prevails at the different stages of hepatocyte transformation: foci, nodule and hepatocarcinoma.[15,82-86] Studies in humans have also shown a shift towards diploid cell growth during hepatocarcinogenesis. An increase in diploid mononucleated hepatocytes with a decrease in polyploid hepatocytes (including binucleated fraction) have been reported in human euploid hepatocellular carcinoma (HCC).[87,88] Diploid cells are also detected in macronodules (dysplasia, high grade) in cirrhotic livers, suggesting an early shift to diploid cell expansion during hepatocarcinogenesis.[89] Therefore, the selective proliferation of mononucleated 2n hepatocytes could be one of the early events of the liver transformation process. Since a diploid genome would be less protected against recessive

mutations than a polyploid genome, a predominance of diploid hepatocytes may predispose to further progression of the lesions toward increasing malignancy.[84]

Conclusion

The onset of polyploidy in the liver has been described for quite some time. Polyploidization is emerging as an important restriction mechanism for hepatocellular growth. However, the biological significance of this original physiological process remains unclear. Presently, little is known about the function and fate of polyploid hepatocytes. Different hypotheses have been put forward: (i) it may protect cells from genotoxic damage by increasing their gene copy number; (ii) it allows the liver to adapt to aging-related cellular loss and still preserve function; and (iii) it may affect the expression profile of specific genes. Further work concerning this fascinating process will offer insights into hepatic pathophysiology.

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Chapter 9

Analysis of Cellular DNA Content by Flow and Laser Scanning Cytometry

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Abstract

This chapter covers several aspects of methodology of DNA content analysis in individual cells that is most commonly used for assessment of DNA ploidy and for enumeration of cells in particular phases of the cell cycle. Briefly presented are general principles of instrumentation and cell analysis by flow- and laser scanning- cytometry. Described are major methods designed to stain DNA with fluorochromes in live cells, in detergent-permeabilized cells, in cells fixed prior to DNA staining as well as in nuclei of cells isolated from paraffin-embedded tissues. Briefly addressed are approaches to estimate cellular DNA content in conjunction with cellular immunophenotype. Discussed are factors that affect accuracy of DNA content measurement such as: (i) differences in chromatin structure of the analyzed cells that restrict DNA accessibility to fluorochromes, (ii) stoichiometry of interaction between fluorochromes and DNA in chromatin and (iii) chemical mass action law defining dependency of fluorochrome binding to DNA in relation to fluorochrome concentration and number of potential binding sites in a sample. Described also are controls used to ensure accuracy of DNA ploidy determination, the principles in ploidy assessment and possible pitfalls in analysis.

Introduction

DNA content is the most frequently measured entity of the cell. Analysis of DNA content reveals cell ploidy, provides information on cell position in the cell cycle and also allows one to estimate frequency of apoptotic cells that are characterized by fractional DNA content. Distribution of cells within the major phases of the cell cycle is based on differences in DNA content between the cells in prereplicative phase ($G_{0/1}$) versus the cells that actually replicate DNA (S phase) versus the postreplicative plus mitotic (G_2 + M) phase cells (Fig. 1). It is generally accepted that DNA content measured by cytometry (DNA ploidy) is defined as DNA index (DI) and for normal (non tumor, euploid) cells in $G_{0/1}$ phase of the cell cycle DI = 1.0. Cells in G_2/M phase of the cell cycle have DI = 2.0 and the S-phase normal cells are characterized by 1.0 < DI < 2.0. Because extensive DNA fragmentation preferential to internucleosomal DNA sections takes place during apoptosis, the low molecular (mono- and oligo- nucleosomal) DNA fragments are extracted during cell preparation for staining and such apoptotic cells can be identified as the cells with fractional DNA content (DI < 1.0). They are often being defined as "sub-G_1" or "sub-diploid" cell population (Fig. 2).[1-4]

Flow- or laser scanning- cytometry (LSC) are the methodologies of choice for cellular DNA content analysis. They provide the means to estimate DNA content in individual cells in large cell populations rapidly and accurately (Figs. 3,4). Historical progression of development of cytometric

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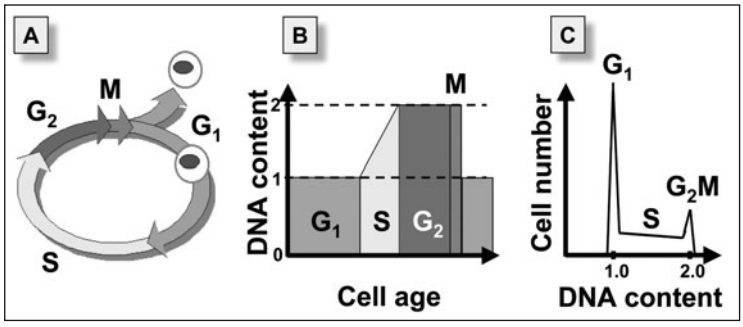

Figure 1. Schematic representation of cellular DNA content changes during cell cycle progression. DNA replication during cell cycle is discontinuous, occurring exclusively during S phase (A), which results that the postreplicative G_2-phase cell has twice higher cellular DNA content compared to the G_1 cell (B). After completion of mitosis (M) the cell divides (undergoes cytokinesis) generating two daughter cells (G_1) each having half DNA content of the mother cell (M). Based on differences in DNA content therefore one can distinguish G_1 from S from G_2M cells. When DNA content is measured in a population consisting of a large number of cells the data can be shown in a form of the cellular DNA content frequency histogram with a characteristic G_1- and G_2M- phase peaks at DNA content DI = 1.0 and DI = 2.0, respectively and S-phase cells are distributed in between the peaks (C).

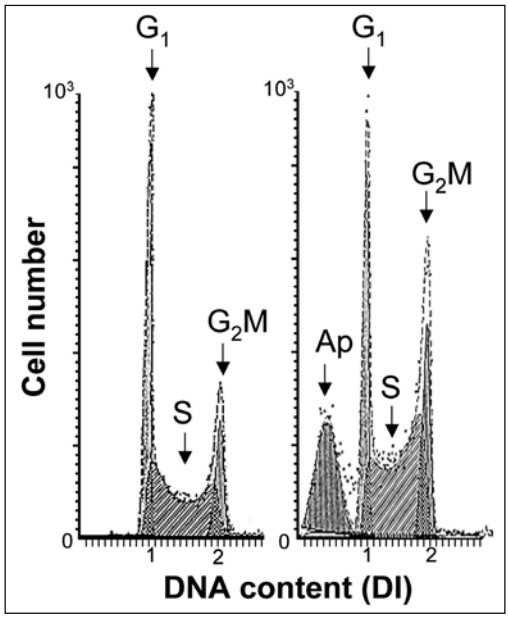

Figure 2. Location of apoptotic cells (Ap) on DNA content histograms. The presence of apoptotic cells manifests as the "sub-G_1" ("sub-diploid") peak on DNA content histograms. This is due to the fact that activation of endonucleases during apoptosis leads to DNA cleavage preferentially at internucleosomal ("linker") sections[1] and the fragmented mono- and oligo-nucleosomal DNA is extracted from cells during their processing and staining.[2,3] It should be noted that apoptotic cells not always have so distinctly lower DNA content and can be well identified. In some instances, particularly if cells in G_2M- and/or S-phase undergo apoptosis, DNA content of apoptotic cells may overlap with that of G_1 cells.[5]

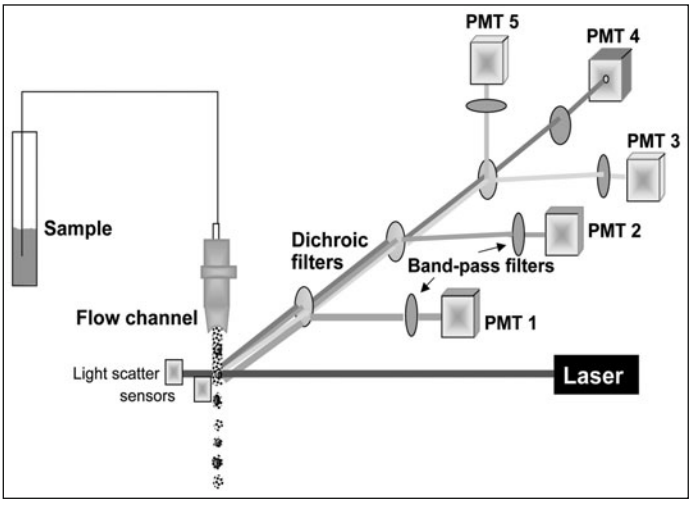

Figure 3. Schematic representation of flow cytometry. Suspension of fluorochrome-stained cells is transported through the cytometer fluidic system in which the individual cells transect the path of laser's beam. Their emission is collected by set of dichroic optical filters which reflect light at a specific wavelength towards the photomultipliers (PMTs) and transmit light at longer wavelength. The band-pass filters located in front of PMTs allow light to pass only at a specific, relatively narrow wavelength range. Intensity of fluorescence emission at these wavelength ranges, integrated over whole cell, is measured by individual PMTs. The light scatter signal generated by the cell when it passes through the laser beam is additionally measured, often at forward and 90° angle ("side scatter"), by separate sensors. The scatter signals provide information about cell size and some morphological features. More than 1,000 cells can be measured per second with an accuracy of fluorescence measurement approaching 1% and sensitivity approaching 200 molecules of fluorescein/cell. Many models of flow cytometers have not one but two or three lasers as excitation source, emitting at UV, blue, green and/or red wavelength. This allows one to select a desired fluorochrome from variety of the available ones. A color version of this image is available at www.landesbioscience.com/curie.

methods and their application for cell cycle and DNA ploidy analysis have been recently reviewed.[5] These methods rely on cells being labeled with a fluorochrome that is expected to stain DNA stoichiometrically and thus to accurately report DNA content. The intensity of DNA-associated fluorescence integrated over the individual cell or cellular nucleus is measured by photomultipliers. The latter offer a wide dynamic range of detection and measurement of fluorescence intensity, much wider compared with the alternative approach of fluorescence measurement, namely the fluorescence image analysis (FIA). A large number of DNA fluorochromes can be used for DNA content analysis and a great variety of techniques have been published during the past three decades.[5] The techniques differ primarily by the mode of cell permeabilization (detergent versus prefixation), choice of the DNA-specific fluorochrome, composition of the stain solution and applicability to different cell preparations.

The results of cellular DNA content measurements are generally presented in the form of frequency histograms (Figs. 2,5). Discrimination of cells in particular phases of the cell cycle based on differences in their DNA content (deconvolution of the histograms) is helped by computer analysis. The software used for this purpose allows one to estimate the percentage of cells in major phases of the cell cycle (G_1, versus S versus G_2/M) as well as the frequency of apoptotic cells with fractional ("sub-G_1") DNA content.[6,7] This software is often included with the purchase of the flow cytometer but is also commercially available (Phoenix Flow Systems, San Diego, CA; Verity Software, Topsham, ME).

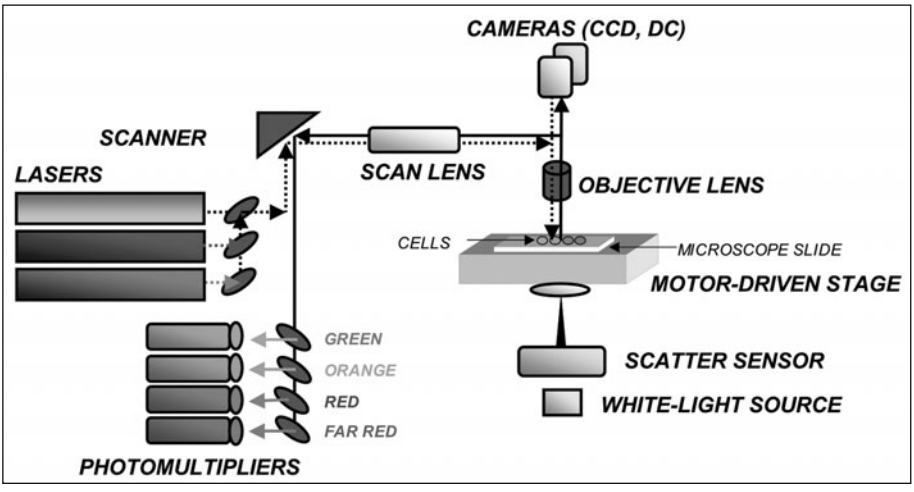

Figure 4. Schematic representation of the laser scanning cytometer (LSC). The microscope is the key part of LSC and it provides structural and optical components. The emission beams from lasers are directed onto computer controlled oscillating mirror, which reflects them through the epi-illumination port of the microscope and images through the objective lens onto the slide. The mirror oscillations cause the laser beam to sweep the area of microscope slide under the lens. The slide is located on the computer-controlled motorized microscope stage which moves perpendicular to the laser beam scan at 0.5 μm steps per each scan. The cell-emitted fluorescence is collected by the objective lens and directed to the scanning mirror. Upon reflection it passes through a series of dichroic mirrors and emission filters to reach one of the PMTs, which records the fluorescence intensity at a specific wavelength range. Laser light scattered by the cell is imaged by the condenser lens and its intensity is recorded by sensors. A white-light source provides transmitted illumination to visualize the objects though an eyepiece or cameras. Up to 100 cells can be analyzed per second with accuracy and sensitivity comparable to that of flow cytometry (Fig. 3). A color version of the figure is available at www.landesbioscience.com/curie.

Supravital Cell Staining

Cellular DNA content can be fluorochrome-stained either in unfixed, usually still live cells, or in the cells following their fixation. Staining of live cells (supravital staining) requires use of a fluorochrome that penetrates the plasma membrane and stoichiometrically stains DNA. Unfortunately, the choice of such fluorochromes is limited. Hoechst 33342 is one of such dyes and when used in combination with the membrane potential sensing dye DiOC5(3) offers relatively good resolution in measurement DNA content of live cells.[8] The dye is excited at UV wavelength (350 nm) and fluoresces in blue (460 nm). Inclusion of DiOC5(3) serves to suppress efflux of Hoechst 33342 from the cell by the active P-glycoprotein pump which otherwise breaks up equilibrium of the binding/staining reaction. Similar effect can be achieved by using the efflux blocker such as verapamil.[9]Another fluorochrome that is being used to supravitally stain DNA is DRAQ5.[10] Its emission can be detected in far-red wavelength (maximal at 670 nm) while the excitation (maximal at 640 nm) is at wide range of the spectrum, stretching down to 488 nm.

The protocols designed to supravitally stain DNA are simple. Generally, inclusion of the fluorochrome into the culture medium for 30-60 min during cell culture is followed by subjecting cells to cytometric analysis without a need for rinsing or centrifugation. However there are cell type (cell line) differences in the rate of the fluorochrome uptake. Occasionally, therefore, several concentrations of the fluorochrome and various time of incubation, different than in the protocols e.g., provided by the vendors of the reagents, have to be tested to optimize staining conditions for

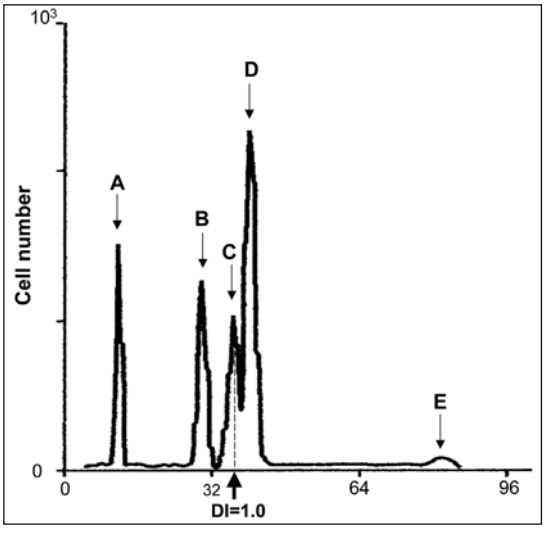

Figure 5. DNA content analysis of human breast cancer biopsy specimen according to the protocol developed by Vindelov et al.[13,14] Cellular DNA content was measured in a sample obtained from a fine needle aspirate of a surgical biopsy of human breast cancer and stained with PI. Chicken erythrocytes and trout erythrocytes were included as internal standard. The peaks from left to right represent chicken (A) and trout (B) erythrocytes, diploid normal nuclei (C; DI = 1.0), hyperdiploid (DI > 1.0) population of tumor $G_{0/1}$ phase cells (D) and G_2 population of tumor cells (E). Under proper conditions of DNA staining, the ratio of the mean DNA content of diploid human cells to chicken erythrocytes is 2.857, the ratio to trout erythrocytes is 1.258 and the ratio of mean DNA content in trout vs chicken erythrocytes is 2.28.[13] Another landmark of linearity in DNA content analysis is the ratio of G_2 to G_1 peaks, which is expected to be 2.0. Modified after Vindelov and Christensen.[13]

a particular cell type. Furthermore, the resolution of DNA content analysis in cells supravitally stained is never as good as that of fixed or detergent-permeabilized cells. The application of protocols designed to supravitally stain DNA is primarily for cell sorting, where the cells selected based on differences in their DNA content can be further subcultured for the purpose of analyzing their growth characteristics, sensitivity to drugs, cloning or expanding their number. It should be noted, however, that exposure of Hoechst 33342-stained cells to UV light during sorting may damage their DNA and be cytotoxic.

DNA Staining after Disruption of Plasma Membrane

Treatment of live cells with detergents causes rupture of the plasma membrane or leads to nuclear isolation which makes DNA accessible to fluorochromes. This approach has been initially used to permeabilize cells to acridine orange, the metachromatic dye that differentially stains DNA and RNA.[11] Exposure of cells to hypotonic salt solution also leads to their lysis and DNA within the nuclei isolated this way is accessible and can be stained with a variety of fluorochromes.[12] Further improvement in the accuracy of DNA content analysis is obtained after controlled proteolysis of detergent-lysed cells. This approach was perfected by Vindeløv and his collaborators who developed a highly accurate method of cellular DNA content measurement, particularly useful for analysis of DNA ploidy in human tumor samples.[13,14] These authors also introduced internal DNA content standards such as nuclei of chicken- and/trout- erythocytes, as intrinsic part of the staining protocol. Their methodology designed for needle biopsy of normal and tumor tissue

is now used worldwide. Figure 5 illustrates DNA content measurement of the specimen of the fine-needle aspirate of human breast cancer specimen by this method.[14]

The accuracy of DNA content measurement for DNA ploidy or cell cycle phase estimate is much greater when isolated nuclei rather than whole cells are analyzed. This is due to the fact that some cytoplasmic constituents may be auto-fluorescent, or contain DNA (e.g., in mitochondria) or nonspecifically stain with DNA-fluorochromes. This background cytoplasmic stainability, thus, lowers accuracy of nuclear DNA determination. Furthermore, the proteolytic step in the Vindeløw's procedure removes some nuclear proteins that are known to restrict the accessibility of DNA to fluorochromes,[15] which additionally leads to improved stoichiometry of DNA staining.[15]

It should be noted, however, that the lysis of plasma membrane of mitotic cells lacking nuclear envelope, that occurs in the detergent or hypotonic-treatment based methods, leads to dispersion of individual chromosomes or chromosome aggregates which are then suspended free in the solution. These methods therefore may not detect mitotic cells, particularly when the cell suspensions are mechanically agitated, pipetted or vortexed. Furthermore, individual or aggregated chromosomes may be erroneously identified as apoptotic cells with fractional DNA content ("sub-G_1" cells). In addition, lysis of apoptotic cells that have fragmented nuclei releases several nuclear chromatin fragments from a single cell. Because each fragment is classified in the cytometer as individual event ("cell") the frequency of "sub-G_1" objects after cell lysis may be much higher than actual frequency of apoptotic cells in a given cell population. This generally precludes application of cell-lysis based methods for analysis of the frequency of apoptotic cells, particularly when mitotic cells are in large proportion (e.g., after arrest in the cell cycle by the mitotic poisons).[16]

DNA Staining in Fixed Cells

Preference for analysis of fixed cells as opposed to the detergent-permeabilized cells is dictated by the need to store or transport samples, for example clinical specimens that cannot be immediately processed. Their storage, unless done at low temperature in cryopreservative media, leads to cell deterioration (autolysis). Fixed cells on the other hand can be stored for months or even years. For analysis of DNA content precipitating fixatives (alcohols, acetone) are preferred over cross-linking agents (e.g., formaldehyde, glutaraldehyde). This is because cross-linking of chromatin constituents impairs stoichiometry of DNA staining with intercalating fluorochromes and thus decreases accuracy of DNA content measurement.[15] It should be noted, however, that highly fragmented DNA such as present in apoptotic cells leaks out from the ethanol-fixed cells during their hydration and staining, but is preserved and remains within the cell upon fixation by formaldehyde. Fixation in ethanol, therefore, rather than in formaldehyde, has to be used to detect apoptotic ("sub-G_1") apoptotic cells. While absolute alcohols or acetone, or a mixture of absolute ethanol and acetone can serve as fixatives (in some instances they may be preferred e.g., for immunocytochemical detection of some antigens concurrently with DNA content) they induce more extensive cell aggregation compared e.g., with 70% or 80% ethanol, which is most commonly used when analysis is limited to DNA content alone.

A variety of DNA fluorochromes may be used to stain DNA in the fixed cells. The most commonly used are 4′,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI) and 7-amino-actinomycin D (7-AAD). Staining with dyes that react with both DNA and RNA, such as PI requires incubation with RNase. The cells may be pre-incubated with RNase and subsequently stained with PI, or RNase (usually at concentration within a range between 10 and 100 µg/ml) is included into a solution containing PI in PBS. In the latter case the cells suspended in that solution are maintained for about 30 min or longer at 37°C or room temperature to allow RNase to digest RNA, before measurement by cytometry. It is of importance that the RNase used is free of DNase activity. If such is not available, one may heat the solution of RNase at 95-100°C for 5 min to destroy DNase- while still preserving RNase- activity. PI is excited in blue light, which is conveniently provided by the 488-nm line of the argon ion laser available on most flow cytometers, while DAPI requires UV or near UV excitation.

Analysis of DNA in Paraffin-Embedded Samples

The method of isolating cell nuclei from paraffin-embedded tissues was developed by Hedley and his colleagues to retrieve archival samples for flow cytometric analysis.[17,18] This methodology enables for retrospective studies to determine the prognostic significance of DNA ploidy or cell cycle distribution (usually frequency of S-phase cells) in tumor progression. The method can also be applied for prospective studies when fresh material is unavailable. One advantage of this methodology is that it offers a possibility to examine by microscopy the tissue sections and thus select the adjacent tumor area of interest to be processed by flow cytometry. The paraffin blocks can then be trimmed to exclude areas of noninvolved tissue in order to diminish the proportion of stromal cells, or of necrotic and hemorrhagic areas to decrease the quantity of debris, as well as to select areas of noninvolved tissue to be used as internal DNA content standard (see further). The accuracy of DNA content analysis of nuclei from paraffin blocks is generally inferior compared to the methods that rely on either ethanol fixation or detergent or hypotonic treatment of fresh tissues. This is due to the fact that the cells embedded in paraffin frequently are usually prefixed in formaldehyde. As mentioned, by cross-linking DNA and proteins formaldehyde fixation impairs stoichiometry of DNA. Because crosslinking by formaldehyde is to some extent reversible, long incubation of the rehydrated nuclei in aqueous solutions, after their isolation from the paraffin blocks, improves resolution of DNA analysis. In nuclei isolated from paraffin blocks DAPI is the preferable fluorochrome since it the least affected, in terms of stoichiometry of DNA staining, by the chromatin structure and thus by protein-DNA crosslinking.[15]

Another factor that lowers accuracy of DNA content analysis and thus identification of aneuploid cells or discrimination of cells in different phases of the cycle in samples of nuclei isolated from paraffin blocks is the presence of debris. Most debris is due to the presence of transected nuclei with incomplete DNA content. Because probability of transecting a nucleus is proportional to thickness of the section and to nuclear size, preparation of thicker sections (≥50 nm) for nuclei isolation is advisable, particularly for tumors with large nuclei such as tetraploid and larger stemlines.

Concurrent Analysis of Cell Surface Antigen and DNA Content

It is often desirable to know the DNA content distribution (histogram) of the particular cell subpopulation identified by its surface immunophenotype. The most common approach, in such a case, is to perform standard immunocytochemical labeling of live cells with the fluorochrome- (most frequently FITC or Alexa Fluor 488) conjugated Ab, which is then followed by short fixing the cells in 0.5-1.0% methanol-free formaldehyde ("paraformaldehyde") in PBS. Because formaldehyde fixation does not adequetely permeabilize the cells it is critical to subsequently have detergent (e.g., Triton X-100) in the staining solution to make DNA accessible to DNA-fluorochrome such as PI or DAPI. Post-fixation in alcohol (methanol or ethanol) following formaldehyde also permeabilizes cells. A gentle fixation with formaldehyde (0.25%) followed by permeabilization in Tween 20 detergent is another procedure designed to preserve both external and internal antigens that can be detected immunocytochemically concurrently with analysis of DNA content.[19] Cellular green (FITC or Alexa Fluor 488) and red (PI) or blue (DAPI) fluorescence is then measured by flow cytometry. During analysis, the cell subpopulation of interest is gated based on its immunophenotype (green fluorescence) and DNA content of this selected subpopulation is then plotted in form of a frequency histogram It is also possible to to combine analysis of DNA content with both cell surface phenotype markers and telomere length.[20]

A simpler approach to concurrently measure DNA content and cell surface immunofluorescence is to combine the supravital staining of DNA with Hoechst 33342 with surface immunophenotyping.[21] For this combination, however, the cytometer with the two- or more- lasers, including one emitting UV light is required. Furthermore, as mentioned, in some cell types it is difficult to obtain high resolution of DNA content analysis after supravital staining of DNA either with Hoechst 33342 or DRAQ5.

Accuracy of DNA Content Measurement

The accuracy of DNA content measurement is reflected by variation in fluorescence intensity between individual cells with identical DNA content, such as G_0/G_1 cells. This variation is being assessed by the value of coefficient of variation (CV) of the mean value of DNA content of the G_0/G_1 cell population. The CV of the DNA-associated mean fluorescence of G_1 cells is thus considered an index of the accuracy of the DNA measurements. High accuracy is required in particular in assessing DNA ploidy to distinguish between DNA diploid and aneuploid cells, which may differ minimally in DNA content. Accurate DNA content measurement is also critical in analysis of cell cycle distributions. There is no formal consensus regarding the acceptable maximal CV value of the mean DNA content of the G_0/G_1 cell population i.e., the acceptable error in cellular DNA content estimate. Most researchers, however, would consider the accuracy to be poor and results unacceptable if CV values of normal, nontumor cells exceed 6%, optimal resolution is achieved when CV is <3%. An exception is analysis of the DNA content of cell nuclei isolated from paraffin blocks, where by the nature of the sample (formaldehyde fixation) good accuracy is difficult, to achieve.

A number of factors can contribute to poor accuracy in DNA content analysis. Most common is inappropriate sample flow and optical adjustment of the flow cytometer. Proper maintenance of the instrument and its careful adjustment prior to analysis, e.g., using fluorescent calibrated standard beads, to maximize the electronic signal intensity and minimize variability of the measurement of the beads, are required to achieve accurate DNA measurements. Problems in sample preparation, either resulting in mechanical damage to the cells or involving incorrect composition of buffers and staining solutions, are another reason of poor resolution in DNA analysis. An excessively large number of cells (DNA) in the sample which leads to significant depletion of the free, unbound fluorochrome in the solution and alters the staining equilibrium (see below), may be still another source of the problems that prevent accurate DNA content analysis. Adjusting samples to achieve a proper fluorochrome to DNA content (cell number) ratio improves the results.

It should be noted that despite good accuracy of DNA content measurements (in terms of proper instrument adjustments and sample staining) the CV of G_1 cell populations may still remains high. This may occur when significant numbers of dead or dying cells are present in the sample, or when the cells were treated with DNA-interacting drugs. Also, in tumors that are polyclonal or have developed drug resistance by gene amplification (e.g., presence of minute chromosomes) the G_0/G_1cell populations may have intrinsically variable DNA content and therefore high CV values of the G_0/G_1cell populations.

Accessibility of DNA in Chromatin to Fluorochromes

The accessibility of DNA to fluorochromes is restricted by chromosomal proteins, predominantly by histones and varies between different cell types. The maximal restriction is seen in cells undergoing terminal differentiation such as during spermatogenesis or erythropoiesis, when DNA stainability (per unit of DNA) is significantly lower compared with other cell types.[15,22] This obviously creates difficulties in assessment of DNA ploidy in such differentiating cells. The degree of reduction varies for individual fluorochromes and DAPI is the least influenced by chromatin structure whereas binding of 7-AAD, an intercalating but more bulky fluorochrome, is affected to a much larger degree. In practical terms, therefore, one may expect intercellular variation in DNA stainability when mixed cell types are measured in the same sample. This can be manifested on DNA content frequency histograms as the presence of pseudo-aneuploid populations, or widening of the G_1 peak (increased CV value). For example under certain conditions of staining monocytes show higher DNA stainability with PI compared to lymphocytes or granulocytes and form a typical pseudo-hyperdiploid peak on DNA frequency histograms. As mentioned, subjecting cells to the detergent methods and in particular the combination of detergent and proteolytic treatment such as in the Vindeløv's procedure,[13,14] increases accessibility of DNA and thereby improves the stoichiometric relationship between DNA content and fluorescence intensity.

There are several ways to estimate stoichiometry of DNA staining. Thus, fluorescence intensity of the cell populations represented by the G_2/M peaks on DNA histograms is expected to be DI = 2.0 as compared to DI = 1.0 for $G_{0/1}$ cells and deviation from this value indicates on problems in DNA quantification.[23] Normal hepatocytes grow at different DNA ploidy levels and therefore may also serve as markers of linearity in DNA measurement. Inclusion of internal standards such as chicken or trout erythrocytes provides still another marker of the stoichiometry of DNA measurement and is highly recommended when DNA ploidy is estimated.[13,14] To demonstrate stoichiometry of DNA staining one has to use linear and not exponential scale for plotting intensity of DNA-associated fluorescence (x-coordinate) and include the origin (point zero) of this coordinate, on the DNA content frequency histogram.

In some instances, however, stoichiometry in DNA staining with fluorochromes cannot be attained. This can be seen when cells were treated with antitumor drugs that modify DNA and/or chromatin structure. Intercalating drugs that interact with DNA fluorochromes by fluorescence resonance energy transfer (FRET), or drugs damaging DNA structure, or crosslinking chromatin, all can alter staining properties of in situ DNA, often in unpredictable ways. As mentioned, the possibility of stoichiometric measurement of DNA content may also be hampered when cells differing markedly in chromatin structure are being compared.

Fluorochrome Binding to DNA—Mass Action Law

Staining of cellular DNA is being done at equilibrium between the ligand (fluorochrome) and the ligand-binding sites in the DNA within the cells sample and thus it follows the chemical law of mass action. Stable level of staining is achieved when there is large excess of the ligand per binding site so a small variation in cell number per sample (binding sites) has no significant effect on the equilibrium. Because it is difficult to have an identical cell number in each sample, the variation is inevitable. One can calculate however approximate concentration of the fluorochrome and relate to the cell number to find out the range within which a decrease in free ligand concentration may not significantly affect DNA stainablility.There are 3×10^9 DNA base pairs per cell (diploid cell in G_1). Most intercalators such DNA-binding fluorochromes, reacting with free (naked) DNA at saturation bind every second base pair. Thus, potentially, in a single diploid cell there are ~1.5×10^9 binding sites. However, because a large portion of nuclear DNA within the cell is inaccessible to the intercalators,[15] only a fraction of the potential binding sites (10-70%, depending on the fluorochrome) can actually bind the ligand (fluorochrome). Thus, there are between 1.5 to 10.0×10^8 sites that actually bind the ligand in a single cell and therefore 1.5 to 10.0×10^{14} binding sites in 10^6 diploid cells, which is approximately a size of average sample subjected to staining. Assuming average MW of most DNA fluorochromes to be about 300, one can estimate (from Avogadro number) that at a concentration 100 μM (30 μg/ml) there are 6×10^{16} molecules of the ligand in 1 ml of the stain solution. Considering the above there is nearly 100-fold excess of the ligand per binding site when 10^6 cells are stained in 1 ml volume at 100 μM dye concentration. One would expect that under these conditions a change in cell number from 1 to 2 million (which alters a concentration of the free, unbound ligand by 1%) should not be reflected by greater than 1% change in stainability of DNA. However, at lower dye concentration (e.g., below 20 μM) or when cell number is drastically changed (e.g., from 1 to 5 million) the change in DNA stainability becomes noticeable. Needless to say, if cells have higher DNA content, (tetraploid, arrested in G_2/M) the equilibrium is shifted even more towards lesser concentration of free dye, which leads to further decrease in DNA stainability. The above estimates have to taken into an account when samples with different cell number are stained to compare DNA ploidy.

Assessment of DNA Ploidy

As mentioned, DNA content measurement by cytometry serves to estimate frequency of cells in particular phases ($G_{0/1}$ versus S versus G_2M) of the cell cycle as well as to assess DNA ploidy. In most situations DNA ploidy is being assessed in hematological or solid tumors; the evidence of aneuploidy by itself is a definitive marker of a presence of the tumor. Often is also considered to

be an prognostic indicator of tumor progression and outcome of the treatment. To assess DNA ploidy of the tumor sample one has to compare DNA content of the $G_{0/1}$ cells population of the presumed tumor cells with that of normal (control) cells. Towards this end most frequently the peak value of the integrated fluorescence (peak channel) of $G_{0/1}$ population of normal cells is being considered to be DI = 1.0 and DNA ploidy of the tumor cells is expressed as a ratio of the peak value (channel) of fluorescence intensity of these cells with respect to that of the normal $G_{0/1}$ cells. It is also common to express DI of the tumor as a ratio of modal rather than the peak value of fluorescence intensity representing DNA content of $G_{0/1}$ population tumor cells to modal value of $G_{0/1}$ population of normal cells. Some authors still prefer to use the mean values of fluorescence intensity of $G_{0/1}$ population rather than the peak or modal values to obtain this ratio. In essence, when DNA measurement is done correctly and accurately, either of these appropaches is expected to yield similar estimate of DI of aneuploid cells.

Normal lymphocytes, including lymphocytes from the same patient whose tumor is being analyzed, or fibroblasts, are often used as standard of DI = 1.0. For comparison with the tumor it is necessary to use normal cells both as external and internal control standards. When used as external control they have to be subjected to identical processing and staining procedure and measured by cytometry under identical laser and detector settings as tumor sample. The external control cells should be measured prior to- and also after- measurement of tumor sample. This double-measurement of control cells allows one to detect the possible shift in fluorescence readout e.g., due to misadjustment in instrument settings in the course of the sequential measurements. In addition to external control, normal cells should also be admixed (e.g., in 1:1 proportion) with the tumor sample cells and used then as internal control in another set of measurements. Often, normal stromal- or tumor infiltrating cells are already present in the tumor sample and they can be used as an internal control of DNA ploidy. In fact, when DNA ploidy is assessed based on measurement of nuclei isolated from paraffin blocks, the internal control provided by the presence of stromal and infiltrating normal cells that provide standard for DI = 1.0 is the only way to assess DNA ploidy of the tumor. This is due to the fact that DNA stainability after formaldehyde fixation and paraffin embedding is markedly altered making external standards useless.

Chicken and trout erythrocytes have been proposed as internal standards for analysis of DNA content by cytometry (Fig. 5).[13,14] Their use is helpful to control and maintain consistency of the staining and measurement procedures. However, one has to be cautious using them as absolute standard for DNA content analysis. Trout, like other fish species, are known to vary in their DNA ploidy level (most species are tetraploid) and it is therefore important to know ploidy of these cells when used as a standard.

Despite the difficulties and potential pitfalls outlined in this chapter, cytometry, including flow cytometry and LSC, is the methodology of choice in analysis of DNA content for DNA ploidy assessment. This methodology is complemented by fluorescence in situ hybridization (FISH) analysis, which provides a possibility to identify individual chromosomes or chromosome components contributing to aneuploidy. It should be noted that LSC, by offering rapid and semi-automatic enumeration of the fluorescent foci within individual nucleus in addition to DNA content measurement can also be used for FISH analysis.[26]

Conclusion

As outlined in this chapter variety of methods are available to estimate cellular DNA content by flow- or image assisted- cytometry. They offer wide choice of analytical capabilities to assess DNA ploidy and cell cycle distributions. The possibility of use of diverse fluorochromes differing in absorption and emission properties as well as in mode of binding to DNA provide the means to optimize DNA content measurement for different cell types. The principles of DNA staining and data analysis described in this chapter will be of assistance for the beginners who initiate DNA content analysis by cytometry. It may also provide useful information for advanced researchers who contemplate change in the methodology to further improve accuracy in assessment of DNA ploidy and cell cycle distribution.

Acknowledgement

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Chapter 1

Polyploidy, Aneuploidy and the Evolution of Cancer

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Abstract

Aneuploidy is a ubiquitous feature of cancer and pre-cancerous lesions, yet its significance is poorly characterized. In this chapter, we review the role of tetraploidy and aneuploidy in progression. We examine how aneuploidy may contribute to the evolutionary dynamics prevalent in neoplastic progression, considering whether aneuploidy itself is selectively neutral or advantageous or if it simply acts as a mechanism for the more rapid accumulation of mutations increasing survival and reproduction of cancer cells. We also review evidence from Barrett's esophagus, a pre-malignant condition, demonstrating that tetraploidy and aneuploidy are correlated with an increased risk of progression to cancer. Ultimately, we aim provide testable hypotheses and methods for understanding the role of aneuploidy in cancer.

Introduction

Most cancers cells are aneuploid, meaning they contain the wrong number of chromosomes. Aneuploidy entails the loss or gain of individual chromosomes or large sections of chromosomes and is defined here as distinct from polyploidy, which involves extra copies of the entire genome, such as triploidy (3N) or tetraploidy (4N). Large-scale chromosomal amplifications and deletions in cancers have been demonstrated using a variety of methods, including comparative genomic hybridization (CGH), karyotyping and fluorescence in situ hybridization (FISH).[1-3]

The frequency with which aneuploidy is observed in cancer leads to a series of important questions: How do cancers become aneuploid? What genes are being targeted by the amplifications and deletions? How can cells survive with such massive perturbations to their genomes? How has the selective pressure of cancer shaped our genomes to sense and respond to these perturbations? Most of these questions have received little attention to date and the answers remain largely unknown. To facilitate research on these questions, we review what is known about each in an effort to frame the questions and hypotheses more precisely and propose methods that might be used to reach an answer.

The Tetraploidy to Aneuploidy Progression in Carcinogenesis

Abnormalities in chromosome content were observed to be common in tumor cells at least 120 years ago.[4] The hypothesis that genomic instability could result from whole genome doublings (tetraploidy) and that this could play an important role in cancer was made more than a century ago by Theodor Boveri.[5] For many years these observations were neglected, as aneuploidy and

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tetraploidy were commonly held to be incidental to tumor evolution. However, within the past several decades the importance of chromosomal abnormalities and chromosomal instability has risen. When aneuploid DNA content became readily identifiable by flow cytometry, it was noted that this finding was more common in higher grade cancers and that aneuploid tumors of many kinds had a more aggressive clinical behavior than their diploid counterparts.[6,7] Today, some authors argue that the evidence points to aneuploidy playing a pivotal role in the chromosomal instability that generates tumor diversity, clonal evolution and malignant phenotypes.[8]

While aneuploidy could in principal be generated by progressive additions to the diploid DNA content by accumulated chromosomal gains, as by mitotic nondisjunction, it would then be puzzling that aneuploid tumor DNA contents are most commonly in the triploid to tetraploid range.[9] Boveri's hypothesis allows that a tetraploid intermediate is a common precursor to aneuploidy and that subsequent chromosomal evolution by loss of superfluous chromosomes or chromosome segments results in the aneuploid chromosomal complement. A conceptual model of the role of the tetraploid intermediate in carcinogenesis was formalized by Shackney et al.[10] Supporting experimental evidence comes from observations of a tetraploid intermediate during murine carcinogenesis.[11,12] Furthermore, when diploid and tetraploid mouse cells from a common mammary precursor were directly compared, the tetraploid cells had greater chromosomal instability and only the tetraploid cells gave rise to malignant tumors when transplanted into nude mice.[13] Perhaps most significantly, tetraploidy has been demonstrated to be a precursor of aneuploidy in several human cancers, including Barrett's esophagus (see below) and cervical carcinoma.[14] The mechanisms that underlie generation of the tetraploid state are now recognized to include the failure of cytokinesis and, in particular, failure of checkpoint control during mitosis.[15] Loss of p53 function plays an important role in augmenting this process, as failure of p53-dependent G1 checkpoint and DNA repair commonly result in G2/M checkpoint arrest; failure of this latter checkpoint, or accommodation or "slippage," allows cells to reenter the cell cycle with a failure of cytokinesis, resulting in tetraploid G1 cells.[15,16]

Tetraploidy and Aneuploidy in Barrett's Esophagus

It is difficult to determine the role of polyploidy and aneuploidy in the development of cancer because most cancers cannot be studied longitudinally. When we detect a neoplasm we either remove it or, if it has metastasized, treat it systemically (which may generate additional aneuploid cells). The same is true for most premalignant neoplasms. This prevents us from studying the effects of ploidy changes on the further development of the neoplasm and from making direct observations of the ordering of events in progression. An important exception is Barrett's esophagus (BE).

Barrett's esophagus is a premalignant neoplasm[17] that predisposes for the development of esophageal adenocarcinoma (EA).[18] Characterized by the presence of specialized intestinal epithelium in the esophagus, it can be recognized endoscopically as a salmon-colored epithelium just above the gastro-esophageal sphincter. Only about 0.5% of people with BE progress to EA per year and most people with BE will die of some other cause.[19] Unlike other premalignant neoplasms, such as an adenomatous polyp in the colon, BE is not removed when detected. Esophagectomies have an 8%-23% mortality rate[20] and thus the risk of progression to EA does not justify the risk of removal of the BE segment. Instead, the standard of care is surveillance with periodic endoscopic biopsies for the early detection of cancer. If EA is detected in an intensive surveillance program, it is often caught prior to metastasis and patients can be treated surgically. For these purely clinical reasons, BE presents a scientific opportunity to study the genetics of how a neoplasm changes over time as it progresses to cancer.

We study BE as a model of neoplastic progression in solid tumors. Aside from the danger of removing it and ease of biopsying it, BE is similar to many other conditions that predispose to carcinogenesis in a variety of respects. Like inflammatory bowel disease, hepatitis, pancreatitis, prostatitis, *H. pylori* infection in the stomach and *Schistosomiasis* infection in the bladder, BE is characterized by chronic inflammation.[21] Similar to other premalignant conditions, only a minority of patients with BE progress to cancer. In addition, neoplastic progression in BE is characterized by some of the most common genetic lesions across all cancers: loss of the tumor suppressor genes p16 (INK4A/CDKN2A) and

p53 (TP53) and the development of tetraploidy and aneuploidy. Studying BE provides us the major advantage of observing the development of these lesions over time. What have these longitudinal studies taught us about the role of polyploidy and aneuploidy in neoplastic progression?

p16

The first genetic and epigenetic lesion commonly observed in BE is loss of the p16 tumor suppressor gene. Tlsty and colleagues have argued that loss of p16 leads to decoupling of the synthesis of DNA and centrosomes in the cell cycle, such that if either is delayed, the cell might enter mitosis with the wrong number of centrosomes or the wrong amount of DNA and aneuploidy could result.[22] It is unclear if this happens in BE. To date, the association between the loss of p16 and ploidy abnormalities has not been adequately studied. We do know that patients can live for many years lacking p16 in their BE neoplasm but never develop aneuploidy.

p53

The genetic lesion that has been associated with the development of both tetraploidy and aneuploidy in BE is loss of the p53 tumor suppressor.[23,24] Our current hypothesis is that although aneuploid cells may arise in a p53 wildtype clone, they normally trigger the p53-dependent DNA damage checkpoint which either leads to senescence or apoptosis and so the aneuploid clone never grows large enough to be sampled. Once the p53 checkpoint is compromised, aneuploid clones are free to proliferate without check. This is why we believe that the loss of p53 precedes the development of both tetraploidy and aneuploidy. Loss of heterozygosity at the p53 locus is also the strongest single predictor of progression and is associated with a 16-fold increased risk of progression to EA[24] as well as a 6-fold increased risk of developing tetraploidy and a 7.5-fold increased risk of developing aneuploidy.

Tetraploidy

Tetraploidy, defined in this case as greater than 6% of cells with 4N DNA content, is also a predictor of progression associated with a 12-fold (95% CI: 6.2-22) increased risk of progression to EA.[25] Sometimes this may be an indication of cells being stalled in the G2 phase of the cell cycle. Other times, the presence of 8N cells in cell cycle analysis suggests that there are viable tetraploid cells in the neoplasm. FISH studies have found that loss of heterozygosity in p53 as detected by microsatellite analysis could be caused by deletion of one allele of p53 or, more often, by duplication of the genome followed by deletion of multiple p arms of chromosome 17 where p53 resides.[26]

Aneuploidy

Most cases of aneuploid clones in BE have DNA content between diploidy and tetraploidy further suggesting that tetraploidy is an intermediate stage of progression followed by selective loss of parts of the genome. This appears to be true of other cancers as well.[9,27-29] We have compiled a survey of 57 esophageal adenomas that were surgically removed prior to therapy and analyzed for DNA content in our study (Fig. 1). This new data agrees with our previously published data[25] that hypodiploids and supratetraploids are rare.

The detection of an aneuploid clone in BE is associated with a 9.5-fold (95% CI: 4.9-18) increased risk of progression.[25] However, the presence of both tetraploidy and aneuploidy is an indication of greater risk of progression than either alone[25,30] and may be a sign of more extensive genomic instability.

It should be noted that in BE, at least, the loss of p53 and the development of aneuploidy is not sufficient to cause cancer. In contrast, loss of p53 is thought to cause malignancy in colorectal carcinogenesis.[31] Although BE patients with both a p53 lesion and a ploidy lesion (either tetraploidy or aneuploidy) are at a very high risk of progressing to cancer, that process can still take years.[30] So there must be other loci that are being targeted by the gains and losses during the further evolution of aneuploid clones. Hopefully, genome-wide analyses of aneuploid BE and EA will reveal the final genetic lesions that cause invasion and metastasis.

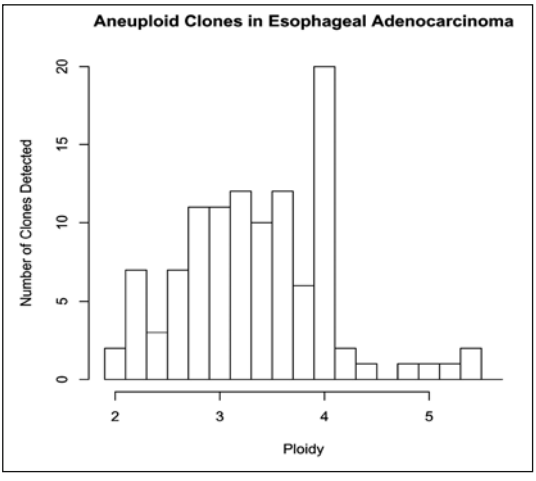

Figure 1. 55 out of 57 esophageal adenocarcinomas in the Seattle Barrett's Esophagus cohort were aneuploid or tetraploid. We detected 107 different aneuploid clones (and 2 diploid clones) in these 57 neoplasms. The frequencies of the different ploidies (2 = diploid, 4 = tetraploid) are shown in the histogram.

Not All Aneuploids Are Equal

Every aneuploid clone is unique. Each contains its own set of gains and losses, some of them probably random, others selected because they gave the clone a competitive advantage in the microenvironment of the neoplasm. Thus, it may be misleading to speak of aneuploidy as a "state". We should expect different aneuploid clones to behave differently during progression and therapy.

It is tempting to think of polyploidy as a state that should not substantially affect the fitness of a cell because the gene dosage is amplified equally across the entire genome. However, that would only be true if the expression of genes was a linear function of concentrations of regulatory proteins. Instead, genetic regulatory interactions, including posttranscriptional modifications, multi-mer regulatory complexes and the interactions of enhancers and inhibitors, are all highly nonlinear. Thus, a gene's expression level may actually decrease with the doubling of the genome if it is overwhelmed by the doubling of an inhibitor gene.[32,33] The effect of increasing the ploidy of a cell on its protein concentrations may be equivalent to genome-wide sequence mutation events. These nonlinear interactions may explain the lethal developmental abnormalities associated with ploidy irregularities in human embryos. Though in most cases gene expression levels do seem to be correlated with gene copy number.[34,35] In fact, the story may be even more complicated because the phenotype of the cell may depend on how it became polyploid. A study in a BE cell line that reliably produces a tetraploid sub-population showed evidence that the cells had somehow passed through mitosis without actually dividing and expressed genes characteristic of both mitosis and G1.[36]

Clones that become malignant may derive from tetraploid cells, for the reasons described earlier, including the fact that tetraploid cells have the genetic buffer that allows them to lose large chunks of their chromosomes and thereby select for different gene dosage effects while retaining enough copies of essential genes to maintain the viability of the cell. Diploid cells that start losing large portions of their genome are probably more likely to suffer a fatal genetic lesion.

If this view is correct, then aneuploidy is a crude mechanism to change gene expression levels. It is crude because copy number alterations often affect large regions of a chromosome, sometimes whole chromosomes and so affect many genes at once. We would predict that some aneuploid states would produce gene product levels that result in a higher fitness for an aneuploid cell than a tetraploid cell. Specifically, we would predict that an aneuploid clone should have a higher

fitness and expand faster than a tetraploid clone from the same neoplasm. This may explain the apparent transient nature of tetraploidy in BE and could be tested in competition experiments in tissue culture.

Because the regions of copy number change affect so many genes in parallel, it is difficult to determine which among those genes are responsible for any increase in fitness of a clone. While gene mapping studies can help pinpoint specific regions commonly amplified or deleted (e.g., location of p16, p53), in general we need better tools to measure the fitness effects of gene deletions and amplifications to clarify the selective effect of copy number changes.

Amplifications and deletions of a few specific genetic regions are known to be associated with cancer, but thus far a census of copy number changes across cancers has not been compiled. There are a variety of challenges associated with such a task. First of all, it is not clear how to calculate the frequency of a copy number change at a locus. Should it be the frequency across cancer patients, in which case lesions in the most common cancers would dominate the results, or should it be the frequency across sub-types of cancer, such that a lesion that appeared in 50% of a rare type of cancer would be weighted equally with a lesion that appeared in 50% of a common type of cancer? Different sub-types of cancer, even within a particular organ system, are likely to have different patterns of copy number changes across the genome. Many CGH studies have not segregated sub-types of cancer since we are still in the process of distinguishing new sub-types. Defining these different sub-types is important because cancers originating in different cell types and organs experience different microenvironments and thus different evolutionary pressures that select for copy number changes in different parts of the genome. Experimentally, if a cancer is aneuploid it is critical to normalize CGH arrays by the number of cells not the amount of DNA, else the average fluorescence reading in a CGH study would appear to be diploid while true diploid regions would be identified as deletions. CGH array studies are also sensitive to the amount of contaminating stroma than can obscure copy number changes in the cancer. Of course, different studies have used different platforms, so any compilation of current studies probably could not resolve copy number changes below the chromosome arm level. If the cancer genome project is successful, it should solve the problems of low resolution and incompatible technological platforms. In fact, the ability to find consistent patterns of genetic alterations in cancer is one of the justifications for the cancer genome project. A further important result of a comprehensive census of copy number changes in cancers would be a determination of which amplifications and deletions are not seen in cancers. These alterations are likely to be deleterious to a neoplastic clone and might lead to the development of new therapies.

Why Do Cancer Cells Survive with Such Massive Alterations to Their Genome?

The genomic instability that characterizes neoplastic progression often involves gains and losses at the scale of chromosome arms and even whole chromosomes. Thus, hundreds of genes are often duplicated or deleted in a single event. In most biological (and technological) systems, a mutation in a functional part is deleterious. How can a cell survive the massive alterations seen in cancers?

Amplification of a gene, while often increasing expression level of the gene, does not necessarily increase the protein level of that gene. There is evidence that if a gene is normally part of a protein complex and the other members of the complex are not over-expressed, the over-expressed, uncomplexed protein may be quickly degraded, resulting in normal protein levels.[34] Thus, cells may be robust to many amplification events.

Devoting metabolic effort for the benefit of the organism and suppressing cellular proliferation are disadvantageous for a neoplastic cell. Those cells that can reproduce faster, avoid differentiation, suppress apoptosis, stabilize their telomeres and stimulate angiogenesis when hypoxia becomes a problem will proliferate faster than competitors that lack those hallmarks of cancer.[37] At the initiation of a neoplasm, cells may not be adapted to the selective pressures of the neoplasm. It is quite possible that many mutations would be beneficial rather than deleterious because they result in the dismantling of the genetic machinery that keeps proliferation in check in a multicellular body.

Perhaps most of the genome of a multicellular organism is devoted to building and maintaining the organism rather than the individual cells[38] and so even large scale copy number changes may not be deleterious for a neoplastic cell.

This hypothesis, that large scale deletions in cells are much more likely to be beneficial to the cell than to an embryo, could be tested experimentally. One could experimentally delete chromosome arms by using the Cre-lox system of site-directed recombination[39-41] (and perhaps even whole chromosomes if one deleted the centromere). The fate of the cell as an orthotopic injection or as an embryonic stem cell could then be compared to wildtype cells. Expression of Cre under an organ-specific promoter would help test the effects of deletions in different organ contexts. It would be important to do the complementary experiment, testing the selective effects of amplifying whole chromosomes or chromosome arms. A recent study generated yeast strains with extra chromosomes through abortive nuclear fusions during mating and showed that an extra copy of a chromosome caused a decrease in fitness, regardless of which chromosome was duplicated.[34] We are not aware of an experimental method to carry out an equivalent study in mammalian cells, though fibroblasts from Downs syndrome patients (trisomy of chromosome 21) grow more slowly than fibroblasts from age-matched controls.[42]

Aneuploidy in Development

Certainly during development most inherited copy number changes are deleterious. The selective effect of ploidy changes appears to be fundamentally different in cancer as compared to ploidy abnormalities present in the germ line. During development, ploidy abnormalities are nearly always lethal. Trisomy (3n) of the entire genome, usually caused by the fertilization of a single egg by two sperm, is lethal, as is tetraploidy (4n), generally caused by the failure of the zygote to divide. Monosomy of individual chromosomes is invariably lethal at a very early stage in gestation, as is trisomy of all but a few chromosomes.[43]

Aneuploidy is common in human embryos and is the leading cause of miscarriage.[44] For unknown reasons, humans have a 10-fold higher rate of aneuploidy compared to other mammals.[45] A few duplications do lead to viable offspring, occurring as rare genetic disorders. Trisomy of chromosomes 13, 18 and 21 are the only whole-autosome trisomies compatible with survival until birth and only trisomy of chromosome 21 (Downs syndrome) allows for survival into adulthood. All are associated with significant developmental abnormalities. Trisomy of chromosome 13 (Patau syndrome) is associated with limb and facial abnormalities, heart and kidney defects and neurological abnormalities including failure of the brain to divide into halves during gestation (holoprosencephaly). Trisomy of 18 (Edwards syndrome) also leads to severe developmental abnormalities.[46] In addition, there are a variety of other conditions associated with ploidy abnormalities (loss or duplication) of various chromosome arms or smaller sections of chromosomes, including trisomy of chromosome arms 10q, 16p and q and monosomy disorders such as 4p- (Wolf-Herschhorn syndrome) and 5p- (cri-du-chat syndrome). While many of these abnormalities have been described, only a few have been genetically characterized. For instance, a 17p- monosomy known as Smith-Magenis syndrome is specifically caused by deletion of the RAI1 (retinoic acid induced 1) gene. The exact function of the gene is unknown, but it is believed be a transcriptional regulator critical for neurological development with disease symptoms the result of haploinsufficiency.[47]

So what is different about cancer that allows for rampant aneuploidy? A copy number change that affects every cell in the body may be likely to have catastrophic effects in at least one organ. In contrast, after development if a new copy number mutation occurs within a single organ, it may be unlikely to be immediately fatal. In fact, cells in any particular organ may not utilize many genes in the genome and so large deletions and amplifications may often be selectively neutral, rather than deleterious. So, large scale copy number changes may only be important during development. Of course, cancer is disadvantageous to the organism as a whole, so perhaps some copy number changes are lethal during development because they essentially cause cancer before the body can even form. One way to test this idea in a model species would be to determine if embryonic cells with extra chromosomes divide at different rates than normal embryos.

Polyploidy in the Evolution of Species

Polyploidization has been hypothesized by some to be a substantive force in genome evolution in both plants and animals.[48] There are a variety of hypotheses for the benefits of polyploidy in genome evolution, though few of these would explain any immediate benefit that would occur on the time scale of a developing cancer. Polyploidy in plants is common, particularly in angiosperms, which raises the question of why polyploidy is detrimental in animal systems but not in plants. It may be a more common phenomenon in plants for the following reasons. First, many plants are self-fertilizing and thus do not have to contend with the problem of reproduction with an individual with the standard chromosome number. Second, polyploidization in animals is thought to be developmentally lethal because of imbalances in protein levels; humans face this issue with their dimorphic sex chromosomes and the X-inactivation mechanism is required for dosage compensation. One would presume that plants would face the same challenges, though perhaps plants have better mechanisms for dosage compensation. In animals, ploidy abnormalities often lead to sterility, a problem not encountered by plants. Plants are also less susceptible to cancer than animals since they lack the circulatory system, migratory (potentially metastatic) cells and vulnerability to organ failure common in animals.

Despite these differences, some insights may be gained by examining the major hypotheses for the frequency of polyploidization in plant systems.[49] Polyploid plants tend to be larger and more robust. This may explain why many domesticated crops are polyploid and why polyploidy is more likely in plants found in extreme environments. Polyploids also have the advantage of gene redundancy: disadvantageous recessive alleles are masked by wild-type alleles and mutations occurring in duplicated alleles may not reduce the total fitness of the organism.[50] In plants, polyploidy also can lead to a loss of self-incompatibility, which may improve reproductive ability. Many animal genomes also show evidence of ancient polyploidization events.[48] Similar to the hypothesized diploid-tetraploid-aneuploid progression in cancer,[16,51] it is thought that genomes may be initially unstable after a polyploidization event and then detrimental duplicated genes are removed or diverge through the process of selection.[52]

Why Is Aneuploidy Common in Neoplastic Progression?

Setting aside the issue of what proportion of copy number changes are beneficial to a neoplastic cell, there is still a question of why aneuploidy and polyploidy are so common in cancer. There are at least three categories of possible explanations for the prevalence of aneuploidy in carcinogenesis: 1) Aneuploidy is a common side effect of lesions that occur during carcinogenesis but is otherwise evolutionarily neutral. 2) Aneuploid clones, by dint of their genetic instability, often generate specific genetic lesions that provide the clone with a competitive advantage over other clones, regardless of their ploidy. 3) Aneuploidy itself provides a competitive advantage over diploid clones.

A Competitive Advantage of Aneuploidy

Several lines of evidence seem most compatible with the third hypothesis: Aneuploidy is observed at high frequencies in many cancers and many different types of cancer.[53] Aneuploidy is more common in higher grade cancers and is associated with more aggressive clinical behavior.[6,7] Aneuploidy does not seem to be logically required for carcinogenesis. Diploid cells can silence tumor suppressor genes by methylation, sequence mutations or localized deletions. They can also activate oncogenes by sequence mutations, local amplifications or over-activation of up-stream genes in the same pathway. This line of reasoning also suggests that aneuploidy may be common because it often provides a direct competitive advantage over diploid cells. Why might this be true?

We argued above that nonlinear feedback effects in gene regulation may make large scale amplifications act like wide spread mutations, up- and down-regulating expression of genes across the genome. Our question then becomes: Given the conventional wisdom that random mutations are expected to reduce fitness, why do the 'mutation packages' resulting from aneuploidy apparently go to high frequency, as if under positive somatic selection? There is a crucial difference between the expected fitness effects of mutations in organisms versus in metazoan cells. Organismal selection has

favored any alleles that increase organismal survival and reproduction. Thus, organisms are typically on or near a peak in their fitness landscape so that most random moves in phenotype space (most mutations) are detrimental to organism fitness.

In contrast, during the progression of a neoplasm, cells are subject not to organismal selection, but to somatic selection among cells.[54] Through their evolutionary history of organismal selection, genomes have not been shaped for optimal fitness in terms of somatic selection. Indeed the opposite may be closer to the truth. Many of the genes in a metazoan genome may function to constrain cellular competition.[55] For example, a history of organismal selection has shaped cell genotypes that limit cell reproduction and increase cell mortality (e.g., through apoptosis).[54] This implies that many mutations may provide a competitive advantage for the mutant cell.[38] Organismal selection is also expected to create in cells mechanisms of 'antiredundancy' or hypersensitivity to the deleterious effects of mutations which help the organism by culling mutant genomes from its cell population.[56]

The combined effect of evolved constraints on cellular competition and evolved antiredundancy mechanisms is that in terms of somatic competition within an organism, cells are expected to be located at or near the bottom of a 'pit' in their fitness landscape.

For organisms located on a fitness peak, random mutations are expected on average to be deleterious. In contrast, for cells located in a fitness pit, random mutations are expected on average to be beneficial.

Since the vast majority of aneuploid and polyploidy cells are supradiploid (greater than 2N chromosomes, Fig. 1), at the very least they carry the burden of having to replicate more DNA during the synthesis phase of the cell cycle than a diploid cell. This should require more cellular resources and perhaps more time than a diploid cell. If the process of genome synthesis is sufficiently parallel and resources are not limiting, then the extra DNA content of a supradiploid cell may not reduce the reproduction rate of the cell. One might be able to test this by generating a tetraploid cell through fusion and comparing its cell cycle time to the diploid cells that were used to generate it. Aneuploids generated in yeast appear to have reduced proliferation rates and require increased glucose uptake to fuel the protein synthesis generated by extra copies of genes which are mostly active.[34] Furthermore, experimental suppression of CENP produces aneuploid cells with a reduced frequency of viable daughter cells but without a measurable change in the net proliferation rate.[57]

One possible explanation for a supradiploid competitive advantage over diploid cells is that supradiploid cells are likely to be more robust to deletions and gene inactivation because they have more copies of essential genes than diploid cells. If an essential gene is haploinsufficient, then a deletion of a single allele in a diploid cell will be fatal. However, a deletion of a single allele in a tetraploid cell would leave three viable alleles and the tetraploid cell would survive. Thus, we would expect environments that cause frequent deletions to select for supradiploid cells. Conditions of chronic inflammation, including Barrett's esophagus, produce oxygen and nitrogen radicals that can result in such DNA damage.[58-60]

Aneuploidy May Generate Advantageous Lesions

The fact that experimentally derived aneuploid cells tend to suffer a fitness disadvantage relative to wildtype cells[34,57] suggests that aneuploidy, as a form of a mutator phenotype,[61,62] may not provide a direct advantage but may increase the probability that one of the genetic variants it generates has a competitive advantage due to the inactivation of a tumor suppressor gene or the amplification of an oncogene. Mutators gain a competitive advantage over nonmutators in at least three cases. First, if beneficial mutations are more likely than deleterious mutations, a lineage that increases its mutation rate will also, on average, increase its fitness. Second, even if deleterious mutations are more common than beneficial mutations, if the occasional beneficial mutation has a dramatic effect, the mutator lineage may generate a variant that out-competes all other lineages even if most of the variants it had produced until that point had been burdened with deleterious mutations. Weaver et al found that although their aneuploid cells produced

fewer viable daughter cells than wildtype cells, aneuploid cells produced more colonies capable of anchorage independent growth.[57] Finally, if the selective environment—including both the microenvironment and the genetic make-up of the competitors—changes frequently, a lineage that produces a diverse set of offspring is more likely to produce at least one that can survive in the changing environment compared to a lineage that produces a homogenous set of offspring. In this case, a mutator lineage is more likely to adapt to the changing environment than a nonmutator. All of these possibilities may apply to neoplastic progression. Experiments to test the fitness effects of various mutations could test the first two cases. Whether an aneuploid cell evolves faster to a changing environment than a diploid cell could be tested in vitro with any number of changing exposures. It is interesting to note that spontaneously occurring mutator strains of *E. coli* do not appear to have a higher fitness than the nonmutator strains.[63,64] We should not be too quick to accept the idea of an indirect benefit of aneuploidy as a mutator phenotype.

Aneuploidy May Be an Evolutionarily Neutral By-Product of Carcinogenesis

The final alternative is that aneuploidy is evolutionarily neutral. It may be a common phenomenon if many genetic and epigenetic lesions can produce aneuploidy or polyploidy by disrupting the cell cycle machinery. Erosion of telomeres leads to bridge-breakage-fusion cycles and so aneuploidy may be a consequence of extensive proliferation in neoplasms.[65,66] If we assume that deletions are more likely to be deleterious than amplifications, then we would predict that most aneuploids and polyploids should be supradiploid. In any case, because the aneuploid clone is hypothesized to be neutral, it should only expand (and contract) by genetic drift, that is, very slowly and then only by chance. If these lesions are common, it is much more likely that a new aneuploid clone would emerge before a previous aneuploid clone could take over the neoplasm. On average, it requires N cell generations for a neutral clone to expand to fill a neoplasm of N cells. If a cell generation takes one day and we assume 1 cm^3 neoplasm (~10^9 cells) has a frequency of 10^{-4} stem cells,[67-72] then an evolutionarily neutral aneuploid stem cell should take approximately 300 years to fill that neoplasm.

The neutral hypothesis of the evolution of aneuploidy predicts that neoplasms will accumulate many aneuploid clones that coexist but mostly derive from different diploid progenitors. Thus, the aneuploid clones should not be closely genetically related. In contrast, the hypothesis that aneuploidy provides a selective advantage predicts that the aneuploid clone should expand in the neoplasm and, if there are multiple clones they will likely be closely related as the aneuploid lineage spins off genetic variants. These hypotheses could be distinguished by taking multiple biopsies from a neoplasm and characterizing their genetics to determine the size and relationships between clones. In BE, we typically see the later pattern, with either a single or closely related aneuploid clones in the neoplasm. This suggests that aneuploidy provides or leads to a selective advantage in BE. However, for unknown reasons, it is rare for an aneuploid clone to take over the entire neoplasm.[17]

Whether aneuploidy and more generally chromosomal instability, provides a competitive advantage for a neoplastic clone may have clinical importance. If aneuploidy provides a competitive advantage, then any surviving aneuploid cells after therapy are likely to replenish and dominate the neoplasm at relapse. If aneuploidy is evolutionarily neutral, a diploid surviving clone is just as likely to grow back to fill the void caused by the intervention. We would predict that aneuploid neoplasms would contain more genetic variants than diploid neoplasms and be more likely to harbor a therapeutically resistant clone. However, neoplasms dominated by genetic drift are more likely to accumulate genetic diversity than neoplasms in which one clone has a competitive advantage and can drive other clones extinct. The current amount of genetic diversity and thus the likelihood of resistance, depends on the interplay between the frequency with which new clones are generated and the homogenizing effects of clonal expansion.[53,73]

DNA Damage Sensing by Linkage

There is a much literature on the mechanisms of DNA damage sensing in the cell cycle, much of it centered on the role of p53.[74-76] This work has been more competently reviewed by others.[77,78] Here we focus on a hypothesis for an alternative and perhaps more primitive, mechanism for sensing DNA damage: linkage between tumor suppressor genes and essential genes.

Some regions of the genome may be protected by the presence of genes that are necessary for the survival of the cell. If enough deletions occur in that region to knock out all alleles of such genes, the cell dies and those genetic lesions are not propagated in the tissue. There is another set of genes, including tumor suppressor genes, which if lost decreases the fitness of the organism but not necessarily the cell. That is, if a clone loses enough alleles of tumor suppressor genes, it might expand but eventually kill its host. Thus, selection at the organism level may have increased the fitness of organisms by shuffling the gene order in a genome to place genes essential for the survival of a cell near to genes essential for the survival of the organism. In this way, if a deletion knocks out the tumor suppressor gene, it is also likely to knock out the nearby essential gene and the cell will die before it can generate a cancer. Thus genes essential for the survival of the cell may act like a crude form of DNA damage sensing, specific to a particular locus in the genome, that triggers apoptosis.

We predict that there has been selection for linking tumor suppressor genes with genes essential for cell survival. Such a linkage would bias against the discovery of the tumor suppressor gene. Linkage with an essential gene would tend to prevent those tumor suppressor genes from being knocked out in neoplasms with chromosomal instability and those tumor suppressor genes would be more likely to be identified in cases where the tumor suppressor gene was silenced by methylation or sequence mutations, leaving the nearby essential gene intact. The observation of at least 245 rearrangements between the human and mouse genomes may be a signature of selection for this kind of linkage.[79] This hypothesis provides one of the first explanations for a selective pressure that may be driving genome rearrangements in species.

Ancient and Recent Cancer Genes

Evolution is often described as a process of accretion. New features are added to old structures. Genes are duplicated and then diverge, adding interactions to an existing gene network. Thus, ancient genes are likely to be in the middle of such a network with many other genes depending on them. Recently evolved genes are likely to be at the periphery with fewer genes depending on them.[80] This leads to the prediction that copy number changes that affect recently evolved genes should be less likely to be deleterious than copy number changes that affect ancient genes. Careful analysis of CGH data from neoplasms might be used to test this prediction. Alternatively, an in vitro system for testing fitness effects[81] might be used to test the prediction in conjunction with the targeted deletion or amplification of either ancient or recently evolved genes.

Conclusion

Aneuploidy is a feature of almost all cancers and many premalignant conditions, including Barrett's esophagus. There are a few exceptions to this rule[27,82] including microsatellite instable colorectal cancer which can generate many genetic lesions without requiring copy number abnormalities.[83] Despite its prevalence, the significance of aneuploidy in cancer is poorly understood. By considering aneuploidy in an evolutionary context and examining its selective effects, we may better be able to understand how this phenomenon contributes to cancer progression. Evolutionary theory predicts that most mutations are deleterious, yet the prevalence of aneuploidy indicates that cancers are able to tolerate an enormous load of mutations and still utilize the body's resources and proliferate faster than normal cells. Evidence in Barrett's esophagus correlates the incidence of tetraploidy and aneuploidy with increased risk of progression. By designing experiments to examine the effects of aneuploidy in cancer and normal cells, we can help determine how aneuploidy develops and spreads in the hopes of developing predictive tools to allow us to better treat patients and prevent cancer.

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Chapter 2

Molecular Mechanisms and Function of the Spindle Checkpoint, a Guardian of the Chromosome Stability

Daisuke Ito and Tomohiro Matsumoto*

Abstract

For equal segregation, chromosomes, which are distributed randomly in the nucleus of interphase, must be aligned at the spindle equator in mitosis before the onset of sister chromatid separation. The spindle checkpoint is a surveillance mechanism that delays the onset of sister chromatid separation while each chromosome is on the way to the spindle equator. Failure in the function of the checkpoint results in aneuploidy/polyploidy, which would be a cause of cancer. Here, we review chromosome dynamics in mitosis, molecular mechanisms of the spindle checkpoint and finally tumorigenesis triggered by missegregation of chromosomes.

Introduction

Mitosis is the final stage of the cell cycle in which chromosomes duplicated in the preceding S phase are segregated equally to two daughter cells. Aneuploidy is a state of the cell with an abnormal chromosome number and is thought to be a cause of congenital hereditary disorders such as Down's syndrome and a trigger for tumorigenesis. It is generally believed that aneuploidy is caused by a failure in chromosome segregation during cell division including both mitosis and meiosis. In the mitosis in vertebrate cells, chromosomes are highly condensed in prophase; following the nuclear membrane breakdown, the condensed chromosomes are captured by spindle microtubules in prometaphase. After the chromosomes are attached to the spindle, they congress on the metaphase plate and are segregated to each daughter cell. In this process, premature separation of a chromosome which is not correctly attached to the spindle microtubules from both poles (called bipolar attachment) leads to a catastrophic consequence: each daughter cell possesses extra or fewer numbers of chromosomes. Usually a surveillance mechanism prevents missegregation of chromosomes during cell division. The spindle checkpoint (also referred to as spindle-assembly checkpoint) is a mechanism that ensures the accurate segregation of chromosomes in mitosis by delaying the onset of anaphase until all the kinetochores of chromosomes are fully attached to the spindle. In this chapter, we provide an overview of chromosome dynamics in mitosis and the molecular mechanism of the spindle checkpoint from the aspect of both its activation and silencing. We also discuss how aneuploidy/polyploidy can trigger tumorigenesis.

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Bipolar Attachment and Chromosome Congression

Chromosomes, which are distributed randomly in the nucleus at the onset of mitosis, must be placed on the metaphase plate before anaphase. To be more precise, all sister chromatids must be bi-oriented (i.e., they attach to the spindle via two kinetochores, each of which interacts with the spindle radiated from one of the two poles) and are positioned on the spindle equator (Fig. 1), a mid point between the two poles. Interaction between the spindle and chromosomes plays a key role in determining the position of each chromosome in mitosis.

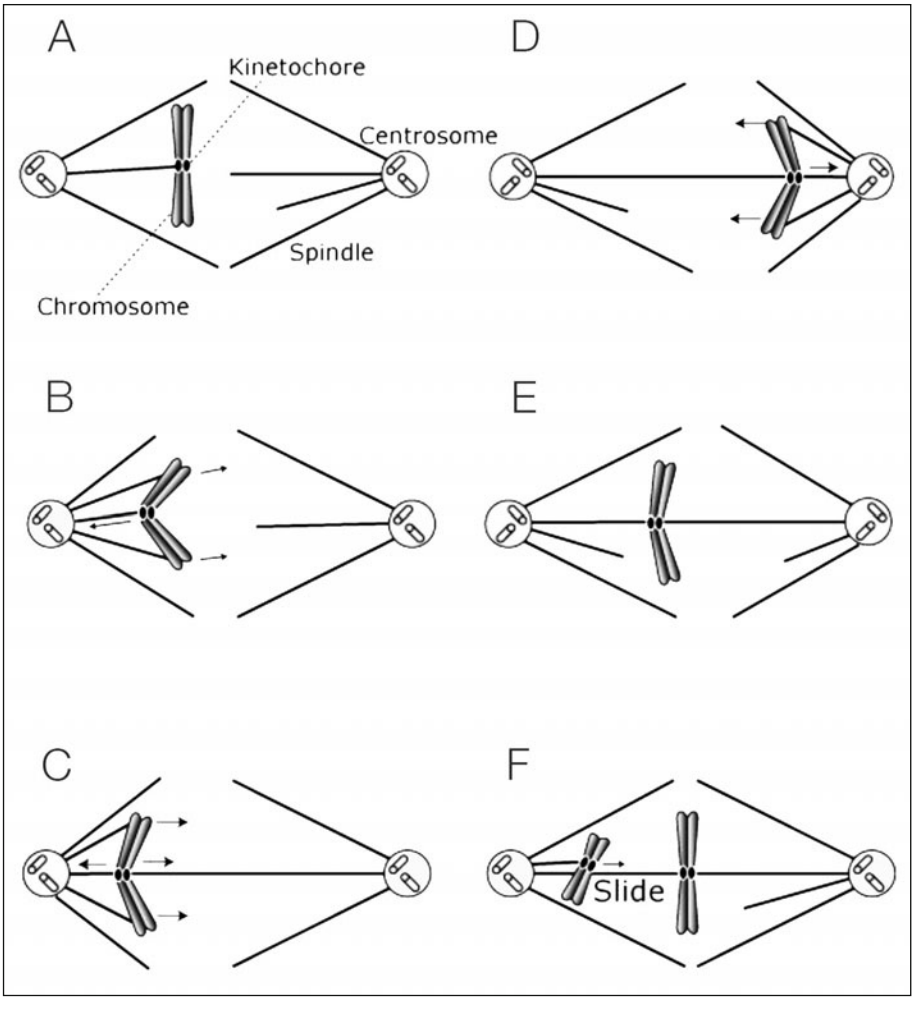

Figure 1. Chromosome dynamics in mitosis. At an early stage of mitosis, sister chromatids interact with the spindle from one pole via one kinetochore (A). These sister chromatids are pulled toward the pole and the chromosome arms are pushed away by interaction between kinesin-related protein and microtubules (B). Upon attachment to the spindle from the other pole, the mono-oriented sister chromatids become bi-oriented (C) and start congression and then they are pulled by the other pole (D). The sister kinetochores switch on and off the poleward and polar ejection force (E). Eventually the sister-chromatids are positioned at the spindle equator (F). In addition, mono-oriented sister chromatids laterally attach to the matured K-fiber between another kinetochore and the pole and slide to the metaphase plate (F).

At an early stage of mitosis, each sister chromatid interacts with the spindle radiated from one pole via one kinetochore (Fig. 1A). This mono-oriented sister chromatid is pulled toward the pole by the attached spindle. At the same time, its arm is pushed away, likely by interaction between kinesin-related proteins, Kid[1,2] and microtubules (Fig. 1B). The two opposing forces, the poleward force and the polar ejection force, act on the leading kinetochore. As it moves closer to the attached pole, the polar ejection force increases. It has been proposed that the leading kinetochore switches off the poleward force when it senses the increasing polar ejection force and thereby allows pole-away movement.[3] When the sister chromatid moves away from the pole and the polar ejection force decreases, the leading kinetochore switches on the poleward force again and moves toward the pole. By repeating this switching process, the leading kinetochore allows oscillation, the movement of the sister chromatid going back and forth around the pole.

Upon the attachment to the spindle radiated from the other pole during the period of oscillation, mono-oriented sister chromatids become bi-oriented (Fig. 1C) and start congression, the movement toward the spindle equator. The kinetochore closer to its attached pole encounters the poleward force through the attached spindle and polar ejection force through both the arm and the sister kinetochore. As the polar ejection force increases, the kinetochore switches off the poleward force. Its sister kinetochore then becomes the leading kinetochore and moves the sister chromatid toward the other pole (Fig. 1D). When the sister chromatid passes the spindle equator, the new leading kinetochore switches off the poleward force due to an increase in the ejection force. The sister kinetochore then switches on the poleward force and pulls the sister chromatid back to its attached pole (Fig. 1E). Repeating these processes results in congression and the sister chromatid is eventually placed at the spindle equator (Fig. 1F).[3,4] Although bi-orientation followed by congression is a major process to place sister chromatids at the metaphase plate, a recent study demonstrated that mono-oriented sister chromatids can migrate to the metaphase plate.[5] A mono-oriented sister chromatid oscillating near the pole may need a long time to find the spindle radiated from the other pole. The kinetochore of such a sister chromatid laterally attaches to the K-fiber that is formed between the pole and another kinetochore (Fig. 1F) and slides to the metaphase plate with the aid of a kinetochore motor, CENP-E. This cooperative process rescues mono-oriented sister chromatids and greatly contributes to placing all sister chromatids at the metaphase plate within a limited time.

Nonetheless, congression is a major event bringing each sister choromatid to the metaphase plate. It is initiated and progresses independently at each sister chromatid. As a result, some sister chromatids arrive at the spindle equator while others are yet unattached or monooriented. Sister chromatids which arrived at the spindle equator earlier do not separate until all sister chromatids arrive at the metaphase plate. A surveillance mechanism termed the spindle checkpoint is responsible for preventing premature sister chromatid separation. Kinetochores not attached to the spindle or attached abnormally activate the spindle checkpoint.

Molecular Basis of the Spindle Checkpoint

The mechanism by which the spindle checkpoint inhibits the premature separation of sister chromatids has been intensively studied until now. As shown in Figure 2, the outline of the spindle checkpoint mechanism is as follows. In the presence of kinetochores which are not attached to the spindle microtubules emanating from the opposite poles, the spindle checkpoint is activated to prevent the premature onset of anaphase. The functional components localize on the unattached kinetochore (reviewed in ref. 6). In particular, one of the most important components, Mad2, forms a complex with Cdc20/Slp1,[7,8] an activator of the APC/C (Anaphase Promoting Complex or Cyclosome) which is an E3 ubiquitin ligase targeting Securin for destruction. As long as the checkpoint is activated, Mad2 stays with Cdc20 and prevents activation of Cdc20-APC/C. Consequently, Securin remains stable and continues to bind to Separase. Securin is a stoichiometric inhibitor of Separase, a protease to cleave the cohesin complex which holds the sister chromatids together.[9] This way, the spindle checkpoint maintains sister chromatids held together until all the kinetochores fulfill the bi-polar attachment. When all the kinetochores fulfill the bi-polar

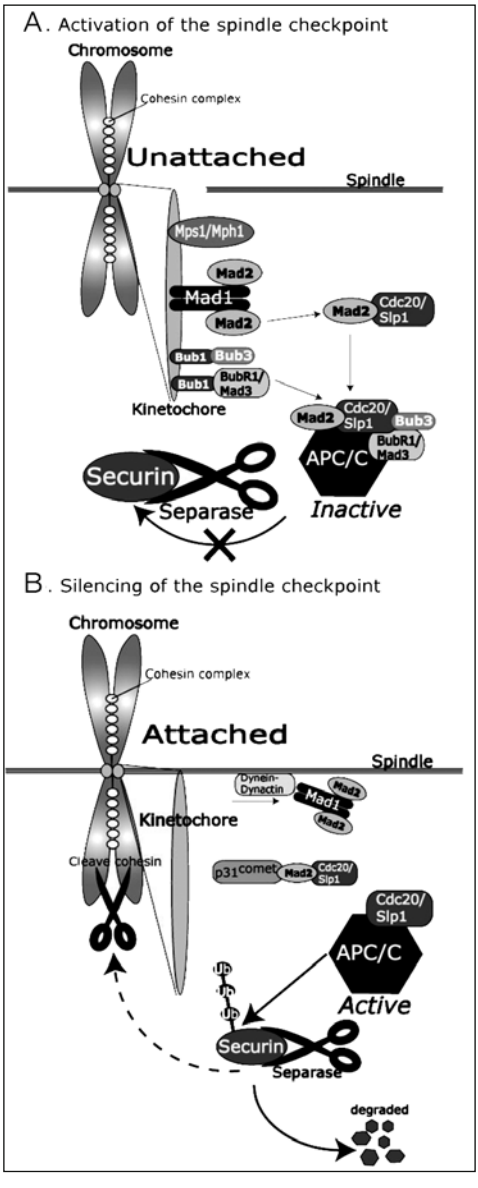

Figure 2. Molecular mechanism of the spindle checkpoint. A) Activation of the spindle checkpoint: In the presence of an unattached kinetochore, the spindle checkpoint is activated to prevent premature onset of anaphase. When the spindle checkpoint is activated, the functional components including Mad1, Mad2, BubR1/Mad3, Bub1, Bub3 and Mps1/Mph1 localize on the unattached kinetochore. In particular, Mad2 forms a complex with Cdc20. Presumably, Mad2, BubR1, Bub3 and Cdc20 form a mitotic checkpoint complex (MCC) to inhibit the activity of APC/C to ubiquitinate Securin. Consequently, Securin continues to inhibit Separase which cleaves the cohesin complex. B) Silencing of the spindle checkpoint: When the last kinetochore is attached to the spindle, the functional components disappear from the kinetochore with the help of the factors such as $p31^{comet}$ and Dynein-Dynactin. Then the APC/C achieves its activity and ubiquitinates Securin.

attachment with the spindle microtubules, the functional components disappear from the kinetochore and Mad2 no longer forms a complex with Cdc20/Slp1. Then APC/C achieves its activity and ubiquitinates its target Securin. Subsequently, 26S proteasome selectively degrades poly-ubiquitinated Securin so that Separase can acquire its activity to cleave the cohesin complex between sisiter-chromatids.[9]

The mechanism for spindle checkpoint activation has been largely elucidated in the past ten years, mainly in experimental systems such as yeast, *Xenopus* egg extracts and mammalian cultured cells. The factors involved in the spindle checkpoint have been identified by genetic screens in the budding yeast *Saccharomyces cerevisiae*.[10,11] The proteins identified include Mad (Mitotic-arrest deficient) 1, 2, 3 and Bub (Budding uninhibited by benzimidazole) 1, 3, which are widely conserved among eukaryotes (Mad3 is BubR1 in higher eukaryotes). In addition to these proteins, Mps1 kinase has been identified as a factor involved in the spindle checkpoint function, which was first discovered in budding yeast as a factor required for the duplication of spindle pole body (SPB), an equivalent organelle to the centrosome in higher organisms.[12,13] Some other proteins involved in the function of spindle checkpoint have been characterized only in higher eukaryotes. These include Rod (Rough deal)-Zw10 (zeste white-10)-Zwilch (RZZ) complex,[14] p31^{comet} (previously known as CMT2)[15] and a minus-end directed microtubule motor protein, CENP-E.[16]

Activation of the Spindle Checkpoint Signaling

When the chromosome is not properly attached to the spindle, the spindle checkpoint is activated to inhibit the progression from metaphase to anaphase. In the spermatocyte of mantids, the cell is arrested in metaphase forever in the presence of an improperly attached free X chromosome. However, when the misattached kinetochore was placed under tension by a micromanipulation needle, the cell entered anaphase.[17] In mammalian PtK1 cells, laser ablation of the last unattached kinetochore relieved the metaphase arrest and the cell entered anaphase.[18] These experiments showed that a single kinetochore not under tension generates a sufficient signal to inhibit the onset of anaphase.

The spindle checkpoint is thought to detect kinetochore-occupancy of the attached spindle and/or tension on the kinetochore/spindle. Because attachment is stabilized by tension,[19] it is difficult to experimentally determine whether a lack of attachment or tension can activate the spindle checkpoint. The components of the spindle checkpoint are known to specifically localize on the kinetochores in response to the spindle checkpoint activation. Mad1 and Mad2 localizes to the unattached kinetochore, not to the attached kinetochore lacking tension.[20] On the other hand, Bub1 and BubR1/Mad3 are recruited to the attached kinetochore lacking tension.[21] However, the mechanism how the components are specifically recruited to the kinetochore remains to be elucidated.

Some of the kinetochore proteins are required for the recruitment of spindle checkpoint components. Localization of Mad1 and Mad2 to the unattached kinetochore depends on Hec1 and Nuf2, components of the outer kinetochore.[22,23] However, the direct physical interaction between the spindle checkpoint proteins and the kinetochore components remains to be clarified.

We have seen that the spindle checkpoint components are recruited to the kinetochore in response to a defect in kinetochore-spindle microtubule association. Now we focus on the downstream events. The downstream target of the spindle checkpoint is the anaphase-promoting complex (APC/C) (reviewed in ref. 9). Mad2 forms a complex with Cdc20 and this association is essential for the function of the spindle checkpoint: inhibit activity of APC/C.[7,8] In mammalian cells, spindle checkpoint components Mad2, BubR1, Bub3 and Cdc20 form a large complex, designated mitotic checkpoint complex (MCC). MCC is a more potent inhibitor of APC/C than only Mad2.[24]

Mad2 Template Model

As mentioned above, Mad2 is one of the most important components of the spindle checkpoint since it binds to Cdc20 to inhibit the activity of APC/C. Protein structural analyses have revealed that Mad2 possesses two conformations, open-form (O-Mad2, also known as N1-Mad2) and closed-form (C-Mad2, or N2-Mad2).[25-27] In this chapter, we use the terms C- or O-Mad2 in attempt for better understanding. The "Mad2 template model" hypothesis is proposed to explain the significance of the two structural states of Mad2.[28] Mad2 holds closed conformation when it is bound to Mad1 or Cdc20 and open conformation when it is free in cytoplasm.[25-29] Cytoplasmic free O-Mad2 changes its conformation to C-Mad2 upon binding to Mad1-bound C-Mad2 at the unattached kinetochore so that it is capable of forming a C-Mad2-Cdc20 complex (Fig. 3). In this model, it is proposed that Mad1-bound C-Mad2 acts as a "template" for the conformation conversion. In this respect, it is hypothesized that the C-Mad2-Cdc20 complex also catalyzes the conformation changes of cytoplasmic O-Mad2 and that consequently the activation signal of the spindle checkpoint is amplified.[28] This hypothesis could reasonably explain why a single unattached kinetochore is sufficient to activate the spindle

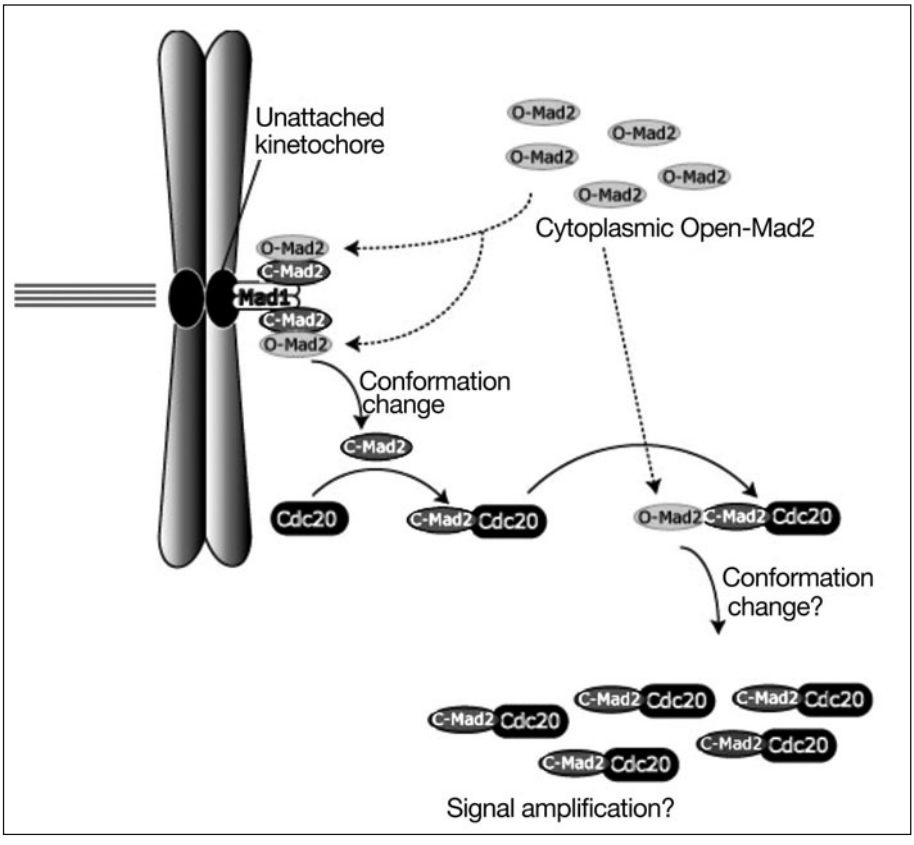

Figure 3. Mad2 template model. Mad2 holds closed conformation when it is bound to Mad1 or Cdc20 and open conformation when it is free in cytoplasm. Cytoplasmic-free O-Mad2 changes its conformation to C-Mad2 upon binding to Mad1-bound C-Mad2 at the unattached kinetochore and forms a C-Mad2-Cdc20 complex. In this model, Mad1-bound C-Mad2 acts as a "template" for the conformation conversion. It is hypothesized that the C-Mad2-Cdc20 complex also catalyzes the conformation changes of cytoplasmic O-Mad2.

checkpoint. Further investigation is required to clarify the mechanism how the Mad1-bound C-Mad2 catalyzes the conformation changes.

Phosphorylation and Spindle Checkpoint Function

Some protein kinases such as Mps1, Bub1, BubR1 and Aurora are involved in the process of spindle checkpoint activation. Thus, it is likely that phosphorylation of certain proteins is a key event for signaling cascade of the spindle checkpoint. Aurora kinases are implicated in many events in the cell cycle: centrosome separation and maturation, spindle assembly and stability, chromosome condensation, congression and segregation and cytokinesis.[30] Its role in spindle checkpoint activation has also been reported. It is proposed that budding yeast Aurora kinase Ipl1 activates the spindle checkpoint in response to tension-defective kinetochore by correcting improper attachment.[31]

Mps1 and Bub1 kinases are known to phosphorylate Mad1 in vitro; however, its significance remains unclear.[32,33] In budding yeast, overexpression of Mps1 causes spindle checkpoint activation without disturbing the formation of the mitotic spindle and leads to the hyper-phosphorylation of Mad1.[32] In the fission yeast *Schizosaccharomyces pombe*, the Mph1 (Mps1p-like pombe homolog) kinase has been identified as a spindle checkpoint component and its overexpression causes a mitotic arrest in a Mad2-dependent manner.[34] In our recent study, we attempted to elucidate the mechanism by which Mph1 kinase activates the spindle checkpoint in fission yeast. *mph1* overexpression did not cause a growth defect attributable to a mitotic arrest in the strains lacking spindle checkpoint components. In addition, when *mph1* was overexpressed, strong Mad2-GFP foci were observed on condensed chromosomes (Fig. 4), which suggested that Mad2 presumably accumulated on the kinetochores upon the activation of spindle checkpoint (unpublished data). These findings indicate that Mph1 acts upstream of the examined spindle checkpoint components and facilitate spindle checkpoint signaling.

It has been shown that a component of the kinetochore is phosphorylated when it is not under tension. The phosphorylation of an unidentified protein is recognized by the 3F3/2 antiphosphoepitope antibody and the phosphorylation is required for the recruitment of Mad2 to the kinetochore.[35-37]

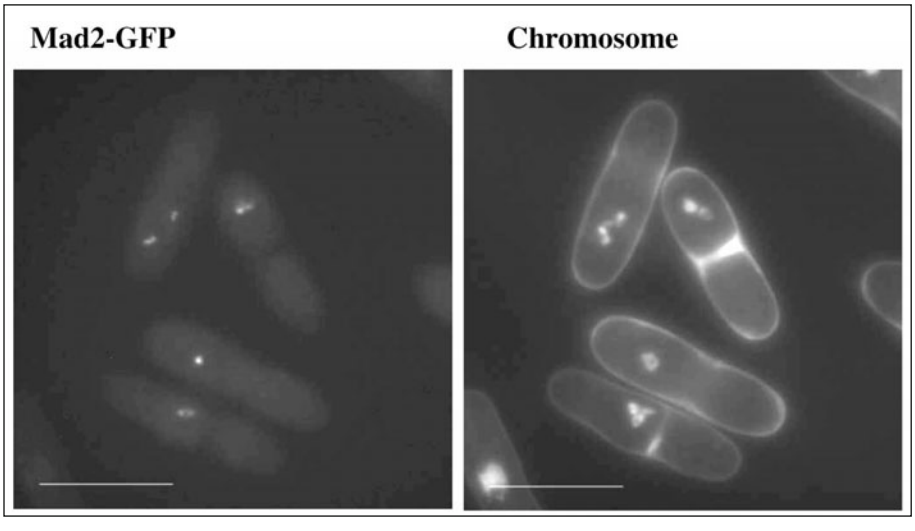

Figure 4. Mad2 on the kinetochore. Mad2-GFP localized on kinetochores when *mph1* was overexpressed. Left panel, Mad2-GFP. Right panel, condensed chromosomes visualized with Hoechst 33342. Scale bars indicate 5 μm.

In order to understand the significance of protein phosphorylation in spindle checkpoint activation, further studies are obviously needed to clarify the substrates of the kinases.

Silencing the Spindle Checkpoint

After all kinetochores are properly attached to the spindle, the spindle checkpoint is turned off within minutes and the chromosomes are synchronously segregated toward each spindle pole.[38] Compared to the activation of the spindle checkpoint, the mechanisms for silencing the checkpoint remain to be ambiguous. To date, through the yeast two-hybrid assay using Mad2 as a bait, Mad2-binding protein p31^{comet} (formerly CMT2) has been identified. In HeLa cells, overexpression of p31^{comet} abrogated the function of the spindle checkpoint and overrode the mitotic arrest.[15] In addition, the association of p31^{comet} with Mad2 coincided with the dissociation of Mad2-Cdc20, which indicated that p31^{comet} plays an important role in silencing the spindle checkpoint.

Recently, two groups reported the role of ubiquitination in spindle checkpoint silencing in mammalian cells. It was shown that the addition of UbcH10, an APC-specific ubiquitin-conjugating enzyme (E2), overrides the mitotic arrest induced by nocodazole.[39] In this process, multi-ubiquitination of Cdc20 by APC/C leads to the dissociation of Mad2 and BubR1 from Cdc20 and this promotes the progression to anaphase. To prevent premature activation of APC/C, deubiquitinating enzyme USP44 deubiquitinates Cdc20 and thereby antagonizes the ubiquitination by APC/C.[40]

In higher eukaryotes, cytoplasmic dynein plays a role in silencing the spindle checkpoint by removing Mad2 and RZZ complex from the attached kinetochores.[41,42] Thus, dynein is thought to be involved in stripping and transporting outer domain proteins away from the kinetochore upon the microtubule attachment. Very recently, through the RNAi screen in *Drosophila*, a novel protein named Spindly has been identified as a factor essential for silencing the spindle checkpoint.[43] The human homologue of the protein was identified and had similar functions. Regarding the silencing mechanism of the spindle checkpoint, the questions such as how kinetochores detect the completion of proper attachments and convey a signal to silence the checkpoint remain to be elucidated.

Additional Surveillance System

The spindle checkpoint is backed up by other surveillance mechanisms in higher eukaryotes. These mechanisms prevent proliferation of cells with extra or missing chromosomes.

A recent study[44] addressed the question of what happens to aneuploids produced by missegregation of chromosomes in mitosis. The authors labeled the nucleus by GFP-tagged histone H2B and monitored its behavior by time-lapse microscopy. They subsequently fixed the cells to analyze chromosome segregation by FISH. The analysis indicated that cells in which chromosomes did not segregate equally became binucleated by furrow regression at a significantly high frequency (Fig. 5A). In contrast, they detected no missegregation events in cells that completed cytokinesis to form two mononucleated cells (Fig. 5B). The results suggest that a surveillance mechanism is responsible for counting the chromosome number at the end of anaphase/telophase and suppresses cytokinesis if two daughter cells contain different chromosome context. Although furrow regression results in production of a tetraploid cell, the chromosome context can be maintained normally. The authors also followed the fate of the tetraploid cells produced by the furrow regression. If diploid human keratinocytes immortalized with telomerase (N/TERT-1) became tetraploids, cell cycle progression was delayed and 50% of them remained in interphase. In contrast, the cell cycle progressed with no delay in the resulting tetraploid HeLa cells. Most of the HeLa tetraploids formed multipolar spindles, which could result in chromosome breakage as well as aneuploidy (Fig. 5A). HeLa cells, which were established from a cervical cancer tissue, may be much more tolerant of tetraploidy or have lost a surveillance mechanism to suppress the growth of tetraploids.

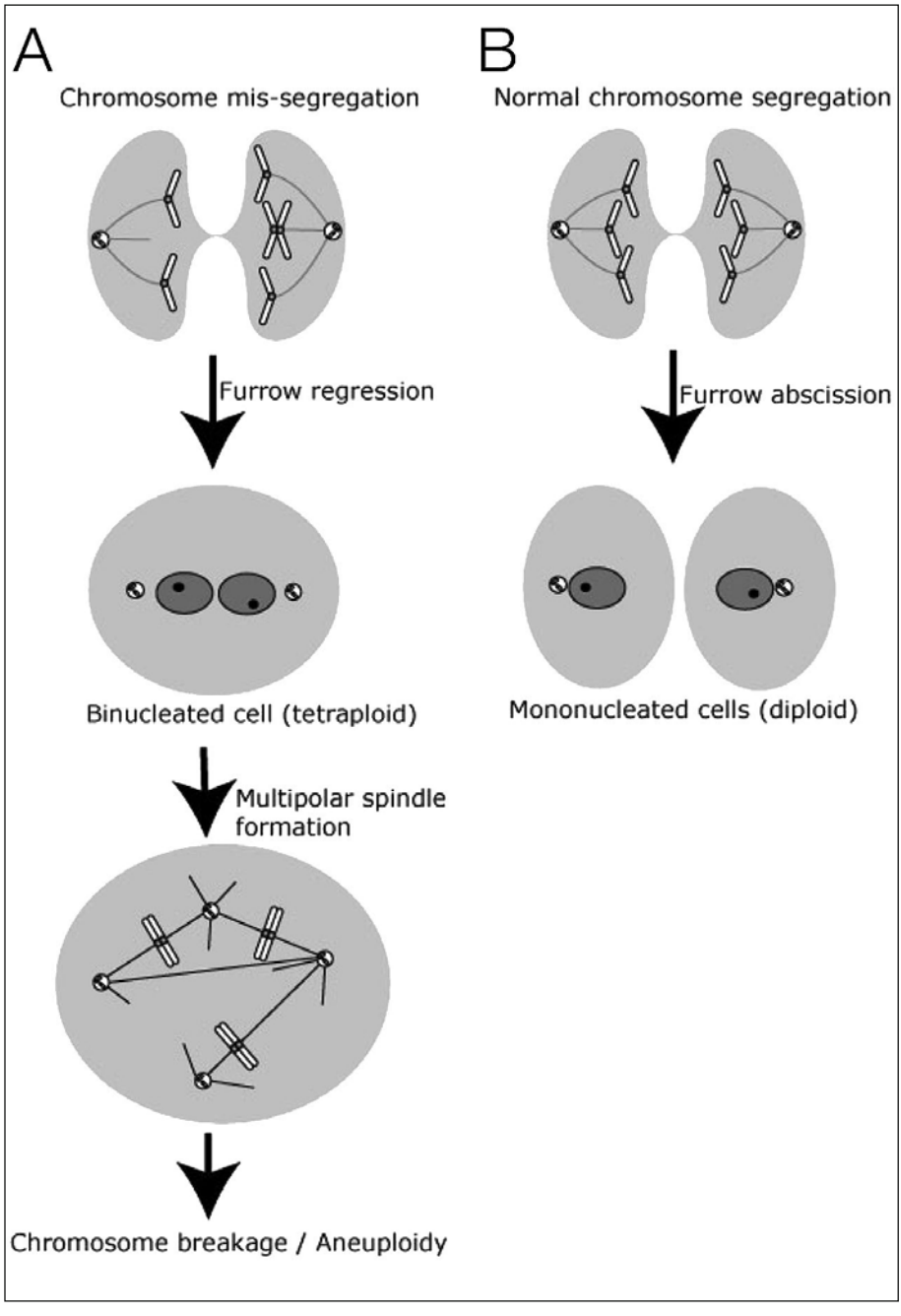

Figure 5. Furrow regression/tetraploidy induced by nondisjunction. A) The cell in which chromosomes are not equally segregated becomes binucleated by furrow regression. The binucleated tetraploid cell subsequently forms a multipolar spindle leading to chromosome breakage and aneuploidy. B) In contrast, the cell with normal chromosome segregation produces two diploid mononucleated cells.

A Trigger of Tumorigenesis

A German cell biologist Theodor Boveri proposed nearly hundred years ago that abnormalities of number and/or structure of the centrosome cause chromosome missegregation and that centrosome abnormalities are expected to affect cell shape, polarity and motility. Deregulation of centrosome number and function thereby may foster both chromosome instability and loss of tissue architecture, two of the most common characters in solid tumors.[45] According to his hypothesis, tetraploids/polyploids with multiple centrosomes would be an intermediate state, which eventually generate aneuploid cancer cells.

Boveri's proposal has been supported by a variety of evidence.[46,47] Importantly, it is supported by clinical studies of patients with a premalignant condition such as Barrett's esophagus (BE). Neoplastic progression in BE is characterized by the development of increased tetraploid population, in which p53 is concomitantly lost. This population, within a year or so, serves as an epicenter that generates a gross aneuploid cell population.[48] In a recent study with the mouse model system, it has also been demonstrated that p53-null tetraploids induced by treatment with dihydrocytochalasin B (an inhibitor of cytokinesis) promote tumorigenesis when transplanted into nude mice.[49]

Tetraploids can be produced by a defect in the spindle checkpoint. If the checkpoint misses a chromosome not attached to the spindle and allows premature sister chromatid separation, a daughter cell may not receive the accurate number of chromosomes. As mentioned earlier, such an event would activate a surveillance mechanism backing up the spindle checkpoint and result in regression of the cleavage furrow and tetraploidy.

A defect in silencing the spindle checkpoint also induces tetraploidy. Overexpression of Mad2 causes a delay in transition from metaphase to anaphase. High dosage of Mad2 would stabilize the Mad2-Cdc20 complex and prevent activation of Cdc20 for the onset of anaphase. In higher eukaryotes, overexpression of Mad2 induces tetraploidy, probably because cells eventually exit from mitosis without separating sister chromatids. In a recent mouse study, it has been shown that chromosome instability in tetraploidy/aneuploidy and tumorigenesis are induced in the transgenic mice overexpressing Mad2.[50]

As shown in Figure 5, in tetraploids mitosis with a multipolar spindle causes chromosome nondisjunction/breakage and thereby chromosome instability. It is, however, likely that chromosome instability in tetraplids/polyploids is induced for other reasons as well. In yeast model studies, it has been demonstrated that as ploidy increases, chromosome stability decreases.[51,52] Notably, a recent genome-wide screen has identified genes that cause ploidy-specific lethality. Loss of function of these genes does not affect growth of a haploid strain, but causes lethality in polyploids.[53] The ploidy-specific lethality genes are categorized into three functional groups—homologous recombination, sister chromatid cohesion maintenance and mitotic spindle function—providing clues to understand the underlying mechanisms to induce chromosome instability in polyploids. In yeast, tetraploids have a high incidence of syntelic kinetochore attachment to the mitotic spindle (a pair of sister kinetochores are attached to the same pole). It has been proposed that such an abnormal interaction is due to mismatches in the ability to scale the size of the spindle pole body (the equivalent structure of centrosome in higher eukaryotes), mitotic spindle and kinetochore. It is also plausible that the length of S phase, which is long enough for haploids to replicate their genome, may not be so for tetraploids. The geometric and/or time constraint in polyploids may be a cause of chromosome instability.

Conclusion

The spindle checkpoint is a unique signaling cascade in that the physical condition such as lack of tension and/or occupancy of the spindle at the kinetochore is a trigger for activation. To date, it has not been elucidated how the kinetochore recognizes the state of interaction with spindle microtubules and how the physical signal is converted to the biochemical signal to inhibit premature progression to anaphase. Future studies should attempt to identify and characterize a kinetochore protein complex responsible for the signal sensing/conversion.

Recently it has been shown that a defect in the spindle checkpoint causes tetraploidy/aneuploidy and leads to tumorigenesis in the mouse model studies. Thus, the spindle checkpoint can be

referred to as a guardian of chromosome stability and a barrier of tumor development/progression. Although spindle poisons such as taxol and nocodazole may be considered as effective anticancer drugs, the cellular effect of these drugs should be reexamined. In the presence of a spindle poison, cells lacking the spindle checkpoint would escape mitosis without separating sister chromatids and become polyploids, some of which could grow more aggressively.

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Chapter 3

Understanding Cytokinesis Failure

Guillaume Normand and Randall W. King*

Abstract

Cytokinesis is the final step in cell division. The process begins during chromosome segregation, when the ingressing cleavage furrow begins to partition the cytoplasm between the nascent daughter cells. The process is not completed until much later, however, when the final cytoplasmic bridge connecting the two daughter cells is severed. Cytokinesis is a highly ordered process, requiring an intricate interplay between cytoskeletal, chromosomal and cell cycle regulatory pathways. A surprisingly broad range of additional cellular processes are also important for cytokinesis, including protein and membrane trafficking, lipid metabolism, protein synthesis and signaling pathways. As a highly regulated, complex process, it is not surprising that cytokinesis can sometimes fail. Cytokinesis failure leads to both centrosome amplification and production of tetraploid cells, which may set the stage for the development of tumor cells. However, tetraploid cells are abundant components of some normal tissues including liver and heart, indicating that cytokinesis is physiologically regulated. In this chapter, we summarize our current understanding of the mechanisms of cytokinesis, emphasizing steps in the pathway that may be regulated or prone to failure. Our discussion emphasizes findings in vertebrate cells although we have attempted to highlight important contributions from other model systems.

Cytokinesis Occurs in Multiple Stages

The process of cytokinesis can be divided into four stages including specification of the cleavage plane, ingression of the cleavage furrow, formation of the midbody and abscission (Fig. 1). Each stage is dependent on the proper execution of the prior stage and thus interference with any stage may result in cytokinesis failure. The first stage of cytokinesis specifies the cleavage plane by recruiting a central regulator of cytokinesis, RhoA, to the site of cleavage. If this step is perturbed, cytokinesis will not initiate properly. In the second stage of cytokinesis, the cleavage furrow ingresses through formation of an actomyosin ring and myosin-dependent motor activity. Failure at this step may lead to a lack of furrow initiation or partial ingression of the furrow followed by regression. The third stage of cytokinesis is characterized by formation of the midbody and stabilization of the cytokinetic furrow. This stage requires proper function of proteins located in the central spindle, a microtubule-based structure that separates segregated chromosomes during anaphase, and on proteins that stabilize interactions between the actomyosin ring and the central spindle. A failure at this stage will lead to regression of the cleavage furrow. The final stage in cytokinesis, abscission, is the step in which the cytoplasmic contents are finally separated from one another. This event requires the presence of a functional midbody, but also additional proteins involved in vesicle trafficking and fusion. Failure at this stage may lead to regression of the cleavage furrow or to formation of a persistent connection between the two daughter cells. Cytokinesis is thus a series of linked processes and a problem at any step of this cascade may be sufficient to induce

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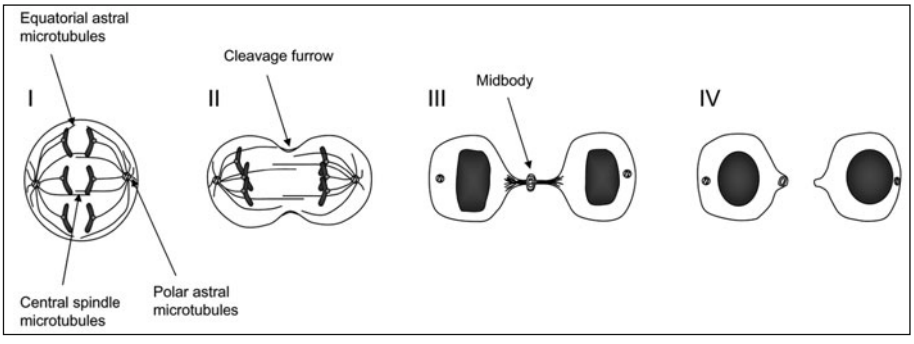

Figure 1. Multiple stages of cytokinesis. Three populations of microtubules first specify the site of cleavage by activating RhoA in a narrow zone between segregating chromosomes (I). Formation and activation of the actomyosin ring next leads to furrow ingression (II). The constricting furrow compacts the central spindle microtubules leading to midbody formation (III). Abscission of the furrow occurs by physically separating the cytoplasm of the daughter cells (IV).

failure. Some proteins participate in multiple steps in cytokinesis and thus perturbation of their abundance or activity may be especially prone to induce cytokinesis failure.

Stage I. Positioning the Division Plane and Initiating Cytokinesis

The Importance of Microtubules

Classic micromanipulation experiments determined that the mitotic spindle dictates the position of the cleavage furrow.[1,2] However, a bipolar spindle is not necessary for induction of a cleavage furrow,[3,4] suggesting that microtubules themselves play an essential role in initiating cleavage. Three separate populations of microtubules have been implicated in the regulation of cytokinesis (Fig. 1; reviewed by ref. 5). First, equatorial astral microtubules, which emanate from the spindle pole to the site of cleavage, may be stabilized in the equatorial cortical region[3] and deliver positive signals that stimulate formation and contraction of the cleavage furrow.[2] In contrast, polar astral microtubules, which emanate from the spindle pole to sites away from the site of the furrow, may help position the cleavage furrow by inhibiting cortical contractility,[6-8] perhaps by spatially biasing the pattern of myosin recruitment.[9,10] Finally, central spindle microtubules, which form an overlapping network between the spindle poles following anaphase, send positive signals that become especially important during later steps of cytokinesis. The signals sent by these distinct microtubule populations are partially redundant, ensuring that selection of the division plane is robust.[11,12]

The RhoA Pathway Plays an Essential Role in Furrow Initiation

What are the positive signals delivered by microtubules that initiate furrowing at the correct place in the cell? A central event is the localized activation of the small GTPase RhoA at the site of the future furrow (Fig. 2; reviewed by ref. 13). RhoA is essential for furrow formation in animal cells[14-17] and activated RhoA localizes to a narrow zone within the furrow.[18-22] Localized activation of RhoA within this narrow zone is thought to be important for efficient furrowing, as perturbations that broaden the zone of RhoA activation often lead to a failure of the furrow to form or to ingress.[19] A narrow zone of activation is established by tethering RhoA activators to the central spindle, delivering a strong yet spatially restricted signal for cytokinesis initiation.

An essential activator of RhoA is the guanine nucleotide exchange factor ECT2,[17,19,23-26] originally identified as a protooncogene.[27] ECT2 is sequestered in the nucleus during interphase (Fig. 2) and released following nuclear envelope breakdown in mitosis, but the protein remains inactive because it exists in an autoinhibited conformation.[24,28] In late anaphase, ECT2 localizes to the central spindle and associates with the centralspindlin complex, composed of the kinesin protein

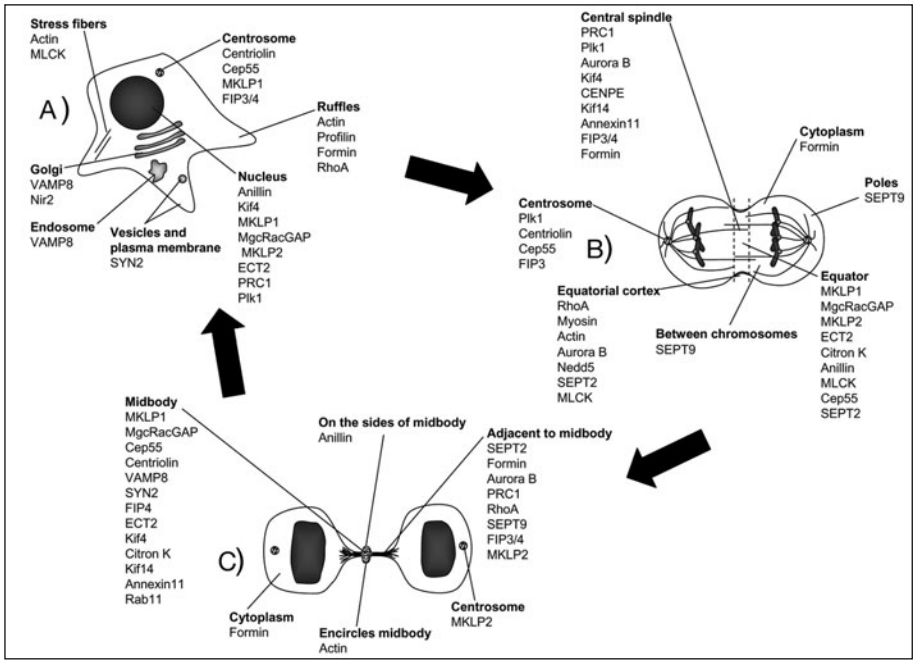

Figure 2. Localization of cytokinesis components. Interphase (A), anaphase (B) and late cytokinesis (C).

MKLP1 and the GTPase activating protein (GAP) MgcRacGAP.[17,19,29,30] MgcRacGAP binds to ECT2 and stabilizes it in an active conformation that permits it to interact with RhoA.[19] Tethering of the centralspindlin complex to the central spindle is thought to restrict activated ECT2 within a narrow zone, resulting in a narrow zone of RhoA activation.[19,23,25,29] Depletion of MKLP1[19] or disruption of the central spindle[31,32] leads to delocalization of ECT2 and MgcRacGAP from the central spindle, broadening the region of RhoA activation.[19] Cells containing a broad zone of RhoA activation fail to form a furrow.[19] Therefore, tethering of MgcRacGAP and ECT2 to the central spindle is not essential for RhoA activation, but is instead important for efficient furrowing by restricting the zone of RhoA to within a narrow zone at the equator of the cell.

These findings suggest that cytokinesis failure could result from failure to properly deliver RhoA activators to the cortex, causing insufficient activation of RhoA. Alternatively, cytokinesis could fail if RhoA is activated too broadly, in regions outside the cleavage furrow. Interestingly, ECT2 deregulation can lead to oncogenic transformation[27,28,33] although it is not clear whether perturbation of cytokinesis is an important component of this phenomenon, as ECT2 may participate in other processes such as spindle assembly[34] and regulation of the Ras/MAP kinase pathway.[35] Like many genes involved in cell division, ECT2 expression is induced by growth factors[36] in a manner that depends on the Rb/E2F pathway.[37] ECT2 is overexpressed in some tumors[38,39] where it could broaden the region of RhoA activation, perturbing proper initiation of cytokinesis. Alternatively, elevated ECT2 could perturb late stages of cytokinesis, as RhoA may need to be inactivated for cytokinesis to be completed.[25] In fact, overexpression of some fragments of ECT2 has no effect on cytokinesis initiation, but specifically blocks later stages of cytokinesis.[24,25]

Other proteins may regulate RhoA activity during cytokinesis.[40] These include additional Rho GEFs such as GEF-H1[41] and MyoGEF,[42] both of which are essential for cytokinesis in mammalian cells. Additional proteins may influence the location and timing of RhoA activation, including the armadillo protein p0071[43] and the Rho effector mDia1, which may sustain RhoA activation

in a positive-feedback loop.[44] In contrast, the protein HEF1, which is upregulated in tumor cells, may impair the RhoA activation cycle.[45]

GAP proteins are also important for controlling RhoA activation and inactivation. As stated earlier, RhoA may need to be inactivated during late cytokinesis to disassemble the cleavage furrow and thus hyperactivation of RhoA could block cytokinesis completion. Two GAP proteins that may inactivate RhoA during cytokinesis are MgcRacGAP and p190 RhoGAP.[46] Although MgcRacGAP plays a critical role in activation of RhoA by recruiting and activating ECT2, phosphorylation of MgcRacGAP by Aurora kinases may stimulate its ability to serve as a RhoGAP,[47] contributing to RhoA inactivation. As its name suggests, MgcRacGAP may also inhibit the GTPase Rac. The activity of Rac is suppressed in the spindle midzone[21] and constitutively activated Rac induces a mutlinucleation.[48] Thus in addition to activating RhoA by recruiting ECT2, MgcRacGAP may inactivate Rac in the furrow to support cytokinesis.[48-51]

Failure of Cytokinesis During Stage I

Together these studies emphasize the importance of microtubules in delivering signals that lead to localized activation of RhoA and possibly suppression of Rac, in the furrow. Recent studies suggest that cytokinesis failure may occur in cells in which spindle elongation or spindle positioning is perturbed, disrupting delivery of activation signals to the cortex. The first example is binucleation of cells in the liver, which may be regulated physiologically.[52,53] In humans, the number of polyploid cells averages 30-40% in the adult liver.[54,55] Studies in rat hepatocytes indicate that tetraploid cells arise from cytokinesis failure in which diploid, mononucleated cells undergo mitosis but do not form a contractile ring.[52] Cells do not undergo anaphase spindle elongation, perhaps because reorganization of the actin cytoskeleton is impaired.[53] Furthermore, astral microtubules fail to contact the equatorial cortex in cells that fail cytokinesis,[53] suggesting that the delivery of RhoA activators to the cortex is impaired. In rat liver, the number of binucleated cells increases following weaning, suggesting there may be important connections between liver physiology and cytokinesis regulation,[53] but how these pathways might impact microtubule organization remains unclear.

The second example is cytokinesis failure that occurs in cells that contain mutations in the APC (Adenomatous Polyposis Coli) tumor suppressor. Some APC mutations may induce cytokinesis failure by interfering with microtubule-dependent anchoring of the mitotic spindle.[56] Although APC has important roles in formation of the mitotic spindle and the spindle checkpoint,[57-60] cells expressing APC mutants become polyploid over time,[56,59,61] indicating that the protein is important for proper cytokinesis. Different APC alleles may have distinct effects on mitosis.[56] For example, in cells expressing a particular C-terminal truncation mutant of APC, microtubules make less contact with the cell cortex, spindles undergo considerable rotation during mitosis and cells do not efficiently initiate cytokinesis.[56] The physiological relevance of these findings was confirmed by the finding that the *Min* allele of APC gives rise to similar mitotic defects and that the frequency of tetraploid cells is greatly increased in *Min*[56]and APC knockout mice.[60] Although it is likely that tetraploidy can arise through multiple mechanisms in tumors carrying different APC alleles, these findings suggest that failure to properly anchor the mitotic spindle can be an important source of tetraploidy.

Stage II. Ingression of the Cleavage Furrow

In the second stage of cytokinesis, activated RhoA leads to recruitment and activation of effector proteins that organize the furrow and stimulate ingression (Fig. 3). RhoA stimulates actin polymerization through activation of formins and stimulates myosin activity by activating kinases such as Rho kinase (ROCK) and Citron kinase. Scaffolding proteins such as anillin and septins also play important roles in organizing the cleavage furrow and promoting cytokinesis. Here we discuss each of these processes and how they might be perturbed to result in cytokinesis failure.

Stimulation of Actin Filament Assembly

Formins are proteins that nucleate formation of unbranched actin filaments in response to stimulation by RhoA (for review, see ref. 62). In the absence of active RhoA, most of the formins

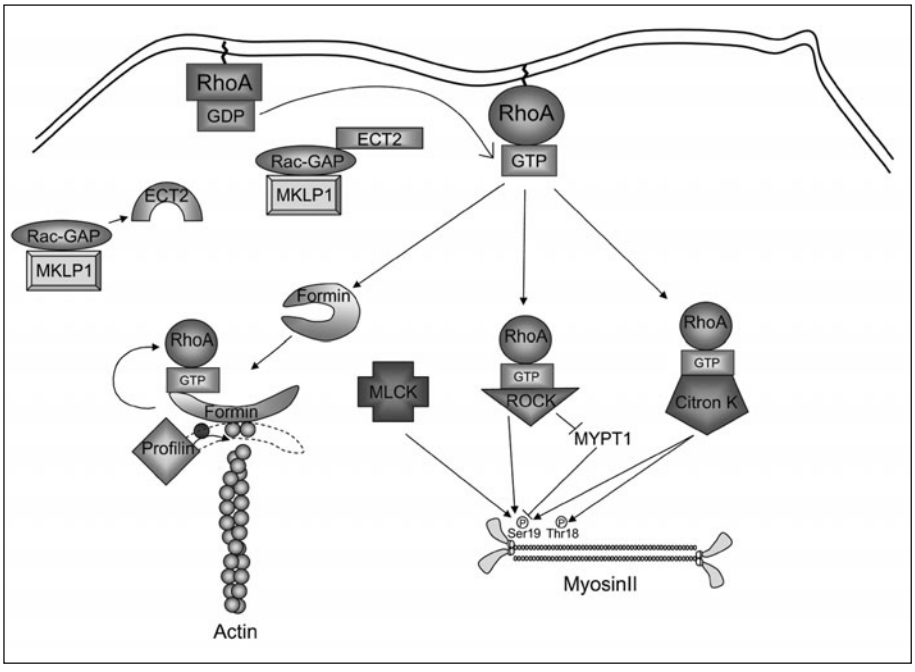

Figure 3. Role of the RhoA pathway in furrow initiation. Autoinhibition of ECT2 is suppressed by association of ECT2 with MgcRacGAP. ECT2 then activates RhoA by stimulating exchange of GDP for GTP. Active RhoA then activates formins to stimulate actin nucleation and binds to ROCK and Citron kinases, stimulating phosphorylation and activation of myosin.

that regulate cytokinesis are autoinhibited.[63] RhoA binding relieves autoinhibition to promote actin polymerization.[64,65] The mammalian formin mDia1 is activated downstream of Rho signaling,[66] and cytokinesis is blocked if mDia1 is inhibited by antibody injection.[67] However, deletion of mDia1 does not perturb cytokinesis in mouse embryonic fibroblasts,[68] suggesting redundant pathways for actin nucleation. Cytokinesis may also depend on the use of preexisting actin filaments that are nucleated outside the furrow.[69,70] Formins may be important in later stages of cytokinesis, as they have been implicated in regulation of Src activity, which has been shown to be important for completion of cytokinesis.[67,71]

Localization and Activation of Myosin

Myosin II (hereafter simply referred to as myosin) is the principle motor protein required for cytokinesis (for review, see ref. 72). Myosin is recruited to the cleavage furrow at early stages of cytokinesis in a RhoA-dependent fashion. Myosin activity and localization are regulated by phosphorylation of its regulatory light chain (myosin light chain or MLC). Because myosin motor activity is directly required for furrow ingression,[73] perturbation of myosin localization or its activity could result in cytokinesis failure.

Phosphorylation of serine 19 of MLC stimulates actin-activated ATPase activity of myosin,[74,75] whereas phosphorylation at threonine 18 promotes myosin assembly. Phosphorylation of myosin at these positions is important for proper localization of myosin to the furrow and for ingression.[76-79] In contrast, phosphorylation at serines 1, 2 and 9 of MLC inhibits myosin ATPase activity[80,81] During mitosis, MLC is phosphorylated at these positions by CDK1.[76,82,83] At anaphase, inactivation of CDK1, controlled by the degradation of mitotic cyclins by the Anaphase-Promoting Complex/Cyclosome (APC/C), is important for MLC dephosphorylation and myosin activation

during cytokinesis. Therefore, failure to degrade mitotic cyclins, or to fully inactivate CDK1, could perturb myosin activation and disrupt cytokinesis.

Three kinases contribute to myosin activation by phosphorylating positions 18 and 19 of MLC (Fig. 3). Two of these kinases, ROCK and Citron kinase, are activated by RhoA. ROCK localizes to cleavage furrows[84,85] and a small molecule inhibitor of ROCK slows cleavage.[84] Citron kinase also localizes to the cleavage furrow and is required for cytokinesis in several systems.[25,50,86-92] Citron kinase can phosphorylate MLC at both ser19 and thr18[93] and its overexpression causes unregulated contraction of the cortex, supporting its role as a positive regulator of myosin activity.[87] Mouse knockout studies suggest that Citron kinase may play an especially important role in neurogeneic and spermatogenic cytokinesis.[94-97] It is likely that ROCK and Citron kinase play partially overlapping roles, explaining why each protein is not essential for cytokinesis in all systems. There is no evidence to suggest that ROCK or Citron kinase is overexpressed or mutated in human tumors, but Citron kinase interacts with the kinesin protein KIF14,[89] which is overexpressed in several tumor types.[98-101] Whether overexpression of KIF14 perturbs Citron kinase function in cytokinesis remains unknown. Knockdown of KIF14 induces cytokinesis failure,[102] perhaps as a result of a failure to recruit Citron kinase to the cleavage furrow.

Myosin light chain kinase (MLCK) is the third and final kinase that has been implicated in direct phosphorylation of myosin light chain. This kinase is activated by calcium/calmodulin and some isoforms of MLCK and calmodulin localize to the cleavage furrow.[103-106] Inhibition of calmodulin or MLCK can disrupt cytokinesis in cultured cells,[106-108] but mice lacking MLCK develop normally, but die after birth, suggesting the kinase is not essential for cytokinesis in all tissues.[109] How myosin light chain kinase is regulated in the cleavage furrow is unclear, but hydrolysis of PIP2 may be important for IP3-induced calcium release, which could stimulate MLCK activity.[110] The mild and varied phenotypes associated with MLCK inhibition again suggest functional redundancy in MLC phosphorylation during cytokinesis.

The overall degree of MLC phosphorylation is also affected by the activity level of myosin phosphatase. This enzyme is inhibited by several mechanisms during cytokinesis to favor MLC phosphorylation. Myosin phosphatase is a heterotrimeric enzyme consisting of a targeting subunit that binds myosin (MYPT1 or MBS), a catalytic subunit (the delta isoform of PP1c) and an additional small subunit. Both ROCK and Aurora B may phosphorylate MYPT1 in the furrow to inactivate the phosphatase.[111-113] In addition, a number of other kinases including Raf-1[114] may negatively regulate myosin phosphatase (reviewed in ref. 72). Thus a number of signaling pathways could converge on myosin phosphatase to regulate cytokinesis.

Organization of Actin and Myosin in the Furrow

How actin and myosin are organized within the cleavage furrow and how contraction occurs, is not well understood (for a more detailed discussion, see refs. 115 and 116). Phosphorylated myosin localizes to the furrow in early anaphase[79] and localization of myosin requires RhoA activation[13,117] but not myosin ATPase activity.[73,118,119] Recruitment may require phosphorylation of MLC, as mutations or inactivation of kinases that perturb MLC phosphorylation also disrupt myosin localization. Other scaffolding components, such as anillin, may be important for maintaining myosin within the furrow, as discussed below.

Both actin and myosin are highly dynamic in furrows and dynamic actin is important for cytokinesis.[108,120] For example, the actin disassembly factor cofilin is necessary for cytokinesis.[121] Cofilin is negatively regulated by the kinase LIMK1 and thus upregulation of LIMK1, or loss of its negative regulator LATS1, is sufficient to enhance actin polymerization and induce cytokinesis failure.[122] Interestingly, inhibition of myosin slows disassembly of actin filaments of the furrow,[123] suggesting that myosin motor activity may help drive actin disassembly.

Scaffolding Proteins in the Furrow

Anillin

Another conserved furrow component is anillin, which may act as a scaffold protein that binds F-actin, myosin, septins and activated RhoA.[124-129] Although anillin localizes to the furrow at early stages of cytokinesis, it is not essential for ingression. Instead, it may stabilize the furrow and be important for later stages of cytokinesis including midbody formation and abscission.[92,127,130-132] However, anillin becomes essential for ingression if the central spindle is disrupted, suggesting it may make early steps of cytokinesis more robust.[128] Anillin interacts with RhoA[128] and its localization to the furrow requires activation of RhoA.[128,129,132,133] MgcRacGAP may also be directly involved in targeting anillin to the furrow,[134] providing a link between the centralspindlin complex and anillin localization.

Anillin contains domains that permit it to interact with phosphorylated myosin,[127] actin filaments and septins.[124,125,135] These features make anillin ideally suited to crosslink the actomyosin and septin cytoskeletons within the contractile ring. Anillin may enhance the robustness of early stages of cytokinesis by promoting the anchoring of myosin in the vicinity of activated RhoA, favoring myosin phosphorylation. Anillin may be essential for asymmetric ingression of the cytokinetic furrow, which occurs when the furrow ingresses from only one side of the cell, rather than circumferentially.[136] Asymmetric ingression may be important in epithelia[137] and embryos[136,138] where it may serve a mechanical function, enhancing the robustness of cytokinesis. Anillin is also important for completion of cytokinesis, as anillin remains in the cytoplasmic bridge even after myosin and actin have dissociated.[127] Interestingly, whereas overexpression of anillin seems to have little phenotype in Drosophila S2 cells,[129] overexpression of anillin in mammalian cells is very toxic,[132] suggesting anillin could have important functions independent of cytokinesis. Levels of anillin appear to be controlled by the ubiquitin-proteasome pathway, as anillin is targeted for ubiquitination and degradation during G1 by the APC/C.[132]

Like the liver, the heart also contains a large number of tetraploid cells that arise through cytokinesis failure. Although it was originally proposed that cytokinesis failure might be a consequence of failure to disassemble myofibrils within cardiomyocytes, recent work suggests this is unlikely to be the case.[139] Instead, cells that fail cytokinesis show complete disassembly of the myofibril, but show abnormal localization of anillin and failure of anillin to concentrate at the midbody.[139] However, these cells also show delays in furrow ingression, suggesting that earlier steps in cytokinesis may also be affected in these cells.

Septins

Septins represent a second class of scaffolding protein that may help to organize proteins within the cleavage furrow. Septins are GTP-binding proteins that can form filaments and localize to the cytokinetic ring.[140-144] Several human septins have been implicated in cytokinesis, including SEPT2 (Nedd5), SEPT9 (MSF) and SEPT12. SEPT12 localizes to the central spindle and midbody during anaphase and cytokinesis, respectively.[145] SEPT2 accumulates in the contractile ring and midbody[146-148] and microinjection of antibodies[146] or antisense downregulation[147]of SEPT2 interferes with cytokinesis. Inactivation of SEPT9 by antibody microinjection or siRNA also induces cytokinetic defects.[149,150]

Septins may participate in several aspects of cytokinesis, including regulation of actin and microtubule dynamics. SEPT2 associates with actin, forming filaments in association with actin bundles and focal adhesions,[146] whereas SEPT9 associates with the microtubule network.[150,151] Septins may also play a direct role in cytokinesis by interacting with anilllin.[124,125,135] Furthermore, SEPT2-containing filaments may provide a molecular platform for myosin and its kinases to ensure the full activation of myosin that is necessary for cytokinesis.[152] Finally, septins may form a barrier that restricts the diffusion of membrane proteins in the furrow,[153,154] thus helping retain activated RhoA within the narrow zone required for efficient initiation of cytokinesis. In mammalian cells, the p85 subunit of PI3 kinase may regulate SEPT2 in cytokinesis,[155] linking cellular signaling pathways with steps in cytokinesis. Septins may be deregulated in tumors, either through gene

fusions[156-158] or by overexpression.[159] The SEPT9 gene is amplified and overexpressed in mouse mammary tumors and human breast cancer cell lines[158] and high SEPT9 expression in human breast cancer cells is associated with oncogenic phenotypes and cytokinesis defects.[160]

Stage III. Formation of the Midbody

The central spindle, also referred to as the spindle midzone, plays an important role in keeping separated chromosomes apart prior to cytokinesis completion, because when microtubules are depolymerized in late anaphase, the nuclei collapse back together.[73] Microtubules in the midzone may be locally nucleated, as the minus ends of the midzone microtubules are decorated with gamma-tubulin.[161,162] As cytokinesis progresses, the constricting furrow compacts the midzone microtubule array. The furrow ingresses until a cytoplasmic bridge is formed that is 1-1.5 microns in diameter. Several kinesin-like motor proteins and chromosomal passenger proteins move along the midzone spindle towards the plus ends and accumulate in the overlapping region, forming a phase-dense structure referred to as the Flemming body, stembody, telophase disc, or midbody (reviewed in ref. 163). Disassembly of the actomyosin ring may be an important step at this stage of cytokinesis, as loss of F-actin accompanies and may trigger midbody formation.[164] Once the cytoplasmic bridge matures and abscission begins, the bridge becomes insensitive to the actin inhibitor latrunculin,[92] implying that the plasma membrane is linked to the midbody by a connection that does not involve dynamic F-actin. Scaffolding proteins such as anillin and septins may stabilize the bridge structure. In almost all systems, central spindle formation is essential for midbody formation, which in turn is necessary for abscission.[16,32,165] In this section, we discuss the components that are required for formation of the central spindle and midbody.

PRC1 is a microtubule bundling protein that is critical for midzone formation in mammalian cells.[32,166] PRC1 accumulates on the central spindle in anaphase and suppression of PRC1 expression causes failure of microtubule interdigitation.[32] In the absence of PRC1, astral microtubules can guide the equatorial accumulation of anillin, actin and chromosome passenger proteins, enabling cleavage furrow ingression, but abscission fails.[167] PRC1 has separate domains that independently target the protein to the midzone and bundle microtubules.[32] PRC1 is targeted to the midzone by the kinesin protein KIF4, which transports PRC1 to the ends of microtubules. Absence of KIF4 leads to a failure to accumulate PRC1 in the central spindle and abolishes central spindle formation.[168] PRC1 in turn recruits the centralspindlin complex and additional mitotic kinesins including CENP-E, MCAK[169] and KIF14.[89] PRC1 also serves as an important docking site for the kinase Plk1 in the central spindle.[170] PRC1 expression may be perturbed in cancer cells or in response to checkpoint signaling pathways. PRC1 upregulation in tumors[169,171] may be a consequence of p53 inactivation, as induction of p53 can inhibit PRC1 expression and interfere with cytokinesis completion.[172,173]

Although the centralspindlin complex (MKLP1 and MgcRacGAP) is important for cytokinesis initiation as described earlier, centralspindlin is also necessary for spindle midzone and midbody formation and ultimately for abscission.[31,165] Centralspindlin is recruited to the midzone by PRC1 (reviewed in ref. 174) and proper localization requires the presence of both members of the centralspindlin complex.[16] A splice variant of MKLP1, called CHO1, includes an additional domain that can interact with F-actin,[175] suggesting that CHO1 could link the actin and microtubule cytoskeletons. Injection of antibodies that target this domain induces failure in late steps of cytokinesis,[175] suggesting CHO1 may stabilize interactions between midbody microtubules and the ingressing cleavage furrow in late steps of cytokinesis. Centralspindlin is also important for recruiting additional proteins to the midbody that are required for abscission and both components of the complex appear to be regulated by phosphorylation. Each of these topics will be discussed in more detail below.

Proteomic approaches have identified a large number of proteins that concentrate at the midbody (Fig. 2)[176] and a functional role for some of these proteins in abscission has been supported by results of RNAi experiments.[92,162,177] Several of these proteins localize to the Golgi apparatus during interphase and are released from the Golgi during mitosis by phosphorylation.[178-180]

Inhibition of Golgi disassembly during mitosis perturbs cytokinesis,[181] perhaps by interfering with release of components that are essential for cytokinesis. Precisely how these proteins function in cytokinesis remains unclear, but one potential function is to recruit mitotic regulators such as Plk1 to the midbody.[179]

Additional proteins that localize to the midbody and are required for cytokinesis include LAPSER1, which may recruit the microtubule severing protein katanin to the midbody,[182] and annexin 11.[183] Annexins are Ca(2+)-binding, membrane-fusogenic proteins with diverse but poorly understood functions. Cells lacking annexin 11 fail to establish a functional midbody and instead remain connected by intercellular bridges that contain bundled microtubules but exclude normal midbody components such as MKLP1 and Aurora B.[183] These data suggest that despite its potential role in membrane fusion, annexin 11 seems to be required at an earlier step for recruitment of MKLP1 and Aurora B to the midbody.

Stage IV. Abscission

Once the midbody is formed, it subsequently organizes the final event of cytokinesis, termed abscission. By the time of abscission, the cytoplasmic bridge has narrowed to 0.2 microns in diameter. At these late stages, microtubule bundles become compacted and begin to disappear.[92,184] In this process, the cytoplasmic bridge is reorganized to permit separation of the daughter cells. A wide variety of proteins involved in vesicle and protein trafficking, membrane fusion and other processes are required for abscission, suggesting the final stage of cytokinesis is just as complex as earlier stages. Human cultured cells may remain connected by the cytoplasmic bridge for many hours before undergoing abscission.[185] In some systems, such as embryos, blastomeres often remain connected by intracellular bridges for many cell cycles. In spermatocytes, the cytoplasmic bridge is in fact stabilized[186] and cytokinesis completion does not occur, enabling communication between the cytoplasm of adjacent cells. Thus in certain circumstances abscission may be the target of physiological regulation.

Membrane Trafficking and Cytokinesis

Membrane trafficking plays a critical role in the process of cytokinesis (Fig. 4). Three pathways have been implicated in the process of cytokinesis. First, the secretory pathway, including Golgi-derived components, may contribute new membranes and proteins to the ingressing furrow and also participate in late steps of cytokinesis completion. Second, the endocytic pathway and recycling endosomes may remodel membranes in the cleavage furrow and also contribute vesicles that may participate in the final steps of cytokinesis. Finally, recent evidence suggests that components of the ESCRT machinery, best characterized for its role in multivesicular body formation, may also be essential for the final stages of cytokinesis. The relative contributions of each of these pathways in the process of cytokinesis is likely to be dependent on cell type.

The Role of the Secretory Pathway

In large embryos, such as *Xenopus* and Sea Urchins, furrow ingression is coupled to insertion of new membrane via microtubule-dependent exocytosis.[187-190] In smaller cells, such as mammalian tissue culture cells, it is less clear whether new membrane insertion is required. Brefeldin A (BFA), which disrupts ER-Golgi-dependent trafficking, blocks cytokinesis completion in some studies[191,192] but not others.[193-196] Further evidence for a role of Golgi-derived vesicles in cytokinesis completion has emerged from studies of the protein centriolin, which may help recruit secretory vesicles to the site of abscission at the midbody.[191] Centriolin was initially identified as a protein that localizes to the maternal centriole during interphase and accumulates on mature centrioles during metaphase.[197] Knockdown of centriolin in mammalian cells causes cytokinesis failure, with the two cells remaining connected by a cytoplasmic bridge.[197] Centriolin localizes to a ring-like structure within the midbody,[191] that also contains gamma-tubulin, GAP-CenA and the centralspindlin complex. Recruitment of centriolin to the midbody ring is dependent on the centralspindlin complex, explaining why centralspindlin may be essential for cytokinesis completion.[191]

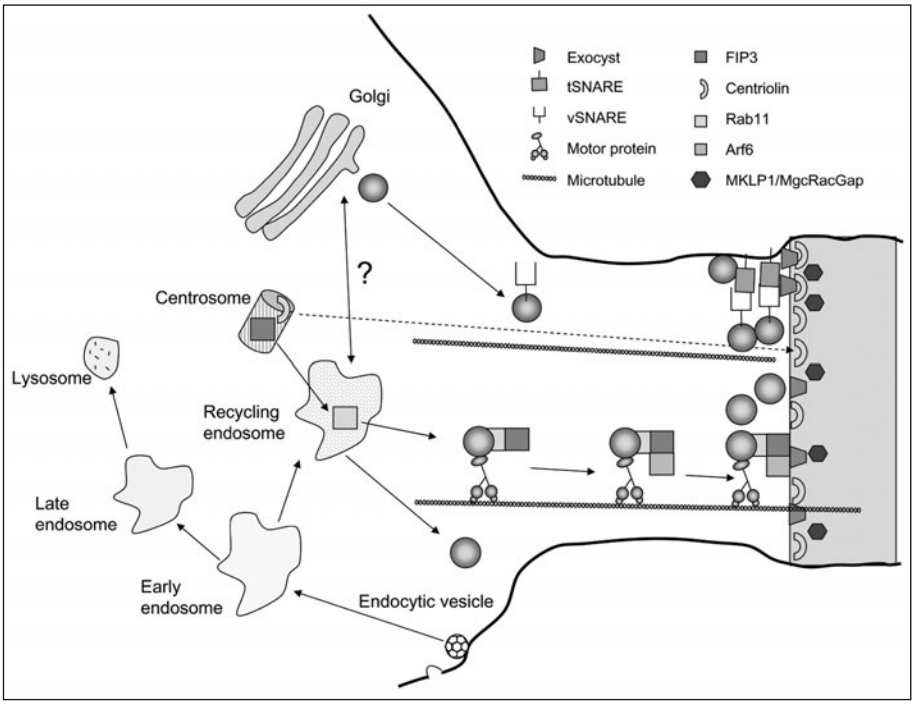

Figure 4. Membrane trafficking in cytokinesis. Secretory vesicles accumulate at the intercellular bridge in a centriolin-dependent manner by SNARE interaction and vesicle tethering by the exocyst complex. Vesicles originating from the recycling endosome and containing the complex Rab11/FIP3 move along microtubules. Interaction of FIP3 with both ARF6 and the exocyst permits vesicle targeting to the midbody. Vesicles then fuse with each other and the plasma membrane, physically separating the daughter cells.

Centriolin may facilitate cytokinesis completion by recruiting to the midbody proteins involved in vesicle tethering and fusion. Centriolin interacts with components of the exocyst,[191] a protein complex that tethers secretory vesicles to the plasma membrane (reviewed in ref. 198). Several components of the exocyst localize to the midbody ring in a centriolin-dependent manner and depletion of exocyst components by siRNA interferes with cytokinesis completion.[191] Centriolin also interacts with snapin, a snare-associated protein and centriolin is required for the recruitment of snapin and SNARE proteins to the midbody.[191]

Secretory vesicles, derived from the Golgi apparatus, accumulate at the intercellular bridge during late steps in cytokinesis.[191] At the time of abscission, the vesicles disappear, suggesting they undergo homotypic fusion with each other and also heterotypic fusion with the plasma membrane, releasing their contents.[191] Interestingly, vesicles seem to accumulate only on one side of the midbody, suggesting that delivery is asymmetric.[191] This finding is consistent with the fact that following abscission, the midbody remains attached to one of the two daughter cells where it may play additional roles in signaling or marking the age of the cell.[191] Why abscission typically occurs on only one side of the midbody remains unclear. It has been suggested that abscission may be triggered by arrival of the maternal centriole from one daughter cell,[184] but this event has not been observed consistently.[191] Asymmetric abscission may be important to enable the midbody to remain attached to a daughter cell, or it may provide an opportunity to regulate the timing of abscission. However, asymmetric abscission may be inherently more prone to failure than if abscission were to occur on both sides

of the midbody. The mechanism and significance of asymmetric abscission is an interesting topic for future investigation.

The Role of Endocytosis and the Recycling Endosome Pathway

Several lines of evidence suggest that the endocytic and the recycling endosome pathways play critical roles in cytokinesis completion. Endocytosis within the furrow may be important for remodeling the plasma membrane during ingression. In addition, endocytosis from other regions of the cell may serve as a source of vesicles destined for delivery to the cleavage furrow either directly or through the recycling endosome. For example, some endocytic vesicles internalized from the polar region are subsequently trafficked to the midbody area during later stages of cytokinesis.[199] Inhibition of proteins essential for endocytosis, including clathrin, dynamin and alpha-adaptin, perturb cytokinesis in several systems[200-204] and inhibitors of clathrin-dependent endocytosis block cytokinesis completion in mammalian cells.[195,199] In addition, there may be direct interactions between the endocytic machinery and proteins required for cytokinesis such as anillin.[205]

Small GTPases that regulate membrane trafficking have been directly implicated in cytokinesis completion. Arf GTPases initiate the budding of vesicles by recruiting coat protein complexes onto donor membranes, whereas Rab GTPases regulate the targeting and docking/fusion of vesicles with acceptor membranes.[206] Two different GTPases, Arf6 and Rab11, have been implicated in regulation of cytokinesis. Rab11 localizes preferentially to the recycling endosome (RE) and is required for proper RE organization and the recycling of vesicles to the plasma membrane. Both Arf6 and Rab11 concentrate near the cleavage furrow and are required for late steps of cytokinesis in mammalian cells.[199,207-210] Both GTPases interact with a common set of effector proteins that assist in delivery of endosomal vesicles to the cleavage furrow, termed FIP3 (Arfophilin-1) and FIP4 (Arfophilin-2).[207-209,211-214] FIP3-containing endosomes accumulate near the cleavage furrow and are required for successful completion of cytokinesis.[209] Recruitment of FIP3 to the midbody requires ARF6 and recruitment of ARF6 to the midbody requires FIP3.[215] Other studies show that Arf6 interacts with MKLP1, suggesting the centralspindlin complex is important for targeting Arf6 to the cleavage furrow.[207,216] The exocyst has also been implicated in targeting of vesicles derived from the recycling endosome. For example, Exo70, a component of the exocyst complex, colocalizes with Arf6 in Rab11-positive endosomes.[208] Exo70 interacts with FIP3 and FIP4 biochemically and depletion of Exo70 impairs FIP3 and Rab11 localization to the furrow and midbody.[208] Together these studies suggest the following model of delivery of endosomal vesicles to the midbody (Fig. 4). Rab11 first recruits FIP3 to endosomes. FIP3 in turn associates with ARF6 and together this complex localizes to the midbody via interactions with the exocyst and MKLP1.

Membrane Fusion During Abscission

Following vesicle targeting to the site of abscission, membrane fusion is necessary to complete cytokinesis. SNARE proteins are critical components required for membrane fusion (reviewed in ref. 217). Several SNARE proteins or associated components have been implicated in cytokinesis completion in different organisms.[92,218-220] In mammalian cells, two SNARE proteins, syntaxin 2 and endobrevin/VAMP-8, localize to the midbody during cytokinesis.[191,221,222] Expression of dominant negative mutants or depletion of SNAREs impairs abscission, but has no effect on ingression of the cleavage furrow, suggesting that SNARE-mediated fusion is required only in the latest steps of cytokinesis.[191,221] Septin proteins may assist in membrane fusion by restricting the diffusion of membrane-associated components such as the exocyst to the region of abscision.[153] Furthermore, septins may assist in abscission by directly recruiting the exocyst[223] and SNARE proteins.[224]

Role of the ESCRT Machinery

Recently, protein subunits of the Endosomal Sorting Complex Required for Transport (ESCRT) that are normally involved in late endosome to lysosome trafficking have also been implicated in abscission. These proteins are best known for their roles in multivesicular body formation (reviewed in ref. 225), where they are important for membrane invagination. ESCRT complexes also play important roles in the topologically equivalent process of viral budding. Because abscission

likely requires changes in membrane organization, a role for the ESCRT complex in cytokinesis is very intriguing. However, the precise mechanism of membrane invagination mediated by the ESCRT complex remains unknown and it is unclear whether the ESCRT pathway functions independently in abscission or whether it assists in secretory- or endosomal vesicle-mediated cytokinesis completion.

Components of the ESCRT machinery localize to the midbody and inhibition of some ESCRT complexes blocks late steps in cytokinesis. For example, CHMP3, a subunit of the ESCRT-III complex, localizes to the midbody and deletion of a C-terminal autoinhibitory domain of CHMP3 inhibits cytokinesis.[226] Other subunits of the ESCRT machinery implicated in abscission include tumor-susceptibility gene 101 (Tsg101), a subunit of the ESCRT-I complex and Alix, an ESCRT-associated protein.[222,227] Alix may interact with actin and microtubules,[228,229] establishing a link between the ESCRT machinery and cytoskeletal components that are present at the midbody. Alix and Tsg101 are recruited to the midbody by interaction with centrosome protein 55 (Cep55), a centrosome and midbody protein essential for abscission.[222,230,231] Interestingly, Tsg101 has been implicated in cancer and may have additional functional roles in cell cycle and transcriptional regulation (reviewed in ref. 225).

Regulation of Cytokinesis

Thus far we have outlined the core pathways and components essential for each stage of cytokinesis. In the remaining part of the chapter, we discuss how these components are regulated to ensure that cytokinesis occurs at the proper place and time. Many regulatory pathways impinge upon the cytokinesis machinery, suggesting that cytokinesis may be responsive to a variety of different cues within the cell. The complexity of cytokinesis regulation suggests that cytokinesis failure could result from alterations in the activity of these regulatory pathways.

Regulation of Cytokinesis by Protein Kinases

Cytokinesis is regulated by mitotic protein kinases, including cyclin-dependent kinases (CDKs), Polo kinase (Plk1) and the Aurora B kinase complex (Fig. 5). Mitotic CDK activity prevents cytokinesis onset until anaphase by phosphorylating cytokinesis components in a manner that inhibits their activity. For this reason, CDK1 must be inactivated for cytokinesis to proceed.[232] In fact, inhibition of CDK1 with a small molecule is sufficient to induce the initial events of cytokinesis,[233-235] suggesting that CDK1 inactivation is the trigger for cytokinesis initiation.

In contrast, Polo kinase and Aurora B kinase positively regulate the events of cytokinesis and must remain active for a period of time following CDK inactivation to promote cytokinesis. This period of the cell cycle, which lasts for about an hour in HeLa cells, has been referred to as "C-phase".[3,236] C-phase is initiated by inactivation of CDK1, mediated by cyclin destruction catalyzed by the APC/C. At later times following anaphase, the APC/C also ubiquitinates other proteins that are essential for cytokinesis, including anillin,[132] Polo kinase[237] and Aurora B.[238] Thus the APC/C may also be responsible for terminating C-phase, an idea that is consistent with the finding that treatment of cells with proteasome inhibitors doubles the duration of C-phase.[73]

Regulation of Cytokinesis by CDK Activity

Because CDK1 is a central negative regulator of cytokinesis, it is possible that failure to fully inactivate CDK1, perhaps as a consequence of failure to fully degrade mitotic cyclins, could inhibit cytokinesis at some step. One setting in which this might occur is in cells that become arrested in mitosis due to persistent activation of the spindle checkpoint, which normally inhibits the APC/C until chromosomes become properly aligned and attached at the metaphase plate.[239] Prolonged activation of the checkpoint may result in abnormal mitotic exit, resulting in incomplete activation of APC/C, or improper timing of degradation of different substrates, leading to cytokinesis failure. There is also evidence that APC/C activity may be spatially regulated within the cell, with the subpopulation of APC/C that is associated with the spindle poles remaining inhibited until later stages of mitosis.[240] It is therefore possible that perturbation of spindle organization could interfere with the timing of degradation of mitotic regulators, thus perturbing cytokinesis.

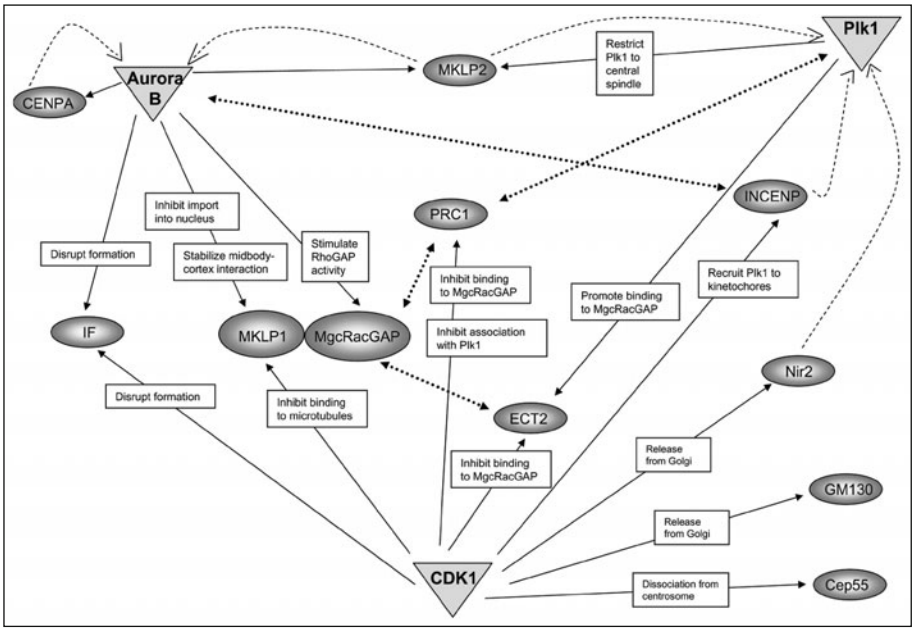

Figure 5. Regulation of cytokinesis by mitotic kinases. A major function of CDK1 is to prevent precocious cytokinesis before proper chromosome segregation. CDK1 thus negatively regulates some of the main players of cytokinesis. At the same time, CDK1 plays a positive role in cytokinesis by releasing cytokinesis proteins from the Golgi apparatus and by facilitating binding of Plk1 to its substrates. Plk1 and Aurora B phosphorylate substrates that are important for both early and late steps of cytokinesis. Solid arrows indicate phosphorylation; dashed arrows indicate changed protein localization; dotted arrows indicate protein interactions. IF, intermediate filaments.

CDK1 activity restrains multiple steps in cytokinesis. Cytokinesis initiation is inhibited because the RhoA pathway is kept inactive. This is a consequence of phosphorylation of ECT2 by CDK1 at a site that blocks its association with MgcRacGAP.[19,26] In addition, myosin light chain is phosphorylated by CDK1 at sites that inhibit myosin activation[82] and high CDK1 activity also inhibits cortical recruitment of myosin.[241] Central spindle formation is also inhibited by CDK1-dependent phosphorylation. Phosphorylation of PRC1 by CDK1 inhibits its ability to bundle microtubules[32,166,242] and its ability to interact with Plk1.[170] CDK1 also phosphorylates MKLP1, inhibiting its motor activity by reducing its affinity for microtubules.[243] Thus CDK1-dependent phosphorylation acts at many steps to block cytokinesis.

Though CDK1 restrains cytokinesis onset, CDK1-dependent phosphorylation is also essential for cytokinesis because it primes Plk1-dependent phosphorylation that occurs during early stages of cytokinesis. CDK1 activity is also important during mitosis to promote dissociation of cytokinesis proteins from cellular organelles that would otherwise sequester the protein. For example, the protein Nir2 is required for cytokinesis and must dissociate from the Golgi apparatus in order to participate in cytokinesis; dissociation is mediated by CDK1-dependent phosphorylation.[179] CDK1-dependent phosphorylation is also important for dissociation of Cep55 from the centrosome and its subsequent phosphorylation by Plk1.[231]

Regulation by Polo Kinase

Polo kinase is an essential positive regulator of cytokinesis in multiple organisms. In mammalian cells, Plk1 localizes to the midzone during anaphase and to the midbody during telophase

and cytokinesis[244] and plays an essential role in the initiation of cytokinesis.[245-248] Plk1 activity is required for recruitment of itself and ECT2 to the central spindle and inhibition of Plk1 with small molecule inhibitors abolishes RhoA GTPase localization to the equatorial cortex, suppressing cleavage furrow formation.[246-248] Plk1 also appears to be important for the interaction between ECT2 and MgcRacGAP.[247] Other evidence suggests that Plk1 may bind to ECT2 in a CDK1-dependent manner.[249] Another study using a distinct Plk1 inhibitor demonstrated that when Plk1 is inhibited, it spreads over the arms of chromosomes, resembling the localization of its binding partner PICH.[245] Therefore, Plk1 activity is required for its own proper localization during cytokinesis and also for recruitment and activation of RhoA.

Plk1 activity may be required for later steps in cytokinesis as well, as Plk1 is targeted to the central spindle by the motor protein MKLP2 and phosphorylation of MKLP2 by Plk1 is required for cytokinesis.[250] Phosphorylation of MKLP2 by Plk1 may be necessary for the spatial restriction of Plk1 to the central spindle during anaphase and telophase, although interaction with PRC1 also appears to be important for docking of Plk1 to the central spindle.[170] Plk1 may also interact with and phosphorylate MKLP1 during cytokinesis,[244,251] although others suggest that Aurora B may be the relevant kinase.[252] Cep55 also appears to be a Plk1 substrate whose phosphorylation is primed by CDK1 but the consequences of this phosphorylation remains unknown.[231] A recent proteomic screen identified a large number of proteins that bind to the Polo-box domain of Plk1, including the Rho kinase ROCK2,[253] where Plk1 and RhoA may function together to enhance ROCK2 activity. Another substrate of Plk1 that may be involved in regulation of mitosis and cytokinesis is NudC,[254,255] a dynein/dynactin associated protein that is essential for midzone formation and cytokinesis completion in C. elegans and mammalian cells.[254]

Plk1 is overexpressed in a broad range of human tumors (for review see ref. 256). Overexpression of Plk1 in HeLa cells leads to an increase of cells with large, often fragmented nuclei or multiple nuclei[257] as well as centrosome amplification,[258] suggesting that increased expression of Plk1 observed in some tumors may have an effect on cytokinesis completion as well as chromosome segregation. This finding has been corroborated in human primary cells.[259]

Regulation by Aurora B and the Chromosome Passenger Complex

The chromosome passenger complex (CPC) consists of the proteins Aurora B, INCENP, survivin and borealin. The complex plays many important roles throughout mitosis and has been implicated in the regulation of cytokinesis (see ref. 260 for review). At the metaphase-anaphase transition, the CPC relocalizes from centromeres to the spindle midzone and the equatorial cortex[261-263] and ultimately concentrates near the midbody, adjacent to the centriolin ring.[191] MKLP2, a kinesin-6 family motor protein, is required for relocalization of Aurora B and also Plk1, to the central spindle in human cells.[250,252,264,265]

Aurora B activity is necessary for several events in cytokinesis (Fig. 5). First, Aurora B is required for proper localization and function of MKLP1. Treatment of human cells with a small molecule inhibitor of Aurora B in early mitosis inhibits localization of MKLP1 (and its binding partner MgcRacGAP) to the central spindle.[266] However, addition of an Aurora inhibitor at later stages of mitosis inhibits phosphorylation of MKLP1 without disrupting its localization,[252,267] yet perturbs cytokinesis completion, indicating that MKLP1 must remain phosphorylated to permit abscission. How phosphorylation regulates MKLP1 is not completely clear, as MKLP1 is phosphorylated at multiple sites that may have distinct effects.[252,267] Phosphorylation of MKLP1 could be important for stabilizing interactions between the cortex and midbody, or be important for recruiting proteins such as centriolin that are necessary for abscission. In addition, phosphorylation of MKLP1 by Aurora B is important to prevent the protein from being sequestered back in the nucleus as cells enter interphase.[252] It is interesting to note that many components required for cytokinesis are located in the nucleus or associated with Golgi apparatus during interphase (Fig. 2) and thus localization of cytokinesis components to the midbody could require sustained phosphorylation that prevents the proteins from being resequestered by these structures as cells exit mitosis.

Another important substrate of Aurora B is MgcRacGAP, whose phosphorylation appears important for completion of cytokinesis.[47,268,269] Phosphorylation of MgcRacGAP has been proposed to stimulate its activity as a GAP for RhoA, which could be important for terminating RhoA activity in late stages of cytokinesis.[47] Another study indicates that phosphorylation of MgcRacGAP by Aurora B at a different site might activate the protein by stimulating release of the GAP domain from an inhibitory interaction with PRC1.[268]

There are several other important Aurora substrates including vimentin, an abundant intermediate filament protein. Intermediate filaments must be disassembled during mitosis to allow cell division and mitotic phosphorylation is important for filament dissociation, as expression of nonphosphorylatable mutants of vimentin leads to cells that show a persistent filamentous bridge.[270,271] Following mitotic exit and during later stages of cytokinesis, ROCK[272] and Aurora B[112,270,271,273] maintain vimentin phosphorylation after CDK1 is inactivated. Aurora B may also promote cytokinesis by inhibiting myosin light chain phosphatase.[112] Aurora B also phosphorylates CENP-A, which appears to play an important role in cytokinesis.[274] Cells expressing mutants of CENP-A that cannot be phosphorylated result in mislocalization of the passenger complex and cause a delay in the final stages of cytokinesis.

Although Aurora B is a critical positive regulator of cytokinesis in vertebrate cells, this role does not seem conserved in yeast, as the budding yeast ortholog Ipl1 and the fission yeast ortholog Ark1 are not essential for cytokinesis. However, in budding yeast, Ipl1 may negatively regulate late steps of cytokinesis in cells with spindle defects,[275,276] perhaps by regulating the localization of anillin-like proteins. This pathway may prevent abscission until segregating chromosomes have cleared the midzone. Whether a similar pathway operates in mammalian cells is not yet clear.

We are just beginning to learn about the mechanisms that regulate Aurora B activation. New work suggests that the TD60 protein may play an important role in activating Aurora B at centromeres.[277] In multiple organisms, components of the mitotic exit network, including the phosphatase CDC14, play important roles in regulating cytokinesis,[278-280] in part through regulation of targeting of the Aurora B complex.[281] Aurora B is also regulated by a Cul3-containing ubiquitin ligase, which is important for removing Aurora B from mitotic chromosomes and allowing its accumulation on the central spindle during anaphase.[282]

In vertebrate cells, Aurora B is expressed in a cell-cycle dependent manner, peaking in the G2/M phase of the cell cycle[283,284] and is highly expressed in a number of cancers.[263,284-291] However, in these tumors, expression of other proliferative markers, such as Ki-67, MCM2, geminin and Aurora A is also increased,[291,292] suggesting that Aurora B upregulation may be part of a broader upregulation of mitotic components in tumor cells. Because Aurora B is a positive regulator of cytokinesis, it is unclear whether its overexpression would perturb cytokinesis. Elevated levels of Aurora B may promote cytokinesis completion in cells that would otherwise undergo cytokinesis failure due to other abnormalities in the mitotic machinery. However, it is possible that perturbing Aurora B expression, or other components of the CPC, could alter the stoichiometry of the complex, perturbing cytokinesis.[293]

Regulation of Cytokinesis by Tyrosine Kinases

Tyrosine kinase signaling pathways may also regulate cytokinesis completion. Small molecule inhibitors of Src, including PP2 and SU6656, inhibit abscission in HeLa cells.[294] Src activity appears to be required in early mitosis, followed by delivery of tyrosine-phosphorylated proteins to the midbody via Rab11-driven vesicle transport.[294] Src colocalizes with the diaphanous-related formins mDia1 and mDia2 in endosomes and midbodies of dividing cells and inhibition of Src blocks cytokinesis.[67] Other tyrosine kinases, such as Fyn and its associated proteins are required for cytokinesis in lymphocytes,[295] through mechanisms that remain obscure.

Regulation of Cytokinesis by Lipids

Several studies indicate that phosphoinositide-containing lipids may be important for cytokinesis, with phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) playing a central role. In mammalian cells, PtdIns(4,5)P2 accumulates at the cleavage furrow and overexpression of proteins

that bind to PtdIns(4,5)P2 perturbs cytokinesis completion but not ingression of the cleavage furrow.[296] Overexpression of dominant negative kinases required for PtdIns(4,5)P2 generation also inhibits cytokinesis completion in mammalian cells.[296]

Hydrolysis of PtdIns(4,5)P2 by phosphlipasec(PLC) yields inositol trisphosphate (IP_3), which stimulates calcium release from internal stores. Inhibitors of PLC can interfere with cytokinesis,[297] which can in some cases be rescued by addition of calcium.[298] Alternatively, PtdIns(4,5)P2 may play a direct role in recruiting membrane proteins required for stability of the furrow. Several proteins required for cytokinesis, such as septin, profilin and anillin can bind to PtdIns(4,5)P2 and overexpression of a protein containing a PtdIns(4,5)P2-binding domain blocks cytokinesis completion by interfering with adhesion of the plasma membrane to the contractile ring at the furrow.[296]

Other studies have shown that the membrane lipid phosphatidylethanolamine (PE) is exposed on the cell surface of the cleavage furrow during late cytokinesis.[299] Addition of a cyclic peptide that binds tightly to PE inhibits cytokinesis completion,[299] perhaps by interfering with contractile ring disassembly.[300] Mutant cell lines that fail to synthesize adequate PE also show defects in cytokinesis completion that can be rescued by PE addition.[300] Proper PE organization may be essential for RhoA inactivation at late stages of cytokinesis, which may in turn be necessary for actin disassembly.[301]

Coupling of Cytokinesis to Other Cellular Pathways

The complexity of cytokinesis regulation provides opportunities for linking cytokinesis to other cellular pathways. Emerging evidence suggests interesting new connections between cytokinesis and the pathways involved in regulation of protein synthesis, DNA replication and DNA damage.

Cytokinesis and Protein Synthesis

Recent work suggests that proper regulation of protein synthesis may be essential for cytokinesis to proceed with high efficiency. Interestingly, of 214 genes identified in a genome-wide RNAi screen in Drosophila S2 cells, 22% were ribosomal proteins and another 5% were involved in translation.[177] Recent work suggests that the protein 14-3-3σ may play an important role in regulating protein synthesis during mitosis.[302] In normal cells, cap-dependent translation is suppressed during mitosis, whereas cap-independent translation is increased. Cells lacking 14-3-3σ do not make this switch, perturbing the pattern of proteins that are synthesized.[302] Downregulation of 14-3-3σ perturbs localization of Plk1 to the midbody and leads to cytokinesis failure.[302] These effects may be a consequence of failure to properly synthesize proteins containing an internal ribosomal entry site during mitosis, such as Cdk11.[302] Interestingly, 14-3-3σ expression is often reduced in tumor cells by targeted degradation or promoter hypermethylation. Loss of 14-3-3σ may in turn result in defective cytokinesis as a consequence of alterations in protein synthesis.

Cytokinesis and DNA Replication

Interestingly, one of the components of the Origin Recognition Complex (ORC), which is required for initiation of DNA replication, may also play a role in cytokinesis in metazoans. In vertebrate cells, Orc6 localizes to kinetochores and to a reticular-like structure around the cell periphery and ultimately to the cleavage furrow and midbody.[303] Elimination of Orc6 induces mutlipolar spindles and formation of multinucleated cells in both human cells[303] and *Drosophila*,[304] suggesting this function is conserved. In *Drosophila*, Orc6 interacts with a septin protein that may be important for cytokinesis. Domains of Orc6 required for DNA replication and cytokinesis appear separable, suggesting that Orc6 has evolved a domain that participates specifically in cytokinesis.[304] How Orc6 might couple the processes of DNA replication and cytokinesis completion remains unclear.

Cytokinesis and DNA Damage

Several lines of evidence suggest that cytokinesis may be regulated in response to DNA damage. Components required for DNA repair, such as BRCA2, may be directly involved in cytokinesis. Other evidence suggests that DNA damage pathways may regulate the expression of cytokinesis

proteins, or regulate their activity by posttranslational modification. Coupling of DNA damage pathways to cytokinesis regulation could be important for preventing the cleavage furrow from cutting damaged DNA that cannot be accurately segregated during mitosis. The existence of such pathways may explain why spontaneous chromosome missegregation is tightly coupled to cytokinesis failure in human cells.[185] In this model, DNA damage, or perhaps incompletely replicated DNA, may trigger pathways that prevent segregation of unreplicated or damaged sister chromatids and at the same time activate pathways that block cytokinesis completion.

BRCA2 is an example of a protein that may play direct roles in both DNA repair and cytokinesis. BRCA2 is required for recombination-based repair of DNA double-strand breaks.[305] However, BRCA2-deficient cells also show centrosome amplification that may be a consequence of defective cytokinesis.[306] BRCA2 localizes to the midbody and inactivation of BRCA2 in murine embryonic fibroblasts and HeLa cells interferes with cytokinesis.[307] BRCA2 may be regulated during mitosis, as it is a Plk1 substrate whose phosphorylation is inhibited in the presence of DNA damage.[308] Interestingly, downregulation of a BRCA2-interacting protein (BCCIP) also leads to defective cytokinesis.[309] Other proteins involved in DNA damage responses may influence cytokinesis regulation by interacting with cytokinesis components. For example, the DNA damage checkpoint kinase Rad53 has been shown to associate with septins in budding yeast.[310] In mammalian cells, Ku70, a DNA-binding protein required for DNA damage repair, forms a complex with ARF6 during mitosis,[311] suggesting a possible link between the DNA damage pathway and completion of cytokinesis.

Transcriptional controls may provide another mechanism for inhibiting cytokinesis in response to DNA damage. The expression of several cytokinesis proteins, including Plk1, ECT2, anillin and survivin, is repressed when DNA is damaged, in a manner that depends on an intact Rb pathway.[312] Other studies suggest that expression of cytokinesis proteins may be inhibited by activation of the p53 pathway.[313] For example, it has been shown that ECT2 expression is repressed by p53 via protein methyltransferases, suggesting that cytokinesis could be more likely to fail under conditions of p53 activation.[314]

Posttranslational modifications may also regulate cytokinesis in response to DNA damage. For example, Aurora B becomes highly poly-ADP-ribosylated when DNA is damaged, a modification that inhibits its kinase activity.[315] Poly(ADP-ribosyl)ation is an immediate cellular response to DNA strand breaks that is catalyzed by NAD^+-dependent enzymes, poly(ADP-ribose) polymerases (PARPs).[316] This effect is mediated by direct interaction between the BRCT domain of PARP1 and Aurora B.[315] Because Aurora B activity is essential for chromosome segregation and cytokinesis, induction of DNA damage could lead to errors in chromosome segregation and failure of cytokinesis.

Conclusion

Cytokinesis is a surprisingly complex process that requires the interplay of many components and regulatory pathways. Cytokinesis failure can arise through defects in any of the four stages in cytokinesis and as a consequence of inactivation or hyperactivation of any of a large number of different components (summarized in Fig. 6). Although many cytokinesis proteins have been identified, we are just beginning to understand how these proteins interact with one another and how they are regulated. Understanding the causes of cytokinesis failure is important, as it may set the stage for genetically unstable tetraploid cells that give rise to tumors.[317] However, cytokinesis failure also seems to occur physiologically in some tissues, even in those that are not tumor prone such as the heart. Understanding how cytokinesis is regulated physiologically in response to different signals, or under conditions of cell stress or damage, remains an important area for future research. Although cytokinesis failure may be accompany certain pathological states such as cancer, it is likely that pharmacologically-induced cytokinesis failure may be an important issue to consider as new medicines are developed. Inhibitors of Rho kinase are being developed for cardiovascular medicine[318] and inhibitors of Aurora kinase are under development as anticancer agents.[319] Because these compounds are likely to induce cytokinesis failure in normal tissues, it will be important to

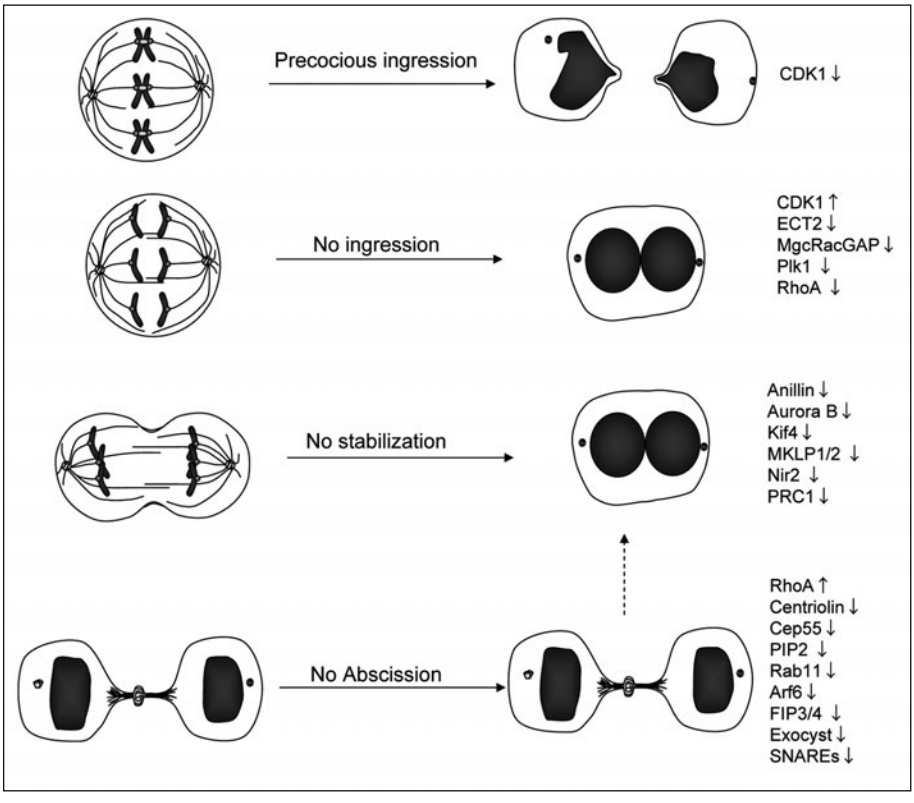

Figure 6. Summary of different phenotypes resulting from cytokinesis failure. Inhibition (downward arrow) or excessive activation (upward arrow) of different cytokinesis components can give rise to distinct phenotypes, including precocious ingression before the chromosomes have been separated, regression of the furrow giving rise to binucleated cells, or stabilization of the cytoplasmic bridge where daughter cells remain connected. The list is not comprehensive; see text for additional examples.

determine how sensitive various tissues are to cytokinesis failure and the consequences of production of tetraploid cells in different tissue types.

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Chapter 4

DNA Damage and Polyploidization

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Abstract

A growing body of evidence indicates that polyploidization triggers chromosomal instability and contributes to tumorigenesis. DNA damage is increasingly being recognized for its roles in promoting polyploidization. Although elegant mechanisms known as the DNA damage checkpoints are responsible for halting the cell cycle after DNA damage, agents that uncouple the checkpoints can induce unscheduled entry into mitosis. Likewise, defects of the checkpoints in several disorders permit mitotic entry even in the presence of DNA damage. Forcing cells with damaged DNA into mitosis causes severe chromosome segregation defects, including lagging chromosomes, chromosomal fragments and chromosomal bridges. The presence of these lesions in the cleavage plane is believed to abort cytokinesis. It is postulated that if cytokinesis failure is coupled with defects of the p53-dependent postmitotic checkpoint pathway, cells can enter S phase and become polyploids. Progress in the past several years has unraveled some of the underlying principles of these pathways and underscored the important role of DNA damage in polyploidization. Furthermore, polyploidization per se may also be an important determinant of sensitivity to DNA damage, thereby may offer an opportunity for novel therapies.

Polyploidization and Cancer

Tumorigenesis is a multistep process that arises from the accumulation of genetic alterations. These genetic changes can come in the form of point mutations that deregulate oncogenes or tumor suppressor genes. On the other hand, drastic gains or losses of whole chromosomes or chromosomal fragments (aneuploidy) are also the norm in cancer. Whether mutation of specific genes or aneuploidy is more critical for tumorigenesis is very much a contentious issue (reviewed in ref. 1).

In one school of thought, which gains prominent shortly after the discovery of oncogenes and tumor suppressor genes, states that these genetic modifications are the main driving force for tumorigenesis, with aneuploidy only as a byproduct of the process. In another school of thought, which origin can be traced to Theodor Boveri nearly 100 years ago, contests that aneuploidy might be a cause of tumorigenesis.[2] For example, weakening of the spindle-assembly checkpoint triggers chromosomal instability and aneuploidy, which appear to be an important stimulus in the initiation and progression of different cancers.[3,4] It is likely that a combination of specific gene mutations and chromosomal instability cooperate to induce tumorigenesis (reviewed in ref. 5).

Polyploidization can initiate chromosomal instability and aneuploidy (reviewed in ref. 6). Tetraploid cells are commonly found in early stages of tumors. Notable examples include Barrett's

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esophagus[7-10] and cervical carcinoma.[11] Several studies have provided evidence that tetraploidization increases chromosome instability in yeast[12,13] and in mammalian cells.[14,15] Moreover, tetraploidy may be an intermediate state in transformation. Cowell and Wigley (1980) found that during transformation of epithelial cells from mouse salivary glands, tetraploids are generated before undergoing a period of chromosome instability.[14] Many viruses can induce tetraploidy via cell fusions. While a direct demonstration between viral-induced cell fusion and human cancer is not yet available, several lines of evidence from in vitro and animal models suggest a link of the two events (reviewed in ref. 16).

It is believed that extra number of chromosomes in tetraploids may provide a buffer for chromosome loss and DNA repair. Moreover, it is generally accepted that the multistep progression of cancer—including initiation, progression, heterogeneity and drug resistance—is a product of evolutionary processes.[17] The extra set of chromosomes in tetraploids may act as a reservoir of genetic materials to allow clonal evolution of tumor. In this connection, parallels can be drawn with the role of whole genome duplication in evolution. Ohno (1970) proposed that whole genome duplication provides the primary source of redundant genes for new evolutionary opportunities.[18] He advanced that two rounds of whole genome duplication by tetraploidization occurred during the evolution of vertebrates, with the first occurred in early chordates ~500 million years ago and the second occurred at the stage of fish or amphibian ~430 million years ago. Although the details of time and number of duplications have been debated over the years,[19] it is generally accepted that polyploidization is able to promote adaptive evolutionary changes. Likewise, it is possible that tetraploidization also contributes to the evolution of cancer cells.

A seminal study by Fujiwara et al (2005) indicates that tetraploids can be generated by transient blocking of cytokinesis in p53-null mouse mammary epithelial cells. Importantly, tetraploidization promotes aneuploidy and tumorigenesis.[15] The presence of p53 normally suppresses the generation of tetraploid cells, presumably by activating the intrinsic apoptotic pathway.[20] Another study reported that chromosome nondisjunction (both copies of a chromosome segregate to the same daughter cells) leads to binucleated tetraploids by promoting cleavage furrow regression; the tetraploid cells then become aneuploidy through further divisions.[21] These and other studies provide strong evidence of the importance of tetraploidization as an early step in tumorigenesis.

How tetraploidization promotes chromosome instability remains incompletely understood. The extra centrosomes in tetraploids are likely to be critical determinants of chromosome instability (reviewed in ref. 22). Indeed, increased centrosome number is a common characteristic of several tumors. The cause of centrosome amplification in tumors is not known, but defects in the control of the centrosome replication cycle or cytokinesis are the likely underlying mechanisms (reviewed in ref. 23). Because centrosomes are microtubule organization centers, cells with supernumerary centrosomes form multipolar mitotic spindles and display other errors during chromosomal segregation. The uneven segregation of genetic materials into the daughter cells may result in different fates, including mitotic catastrophe, aneuploidy and transformation.

Although polyploid cells frequently contain multiple centrosomes, multipolar mitosis can be suppressed either by functional silencing of extra centrosomes or by centrosome clustering.[24-28] Indeed, Ganem et al (2009) found that the fraction of cells undergoing multipolar mitosis is markedly less than that possessing extra centrosomes in a variety of cancer cell lines.[29] A genome-wide RNA interference screen in *Drosophila* S2 cells revealed that a variety of proteins, including those that organize microtubules at the spindle poles and components of the spindle-assembly checkpoint, are required for centrosome clustering.[26]

Mechanisms of Polyploidization

Polyploidization may arise from diploid cells through a number of different mechanisms, including cell fusion, endoreduplication, mitotic slippage and cytokinesis failure. Before focusing on the role of DNA damage in polyploidization, we will first review the various mechanisms that can generate polyploid cells.

Multinucleated cells can be produced by fusion of different cells (called heterokaryon). Cell fusions are important in several physiological processes, including fertilization, development, immune system defense and tissue repair. Infections with many viruses can also induce cell fusions (reviewed in ref. 16). Enveloped viruses enter cells with the help of viral proteins that fuse biological membranes. A side effect of this mechanism is the ability of viruses to fuse different cells together. Cell fusions is believed to be important to cancer development and progression. Classic studies by Barski et al (1960) revealed that synkaryons (cells formed by fusion and subsequently display a single nucleus) formed in vitro could form tumor when implanted in mice.[30] Fusion of tumor cells with other cells in vivo has also been documented, for instance between human glioma cells with hamster cells[31] and tumor cells with myeloid cells.[32]

Tetraploid cells are also frequently generated after mitotic failure. Unscheduled exit from mitosis is normally prevented by the spindle-assembly checkpoint until all the kinetochores are properly attached to the spindles (reviewed in ref. 33). After prolonged activation of the spindle-assembly checkpoint, however, cells can exit mitosis precociously by a process termed mitotic slippage (also called adaptation).[34,35] In cells that undergo mitotic slippage, CDK1 is inactivated and the cells enter G_1 phase without chromosome segregation and cytokinesis. The nuclear envelope then randomly reforms around groups of chromosomes, generating cells that contain tetraploid DNA contents and two centrosomes. Although the exact mechanism of mitotic slippage is not known, the central event seems to be a slow but continuous degradation of cyclin B1.[36]

A p53-dependent "postmitotic checkpoint" is activated after mitotic slippage.[37] Activation of the p53-p21$^{CIP1/WAF1}$ axis leads to the inhibition of CDK2 and delays S phase entry. The prolonged block in mitosis prior to slippage ensures the accumulation of p21$^{CIP1/WAF1}$ before the synthesis of cyclin E-CDK2.[38] Other p53-independent mechanisms may also contribute to the postmitotic checkpoint. For example, expression of human papillomavirus E6 mutant that is defective in targeting p53 for degradation can partially induce polyploidy.[39] Proper function of the spindle-assembly checkpoint is also required for the postmitotic checkpoint. Vogel et al shows that spindle-assembly checkpoint-compromised HCT116 cells failed to arrest at the postmitotic checkpoint after nocodazole treatment.[40]

In addition of mitotic slippage, a failure in cytokinesis after anaphase also produces binucleated tetraploid cells. Successful cytokinesis requires the complete clearance of chromatin from the cleavage plane. Conditions including chromosome nondisjunction[21] and chromosomal bridge[41] can severely delay cytokinesis and promote cleavage furrow regression and tetraploidization. Such chromosomal segregation defects have been estimated to occur at a remarkably high frequency of ~1% in dividing somatic cells and at even higher incidence in transformed cells.[42,43]

A p53-dependent "tetraploidy checkpoint" has been proposed to prevent S phase entry in cells that have undergone mitotic slippage or aborted cytokinesis.[44] The checkpoint is believed to sense the increase in chromosome number and halt the cell in tetraploid G_1 state. However, the function of the tetraploidy checkpoint is contentious and its existence has been disputed.[15,45,46] One possibility is that the p53-dependent arrest after tetraploidization is mainly due to DNA damage or centrosomal stress during the aberrant mitosis (reviewed in ref. 6). Indeed, DNA damage can be readily detected in cells undergoing prolonged mitotic arrest.[47,48] Another possibility that has been proposed is that as transcription is turned off during mitosis, the lack of transcription during a protracted mitotic arrest can trigger subsequent stress and cell cycle arrest.[49] Irrespective of the precise signals that activate p53, cells with defective p53 pathway are expected to be prone to polyploidization following mitotic slippage or aborted cytokinesis.

While DNA reduplication is stringently prevented in the normal cell cycle, multiple rounds of genome reduplication, called endoreduplication, occur in cell types such as megakaryocytes and trophoblast giant cells. The mitotic CDK1 is typically inactivated to restrain mitosis during endoreduplication cycles. This has been observed in a wide range of endoreduplication

cycles, including maize endosperm,[50] *Drosophila*[51] and megakaryocytes.[52] Likewise, extensive genome reduplication can be triggered by disruption of CDK1 expression in mammalian cells.[53] Although the molecular basis of how CDK1 inactivation contributes to genome reduplication remains to be defined, the prevailing view is that APC/C plays a salient role in preventing rereplication. Unscheduled activation of APC/C reduces the concentrations of mitotic cyclins and geminin, resulting in rereplication.[54,55] To what extent does this pathway plays in the polyploidization of cancer cells remains to be deciphered. It is conceivable that DNA reduplication can occur in situations where CDK1 activity is inhibited for an extended period of time, such as after DNA damage. In fact, a connection between DNA damage and polyploidization is well established. Before describing the evidence of linkages between DNA damage and polyploidization, we will first review the current understanding of the DNA damage checkpoints in mammalian cells.

The DNA Damage Checkpoints

Surveillance mechanisms termed the DNA damage checkpoints prevent precocious entry into the cell cycle after DNA damage (Fig. 1). In essence, DNA damage activates sensors that facilitate the activation of the PI-3 (phosphoinositide 3-kinase)-related protein kinases ATM and ATR. ATM/ATR then activates CHK1 or CHK2, which in turn inactivates CDC25s (for the intra-S DNA damage checkpoint and the G_2 DNA damage checkpoint) or activates the p53-p21$^{CIP1/WAF1}$ pathway (for the G_1 DNA damage checkpoint), culminating in the inhibitory phosphorylation of CDKs and a halt in cell cycle progression (reviewed in ref. 56).

Following exposure to ionizing radiation or other genotoxic insults that elicit DNA double-strand breaks, ATM is autophosphorylated at Ser1981, leading to dimer dissociation and

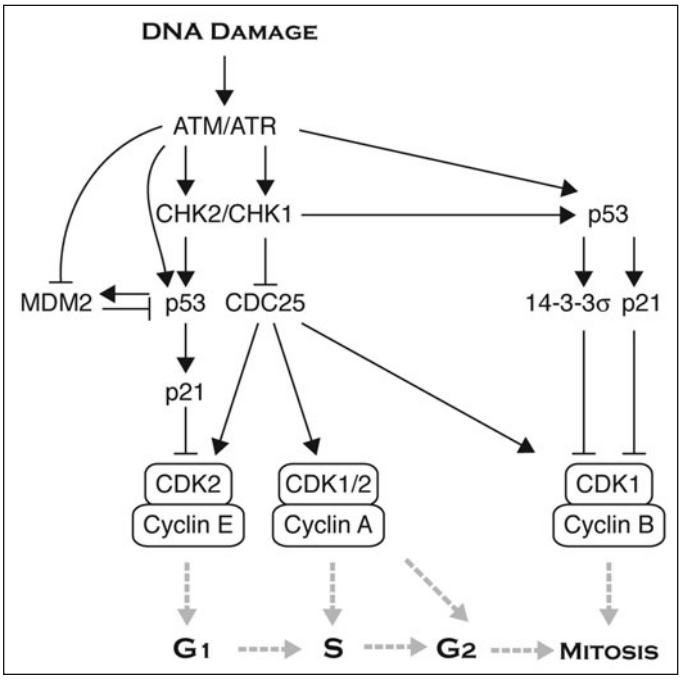

Figure 1. The DNA damage checkpoints. A simplified version of the major pathways of the DNA damage checkpoints is shown. Uncoupling the checkpoints promotes mitosis in the presence of DNA damage. See text for details.

activation of the kinase. ATR is activated by a broader spectrum of stress including ultraviolet irradiation, hypoxia and replication stress. ATM and ATR phosphorylate residues in the SQ/TQ domain of CHK1/CHK2, thereby stimulating the kinase activity of these effector kinases.

The upstream sensors that initiate the activation of ATM/ATR consist of an intricate network of large protein complexes, of which many components contain the BRCT domain. These include the RAD9-HUS1-RAD1 (9-1-1) clamp and the RAD17-RFC clamp loader that facilitate ATR-mediated activation of CHK1.[57] Another large complex that participates in ATM/ATR activation is the so-called BRCA1-associated genome surveillance complex composed of BRCA1, BLM and MRN (MRE11-RAD50-NBS1).[58,59] Stalled replication forks mainly activate the ATR-CHK1 pathway. Replication fork progression can be impaired by insufficient nucleotide supply or lesions and obstacles on the DNA. Several proteins including ATRIP (ATR-interacting protein), TopBP1 and Claspin appear to be required for recruiting ATR to single-stranded DNA present at stalled replication forks to phosphorylate CHK1.[60] The ATR-CHK1 pathway is essential even in the absence of exogenous stresses during unperturbed S phase, probably for maintaining high rates of replication fork progression.[61] Claspin is usually degraded by $SCF^{\beta\text{–}TrCP}$-mediated ubiquitination following the phosphorylation of Claspin by PLK1. This pathway is inhibited after DNA damage.[62] In response to genotoxic stress in G_2 phase, the phosphatase CDC14B translocates from the nucleolus to the nucleoplasm and activates APC/C^{CDH1}. This degrades PLK1 and consequently stabilizes Claspin, allowing the G_2 DNA damage checkpoint to be maintained.[63]

Once the ATM/ATR-CHK1/CHK2 cascade is activated, the G_2 DNA damage checkpoint is believed to be carried out by the inactivation of all three isoforms of the CDC25 family (CDC25A, CDC25B and CDC25C) by CHK1 and CHK2.[64] Phosphorylation of $CDC25C^{Ser216}$ by CHK1/CHK2 inactivates its phosphatase activity either directly or indirectly through the creation of a 14-3-3 binding site. Binding of 14-3-3 masks a proximal nuclear localization sequence and anchors CDC25C in the cytoplasm, preventing efficient access of CDC25C to cyclin B1-CDK1. Interestingly, phosphorylation of a proximal site (Ser214) by cyclin B1-CDK1 inhibits further phosphorylation of $CDC25C^{Ser216}$. This provides an elegant mechanistic explanation for the suppression of DNA damage-mediated CDC25C inactivation during mitosis.[65]

CDC25B is believed to possess a unique role in activating cyclin B1-CDK1 at the centrosome. CHK1 may shield centrosomal cyclin B1-CDK1 from unscheduled activation by CDC25B during normal G_2 phase and presumably also during the G_2 DNA damage checkpoint. The molecular basis of this activity may be due to CHK1-dependent phosphorylation of $CDC25B^{Ser323}$, creating a docking site for 14-3-3 that prevents access of substrates to the catalytic site. Dissociation of CHK1 from the centrosomes at the end of G_2 phase, together with positive regulatory phosphorylation of $CDC25B^{Ser353}$ by Aurora-A, enables CDC25B to activate the centrosomal cyclin B1-CDK1 and initiate mitosis.[66]

CDC25A is arguably the most important member of the CDC25 family due to its nonredundant role in mouse cells. CDC25A is targeted for rapid degradation by CHK1/CHK2 through a ubiquitin-mediated mechanism. CDC25A stability is controlled by APC/C^{CDH1} complexes during mitotic exit and early G_1 and by $SCF^{\beta\text{-}TrCP}$ complexes during interphase. Importantly, the $SCF^{\beta\text{-}TrCP}$-dependent turnover of CDC25A is enhanced in response to DNA damage. Phosphorylation of $CDC25A^{Ser76}$ by CHK1 is required for the phosphorylation of a phosphodegron centered at Ser82 (by an as-yet-unidentified kinase), creating a binding site for β-TrCP. Interestingly, β-TrCP also binds to a separate nonphosphorylated sequence in CDC25A (the DDG motif) and plays a role in CHK1-induced ubiquitination and degradation of CDC25A.[66]

There is also evidence that CHK1 can phosphorylate and activate WEE1 by promoting 14-3-3 binding.[67,68] Suppression of CDC25s or activation of WEE1 promotes $CDK1^{Thr14/Tyr15}$ phosphorylation, thus preventing damaged cells from entering mitosis. Other mechanisms are also known to play critical roles in the G_2 DNA damage checkpoint. For example, the p53

downstream target 14-3-3σ is involved in sequestering cyclin B1-CDK1 in the cytoplasm after DNA damage.[69]

Cells have also evolved checkpoints in S phase to prevent replication of damaged DNA. One of the better-understood S phase checkpoints is the intra-S DNA damage checkpoint, which is important for the responses to double strand breaks. A hallmark of the intra-S DNA damage checkpoint is that it slows down but does not stop DNA synthesis. In addition, there is no strong correlation between the sensitivity to DNA damage and the loss of the checkpoint.[70] For these reasons, it has been suggested that the intra-S DNA damage checkpoint may be involved in tolerating damage during replication rather than actually repairing the damage.[71] The checkpoint affects two distinct processes: origin firing and the rate of replication folk progression. As a global response, origins distant from the site of DNA damaged are prevented from firing through checkpoint activation. The mechanism involves ATM/ATR-dependent activation of CHK1/CHK2, which then phosphorylate CDC25A, leading to its rapid degradation. This prevents the dephosphorylation of $CDK2^{Thr14/Tyr15}$ and inhibits S phase progression by preventing the loading of replication initiation protein CDC45 onto the origin.[72,73,74]

Replication fork slowing may represent a more local response to DNA damage during S phase. The current paradigm states that two separate pathways downstream to ATM, namely CHK2 and MRN complexes, are required for folk slowing. Mutations in either pathway results in radioresistance DNA synthesis. However, activation of CHK2, degradation of CDC25A and inactivation of CDK2 occur normally in irradiated MRN complexes-defective cells.[74] Precisely how MRN complexes reduce the rate of replication folk progression is still unclear. One of the downstream effectors may be SMC1, which functions in the cohesion of sister chromatids following DNA replication and homologous recombination DNA repair.[75,76] It has been shown that SMC1 is phosphorylated by ATM upon ionizing radiation-induced DNA damage, but the details of the mechanism await further clarification.[77]

When cells suffer DNA damage during G_1 phase, it is critical for them to halt the entry into S phase until the DNA is repaired. It is well established that the G_1 DNA damage checkpoint involves the stabilization and activation of p53, which in turns transcriptionally activates the CDK inhibitor $p21^{CIP1/WAF1}$, leading to the inhibition of cyclin E-CDK2 complexes and G_1 arrest. The activity of p53 is highly regulated by posttranslational mechanisms including protein-protein interaction, acetylation, neddylation, phosphorylation, sumoylation and ubiquitination.[78] In unstressed cells, p53 is restrained by binding to MDM2, itself a transcriptional target of p53, in a negative feedback loop. MDM2 directly binds to the NH_2-terminal transactivation domain of p53 to inhibit its transcriptional activity and shuttles p53 out from the nucleus by the virtue of its nuclear exporting signal. In addition, MDM2 is also a ubiquitin ligase that targets p53 for ubiquitin-mediated proteolysis.

The crucial event in p53 activation and stabilization is the phosphorylation of the NH_2-terminal residues by checkpoint-stimulated protein kinases. Upon DNA damage, ATM and ATR are activated and phosphorylate $p53^{Ser15}$, which inhibits the interaction of p53 with MDM2, resulting in p53 stabilization.[79,80] Apart from directly phosphorylating p53, ATM also induces $p53^{Ser20}$ phosphorylation indirectly via CHK1 and CHK2.[81-83]

In addition to its well-known role in the G_1 DNA damage checkpoint, a growing body of evidence also indicates the importance of the p53-$p21^{CIP1/WAF1}$ axis in the G_2 DNA damage checkpoint.[84] In a recent study, $p21^{CIP1/WAF1}$ was found to downregulate EMI1.[85] Since EMI1 is an inhibitor of APC/C, it is possible that $p21^{CIP1/WAF1}$ contributes to the maintenance of G_2 arrest by stimulating the degradation of the mitotic cyclins.

Polyploidization Induced by DNA Damage

It is vital to prevent the precocious activation of cyclins-CDKs to provide sufficient time for DNA repair. It is well known that bypass of the classic DNA damage checkpoint pathways described above promotes premature entry into mitosis. Checkpoint-uncoupled cells then

undergo mitotic catastrophe, a special form of cell death during mitosis. For instance, cells lacking p53, $p21^{CIP1/WAF1}$, or 14-3-3σ fail to arrest in G_2 after DNA damage and undergo mitotic catastrophe.[69,84] However, a significant proportion of checkpoint-bypassed cells survive the aberrant mitosis. While these cells are able to enter and exit mitosis, they often fail to complete cytokinesis properly, giving rise to tetraploidy.[84,86-88] In support of this, a population of polyploid cells can frequently be detected in malignant tumors.[89] Polyploid cells are also observed in colonies that survive after treatments with DNA damaging agents.[90,91] The spindle-assembly checkpoint is required for mitotic catastrophe induced by abrogation of the DNA damage checkpoint,[40,92] suggesting a trap in mitosis is required for these types of cell death. Thus a weakened spindle-assembly checkpoint may potentiate with the bypass of the DNA damage checkpoint to induce polyploidization.

How DNA damage leads to polyploidization is still not completely understood. A likely explanation is that mitosis occuring in the presence of damaged DNA generates either chromosome fragments or entire lagging chromosomes, leading to cytokinesis failure or cell fusion.[87] Indeed, it has been demonstrated that lagging chromosomes are able to promote cleavage furrow regression and tetraploidization.[21] The frequent presence of micronucleus (which are formed from chromosome fragments and lagging chromosomes) in polyploid cells also reflects the role of chromosomal damage in polyploidization (reviewed in ref. 93). Furthermore, incorrect fusion of chromosomes during repair of double strand breaks can lead to formation of chromosome bridges.[94] Aurora B appears to be part of a sensor that responds to unsegregated chromatin at the cleavage site.[41]

Uncoupling of the DNA damage checkpoint is thus a key event in polyploidization. In fact, the G_2 DNA damage checkpoint is partially impaired in many cancer cells.[95] They are unable to maintain G_2 arrest and eventually undergo aberrant mitosis.[96] Uncoupling of the ATM/ATR-CHK1/CHK2 axis is well documented. Ablation of the G_2 DNA damage checkpoints induces unscheduled activation of cyclin B1-CDK1 and premature entry into mitosis.[69,97,98] Cells that contain defective ATM, such as those derived from ataxia-telangiectasia, often exhibit radio-resistant DNA synthesis.[99] Likewise, IR-induced G_1 arrest is impaired in $CHK2^{-/-}$ mouse embryonic fibroblasts.[100] Studies using conditional CHK1 knock-out mice also revealed that CHK1 deficiency causes inappropriate S phase entry, accumulation of DNA damage during replication and premature entry into mitosis.[101,102] Since the ultimate effect of the ATM/ATR-CHK1/CHK2 pathway is the inhibitory phosphorylation of CDK1, it is not surprising that expression of a nonphosphorylatable mutant of CDK1 can also trigger premature entry into mitosis.[102-104]

Chemical agents that inhibit the ATM/ATR-CHK1/CHK2 pathway can induce checkpoint bypass and many are potential chemotherapeutic agents. Caffeine is a classic inhibitor of ATM/ATR.[105-107] The checkpoints can also be uncoupled with CHK1 inhibitors such as UCN-01.[108-110] Inhibition of CHK1 with UCN-01 after DNA damage overcomes the DNA damage checkpoints, inducing premature activation of cyclin B1-CDK1 and mitotic catastrophe. However, UCN-01 is also a potent inhibitor of Protein Kinase C, CDKs, MK2, AKT (through inhibition of phosphoinositide-dependent kinase 1) and other kinases. This promiscuous nature of UCN-01 makes defining its precise role difficult. In fact, the two kinases that can phosphorylate $CDC25C^{Ser216}$—C-TAK1 and CHK1—can both be inhibited by UCN-01.[111] Likewise, inhibition of CHK2 promotes premature entry of mitosis after DNA damage.[112]

Another possible mechanism that can promote polyploidization after DNA damage is due to the inhibition of CDK1. As CDK1 is turned off by inhibitory phosphorylation after DNA damage, it is possible that prolonged inhibition of CDK1 may induce endoreduplication cycles, similar to those in cells such as megakaryocytes (see above). However, there is little experimental support of this hypothesis at this stage.

Similarly, defects of the MRN complexes can also ablate the intra-S-phase DNA damage checkpoint and induce polyploidization. Nijmegen breakage syndrome is a rare autosomal

recessive disorder characterized by microcephaly, immunodeficiency and predisposition to hematopoietic malignancy, sharing a wide range of clinical features with ataxia telangiectasia. The disorder is caused by mutation of the *NBS1* gene, which encodes a member of the MRN complex. Hypomorphic mutations have also been found in MRE11.[113] Complete inactivation of MRE11, RAD50, or NBS1 leads to early embryonic lethality in mice through accumulation of double strand breaks during development.[114-116] Furthermore, cells derived from mice that lacking functional NBS1 share similar phenotype with ATM-defective cells, showing an impairment of the intra-S DNA damage checkpoint after ionizing radiation.[117] Moreover, NBS1-deficient B lymphocytes show defective intra-S phase checkpoint, chromosomal translocation and tetraploid DNA content.[118] Finally, it has been well known that simian virus 40 induces host endoreduplication by large T antigen.[119] In fact, large T antigen interacts with NBS1 and disrupts the function of MRN complexes, leading to DNA rereplication and tetraploidization.[120]

The Sensitivity of Polyploid Cells to DNA Damage

As described above, a growing body of evidence indicates that tetraploidization promotes chromosome instability and transformation. Nevertheless, tetraploidy appears to be a relatively more stable state than other aneuploidy.[6] Artificially generated tetraploids can be maintained in culture for a long period without any obvious collapse of the ploidy.[121,122] However, there is also evidence that polyploids are under more stress and are less robust than diploids. In budding yeast, diploids take over tetraploids in long-term culture.[123] Tetraploid yeasts are notably genetically unstable, with high levels of both chromosome loss and interhomolog recombination.[13] Similarly, in chimeric mice produced from the combination of diploid and tetraploid cells, the tetraploid cells are out-competed and ultimately produces fetuses completely composed of diploid cells.[124] Mammalian tetraploid cells exhibit an increase in the basal expression of p53 and an enhanced rate of apoptosis.[121] This may reflect an elevated level of stress in tetraploid cells. In budding yeasts, several genes involved in DNA repair are essential for the viability of polyploid cells (but not in diploids), suggesting that polyploidization may elevate the levels of DNA damage.[13]

Whether ploidy influences the responses to genotoxic stress remains incompletely understood. It is likely that the increase amount of DNA per cell may raise the chance of receiving damage. Given that tetraploid mouse mammary epithelial cells were more prone to transformation after exposure to a carcinogen than diploid cells,[15] one possibility is that the increase in sensitivity to DNA-damaging agents in tetraploid cells may increase mutagenesis. This is supported by the findings that tetraploidization of Hep3B cells or human fibroblasts sensitizes cells to genotoxic stress inflicted by ionizing radiation and topoisomerase inhibitors.[122] Tetraploid cells contain higher number of γ-H2AX foci after ionizing radiation than their diploid counterparts. However, results described by Castedo et al indicate that tetraploid HCT116 and RKO cells are more resistant to DNA damaging agents (camptothecin, cisplatin, oxaliplatin, gamma- and UVC-irradiation) than their diploid counterparts.[121,125]

An additional factor that may affect the sensitivity of polyploid cells to genotoxic agents is the increase in cell volume. Ploidy is one of the key intrinsic factors that influence cell volume (reviewed in ref. 126,127). For instance, *Drosophila* polyploid salivary gland cells are more than 1,000 times larger than diploid cells and cells from tetraploid mice are about twice the size of those of diploid cells.[128] The increase in cell volume is believed to provide a metabolic growth advantage for polyploid cells.[129] It is possible that the increase in cell volume and surface area may allow polyploid cells to receive a higher dose of genotoxic agents.

Polyploidization and Cancer Therapies

Polyploidization can be a double-edged sword in cancer research. On the one hand, as we have discussed in detail above, aberrant polyploidization is believed to be a critical factor of tumorigenesis. Deciphering the mechanism of polyploidization will help us to understand the basis of tumorigenesis. On the other hand, polyploidization could be exploited as a strategy to induce cell death in cancer therapies.

For many types of potential therapeutic genotoxic agents, it is often not immediately obvious why they should selectively target cancer cells but spare normal cells.[130] Many effective anticancer agents are believed to take advantage of the severe imbalance of various cellular regulators and components in cancer cells. The obvious doubling of DNA and other cellular components in polyploid cells in relation to their diploid counterpart may offer an opportunity for designing novel therapeutic approaches.

If tetraploid cells are indeed more sensitive to DNA damage,[122] an implication for chemotherapeutic intervention is that some cancer cells can be sensitized to genotoxic agents by a preceding step that induces polyploidization. This will probably be especially apt for cells that contain a weakened or defective spindle-assembly checkpoint. Treatment of cancer cells with spindle poisons can induce mitotic arrest and apoptosis. However, cells can also undergo mitotic slippage and enter a tetraploid G_1 state. If additional checkpoint is lacking (such as being p53 defective), tetraploid cells can further undergo DNA replication and become polyploids. Thus it may be of advantage in cancer therapy to first induce polyploidization before treatments with DNA-damaging agents. In this scenario, sequential rather than simultaneous treatment with spindle inhibitors and DNA damaging agents will be critical, as cells are sensitized to DNA damaging agents only after mitotic slippage.

Mitotic slippage can also be promoted with inhibitors of mitotic kinases such as CDK1.[131] Mitotic slippage per se does not appear to be toxic, but a substantial portion of cells may be killed during the subsequent multipolar mitosis.[131] CDKs themselves are important targets for cancer therapies. Several small chemical inhibitors (purine analogs such as flavopiridol, BMS-387032, E7070 and roscovitine) have shown preclinical and clinical anticancer activity. In particular, roscovitine (Seliciclib, CYC202 or Cyclacel) is a potent chemotherapeutic agent and has been tested in clinical trials for a variety of cancers.[132] Hence it is possible that sequential treatment of a spindle poison followed by roscovitine and DNA damaging agents may prove effective against some cancers.

There are also other reports indicating that tetraploid cells are more resistant to DNA damaging agents.[121,125] The presence of polyploid giant cells in cancers may also account for resistant to cancer therapy. Following DNA damage (in particular with relatively low dose of DNA damaging agents), many polyploid cells appear after an initial phase of mitotic catastrophe and survive for weeks as mono- or multi-nucleated giant cells.[90,91] Whether these cells still retain proliferative potential is controversial. Although some studies indicate that giant cells have reduced or no proliferative potential,[133] other studies have shown that giant cells can undergo multipolar mitosis or de-polyploidization to return to near diploid state.[134] The latter studies suggest that the multistep process of escaping cell death through polyploidization and then depolyploidization may account for tumor relapse after initial efficient cancer therapy.

Conclusion

Several intricate DNA damage checkpoints ensure that cell cycle progression is delayed after DNA damage. Defects of the checkpoints in several disorders permits mitotic entry even in the presence of DNA damage. Likewise, aberrant entry into mitosis can be induced by chemicals that uncouple the checkpoints. Forcing cells with damaged DNA into mitosis causes severe chromosome segregation defects, including lagging chromosomes, chromosomal fragments and chromosomal bridges. The presence of these lesions in the cleavage plane is believed to abort cytokinesis. If this is coupled with defects of the p53-dependent postmitotic checkpoint pathway, cells can enter S phase and become polyploids. Several lines of evidence indicate that polyploidization triggers chromosomal instability and contributes to tumorigenesis (Fig. 2). Other mechanisms, including the prolonged inhibition of CDK1 activity and defects of the intra-S checkpoint, may also provide a link between DNA damage and polyploidization. These recent advances raise several important issues that require further investigation. Outstanding issues include the need of more compelling evidence of the linkage between DNA damage and polyploidization, as well as a direct demonstration of the importance of polyploidization in human cancers.

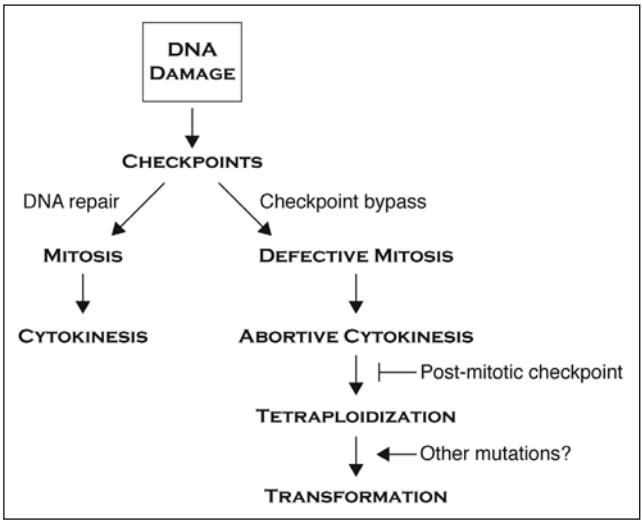

Figure 2. Promotion of polyploidization and transformation by DNA damage. The DNA damage checkpoints delay cell cycle progression. After the damaged DNA is repaired, the checkpoints are inactivated and the cell cycle can proceed. If the checkpoints are bypassed, however, cells can enter mitosis containing damaged DNA. This results in chromosome segregation defects that include lagging chromosomes, chromosomal fragments and chromosomal bridges, thereby preventing the success completion of cytokinesis. These cells can become tetraploids if the p53-dependent postmitotic checkpoint is defective. Tetraploidization (possibly coupled with other mutations) triggers chromosomal instability and contributes to tumorigenesis.

Polyploidization of cancer cells may offer an opportunity for drug intervention. Different strategies that trigger mitotic arrest, mitotic slippage and DNA damage should be explored to see if they sensitize various types of cancer cells. More vigorous studies are also required to provide a comprehensive picture of whether polyploidization sensitizes cancer cells to DNA damaging therapeutic agents.

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Chapter 5

Role of the p53 Family in Stabilizing the Genome and Preventing Polyploidization

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Abstract

Cellular defects resulting in chromosomal instability and aneuploidy are the most common features of human cancers. As a major tumor suppressor and intrinsic part of several cellular checkpoints, p53 contributes to maintenance of the stability of the genetic material, both in quality (ensures faithful replication) and quantity (preservation of diploidy). Although the exact trigger of p53 in case of numerical chromosomal aberrations is unknown, the absence of p53 allows polyploid cells to proliferate and generate unstable aneuploid progeny. A more recent addition to the p53 family, p73, emerged as an important contributor to genomic integrity when p53 is inactivated. p73 loss in p53-null background leads to a rapid increase in polyploidy and aneuploidy, markedly exceeding that caused by p53 loss alone. Constitutive deregulation of Cyclin-Cdk and p27/Kip1 activities and excess failure of the G2/M DNA damage checkpoint are important deficiencies associated with p73 loss.

p53—Tumor Suppressor

The p53 tumor suppressor gene encodes a multi-functional protein involved in the comprehensive control of cellular responses to genotoxic stress.[1,2] Its tumor suppressor effects are mediated by a variety of mechanisms including cell cycle arrest, apoptosis and cellular senescence that prevent cells with damaged DNA to pass on their genomes to progeny.[3] In unstressed cells, p53 is maintained at very low levels, but it becomes rapidly stabilized and activated in conditions of genotoxic stress. In the absence of p53, cells with damaged DNA fail to properly respond to DNA damage checkpoints but instead continue to proliferate, which results in random mutations, gene amplifications, chromosomal re-arrangements and aneuploidy. This is frequently associated with tumorigenesis. Consequently, p53 is functionally inactivated in more than half of human cancers ranging from carcinomas, sarcomas and lymphomas.[4-8] Moreover, alterations of the p53 gene occur not only as acquired somatic mutations in human cancers but also as germline mutations in patients with the cancer-prone Li-Fraumeni syndrome.[9-11]

The importance of p53 in tumor suppression was confirmed by animal models. p53 knock-out mice are highly prone to spontaneous tumor formation (T-cells lymphomas and fibrosarcomas).[12] Moreover, a single dose of 4 Gy γ-irradiation dramatically decreases the latency for tumor development in $p53^{+/-}$ heterozygous mice.[13] Cells derived from $p53^{-/-}$ null mice show signs of spontaneous genomic instability. For instance, mouse normal embryonic fibroblasts (MEFs) and pancreatic cells obtained from $p53^{-/-}$ mice exhibit a high degree of aneuploidy.[14-16] Murine models also proved

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that p53 loss synergizes with (proto)oncogenes (such as Myc, Ras, Wnt-1) in accelerating tumor formation.[17-21]

Analysis of human tumors revealed that the p53 gene is mainly targeted by missense mutations, which generate abnormally stabilized protein, while complete gene deletions are relatively rare.[22] Recent advances in clarifying the role of p53 hotspot mutants (i.e., which recur over and over in many patients) in a physiological context were offered by two knock-in mice that harbor the structural mutant p53R172H and the contact mutant p53R270H (corresponding to codons 175 and 273 in humans). p53R270H/+ and p53R172H/+ mice model the Li-Fraumeni syndrome. Of note, they developed allele-specific tumor spectra that were distinct from $p53^{-/+}$ mice. Moreover, p53R270H/– and p53R172H/– mice again developed a different tumor spectrum compared to $p53^{-/-}$ mice, including more frequent carcinomas and endothelial tumors. These results demonstrate that missense mutant p53 alleles expressed under physiological control have enhanced oncogenic potential (gain-of-function) that goes beyond the simple loss-of-function of p53 null alleles.[22] As the underlying mechanism it was suggested that p73 and p63 protective functions are concomitantly disabled by binding to the missense mutant p53 protein.[22,23]

p53 and Genomic Stability

p53 and Cell Cycle Checkpoints

p53 is one of the main effectors of cell cycle checkpoints. However, the precise mechanisms of its actions are still controversial. Clearly, p53 mediates G1 arrest in response to DNA damage, thus preventing DNA synthesis from damaged templates.[24] Apart from this, p53 is involved in regulating the cell cycle at transitions of G1/S and G2/M and within S-phase.[25-27] Evidence for a possible role of p53 in M-phase came from observations that p53 contributes to the control of centrosome duplication[28,29] and to the prevention of DNA rereplication when chromosome segregation is impaired by spindle inhibitors.[28,29] The various cell cycle checkpoints and a simplified view of p53 contribution to them are summarized below:

G1/S Checkpoint

Blocks replication of damaged DNA (e.g., if the nucleotide pool is inadequate for genome duplication[30,31] or under drugs that produce DNA damage).

p53 Model

DNA double-strand breaks activate ATM. ATM phosphorylates downstream effectors, either directly or through its immediate target Chk2. Phosphorylated histone H2AX (γH2AX) marks the chromatin site of the actual damage. p53 can be phosphorylated either by ATM or indirectly by Chk2. This contributes to p53's stabilization and activation. Through its transcriptional targets (such as cyclin-dependent kinase inhibitor p21) p53 delays cycle progression.[32-34]

Intra-S Phase Checkpoint

Stops DNA synthesis if the damage occurs in S-phase or cells with damaged DNA slipped through a G1/S block.

p53 Model

Intrinsic events or genotoxic stress (e.g., hydroxyurea and UV) during S-phase activate a checkpoint that prevents the progression of replication forks. The main player in this checkpoint is ATR. ATR phosphorylates and activates Chk1, γH2AX and BLM helicase. p53 becomes activated by ATR or Chk1 and transported by BLM to sites of stalled replication forks. Through direct interactions with components of the replication machinery and through activation of target genes, p53 is able to slow down replication.[34-37] p53-proficient cells show a lower level of double strand breaks (DSB), while in the absence of p53, ssDNA regions associated with stalled replication forks turn into DSB and generate major chromosomal abnormalities.[38]

G2/M Checkpoint

Employed when cells containing wrongly replicated (under- or overreplicated) DNA exit S-phase. The G2/M restriction point prevents mitosis of such cells. Loosening the G2/M checkpoint is one of the hallmarks of malignant transformation. This checkpoint is the "last barrier" prior to mitotic division that can block the perpetuation of mutations or an unstable genome.[39] The G2/M transition is under very strict control and integrates multiple pathways that are p53 dependent and independent.

p53 Model

It is thought that p53 is able to block cells in G2 via indirect inactivation of CDK1. Gadd45, p21 and 14-3-3σ, all p53 transcriptional targets, are able to inhibit CDK1. On the other hand, cyclin B, the regulatory subunit of CDK1, can be transcriptionally repressed by p53.[40] However, alternative pathways (ATM/ATR-dependent, caffeine and UCN-01 inhibitor sensitive) explain why p53-null cells are still able to arrest in G2/M.[41] Cells can stop the cell cycle in G2 by activating the ATM/ATR pathway and downregulate CDK1 via Chk1 and Chk2.[40] The Chk kinases inactivate Cdc25C, the phosphatase responsible for eliminating the inhibitory phosphorylations on CDK1.

Mitotic Checkpoint or Spindle Assembly Checkpoint

Does not allow anaphase to proceed until all chromosomes are properly attached to the spindle microtubule apparatus. Checkpoint proteins are components of the kinetochore, a macromolecular complex that resides at centromeres of chromosomes that establishes connections with spindle microtubules. Mitotic exit with abnormal chromosomes results in arrest.[41,42]

The p53 Model Is Controversial

p53-deficient fibroblasts, but not their wild-type counterparts, fail to arrest in response to spindle inhibitors and undergo another round of replication without mitosis to become polyploidy.[28,43] However, more careful studies revealed that p53 acts, in fact, at the subsequent G1 step to induce arrest. In response to spindle inhibitors, both wild-type and p53-null MEFs moved equally well from M into G1. The difference was that wild-type cells remained arrested, while p53 null cells were able to restart DNA synthesis and thus became polyploid. Eventually, some of these cells would escape the postmitotic block and divide, generating aneuploidy.[44]

However, p53 might still play some role in the mitotic checkpoint proper through its reported colocalization with centrosomes and a direct involvement in preventing multipolar mitotic spindles and centrosome amplification.[29] Loss of p53 was reported to be associated with accumulation of centrosome abnormalities, multiple spindle poles and missegregation of chromosomes into daughter cells in a fraction of mouse fibroblasts.[29]

p53 in DNA Repair

In addition to DNA damage-induced transcription-dependent p53 functions, evidence has accumulated for a direct role of p53 in DNA repair, DNA replication and DNA repair associated with active replication.[45] Genetic studies using KO mice for different genes involved in nonhomologous end joining (NHEJ) showed that p53 is a negative regulator of error prone NHEJ.[42,46-48] Additionally, p53 has been reported to have intrinsic Mg^{2+}-dependent exonuclease function.[49] p53 colocalizes with PCNA, DNA polymerase α, DNA ligase and RPA in the nuclei of Herpes virus—infected cells[50] and binds recombinases (such as Rad51[51] and Rad54[52]) in human cells. Thus, p53 is likely to negatively modulate homologous recombination.[53] Moreover, p53 null mice show an increased frequency of homologous recombination at different stages of development.[54] Recent studies of human cells revealed a requirement for p53 in global modulation of chromatin structure upon localized subcellular UV irradiation.[55] Thus, p53 has a role in increasing global chromatin accessibility, potentially through histone acetylation.

Deficiencies in cell cycle checkpoints or in the system that detects and repairs DNA damage have a deep impact on genomic stability and increase the probability of tumor formation.[24] Thus, p53 may help maintain genomic stability by preventing DNA replication of damaged DNA,

preventing replication in conditions that could harm the DNA and preventing rereplication of DNA that could lead to aneuploidy.[24]

Although its precise role in tumorigenesis is still controversial as to being cause or consequence, aneuploidy is a hallmark of cancer development. While aneuploidy can arise directly from defects in duplication, maturation or segregation of centrosomes,[56] it is generally thought that the precursor of aneuploidy is a polyploid state. One proposed route to aneuploid cancer cells is through an unstable tetraploid intermediate.[57,58] Supporting this idea, recent studies demonstrate that tetraploidy promotes chromosomal aberrations and tumorigenesis in vivo.[59-61]

Mechanisms of Polyploidization

Without excluding the possibility of other ways, it is considered that a diploid organism can acquire polyploid cells through several general mechanism: cell fusion, endoreplication (also called endomitosis) and a variety of defects that result in a nondividing cell cycle (nonmitotic, abortive cell cycle).[62]

DNA endoreplication is widely observed in the plant kingdom and selectively occurs in many animals as a response to developmental needs.[63] In humans, endoreplication can occur during differentiation (e.g., in megakaryocytes, hepatocytes and trophoblasts) or as a physiological response to metabolic stress (muscle cells). Megakaryocytes are bone marrow precursors that generate platelets. Specifically, megakaryocytes traverse the initial stages of mitosis (anaphase A) including centrosome duplication, but skip anaphase B and cytokinesis, resulting in polyploid cells.[62] Metabolic stress also facilitates polyploidy in several tissues. An increase in the percentage of polyploid hepatocytes is seen in ischemic lesions of the liver, regrowth of the liver after partial hepatectomy and in advanced age.[64,65] Hypertensive humans or rats show polyploid heart muscle and vascular smooth-muscle cells.[66] Another example are tetraploid fibroblasts frequently observed during wound healing in biopsies.[67] Thus, an increase in the amount of DNA and, consequently, in cell volume is regarded as beneficial for cells that have high metabolic rates like liver or muscle cells. Of note, this tetraploid state does not trigger any p53-depedent checkpoint. This led many authors to conclude that although mechanistically very similar, endoreduplication and polyloidization are not the same phenomenon.[63] Along the same lines, it is unclear if polyploid cells resulting from physiologic endoreduplication are more prone to genomic instability than their diploid parental tissue and if they associate with future organ pathologies.

From an evolutionary point of view, polyploidy could be advantageous for the entire organism due to better use of heterozygosity, the buffering effect of gene redundancy on mutations and, in certain cases the facilitation of reproduction through self-fertilization or by asexual means.[63]

Polyploidization resulting from an abnormal cell cycle, however, puts cells at risk for aberrant mitotic divisions and for subsequent mitosis with multipolar spindles, which eventually lead to aneuploidy. In conditions of abnormal DNA replication, sister-chromatid nondisjunction, mitotic spindle dysfunction or defective cytokinesis, cells are not able to proceed through a proper mitotic division. Regularly, many of these defects that result in abortive cell cycles trigger checkpoint responses that block cell-cycle progression or, in some cases, trigger apoptosis. However, checkpoint activation often produces only transient delays in cell-cycle progression. So, even if the initial insult persists, the possibility exists where some cells 'slip' past the arrest, exiting as a tetraploid from the defective cell division.[62] One of the important checkpoint responses to abnormal passage through mitosis is the activation of p53 in the next G1 phase.[68]

Disadvantages of Polyploidy

The obvious consequences of increasing the DNA content of a cell or organism include the disrupting effects of nuclear and cellular enlargement, the propensity of polyploid mitosis and meiosis to produce aneuploid cells and the epigenetic instability that affects gene regulation.[63] Increasing the genomic content of an organism usually increases cell volume, with a subsequent change in the spatial relationships between various components of the cell. Recent studies suggest that any imbalance of the ratio between the internal and the surface components of the nucleus has regulatory repercussions.[63] For instance, the volume of budding yeast cells increases linearly with

each extra chromosome pair.[62,69] In the nucleus, the precise localization of telomeric and centromeric heterochromatin ensures proper organization of chromosomes in the nucleus.[70] Lamins, which form a fibrous network that lines the inside of the nuclear envelope, interact with heterochromatin and have a function that is vital to the cell. This is demonstrated by the phenotypic effects of laminar abnormalities on human health.[71]

Chromosomes in polyploid cells of Arabidopsis thaliana have a greater mobility within the interphase nucleus relative to chromosomes in diploids, due to loss of the nuclear substructure that normally restricts chromosome movement.[70]

Most interestingly, when compared with haploids or diploids, tetraploid budding yeast cells have significantly increased rates of chromosome loss and recombination.[72] They also show increased sensitivity to gamma-irradiation and to other DNA-damaging agents.[62] Likewise, polyploid fission yeast undergoes chromosome missegregation at a high frequency.[73] Also, p53 null tetraploid cells are highly competent to induce tumors in nude mice. Polyploidization sensitized cells to genotoxic stress imposed by ionizing radiation and topoisomerase inhibitors.[74] These findings raise the possibility that an increase in ploidy generally impairs genomic stability.[61]

Tetraploidy Checkpoint Theory

Several views exist on the possible fate of polyploid cells. In the best case scenarios, multipolar mitosis of tetraploid cells can lead to the formation of diploid cells through a poorly understood process, known as 'reduction mitosis'. Alternatively, some cells 'adapt' to multiple centrosomes by clustering them at the spindle poles. This allows a bipolar mitosis to occur, which seems to progress normally. This mechanism is employed by many cancer cells as a way to avoid mitotic catastrophe.[75,76]

Another view suggests that tetraploid cells undergo cell cycle arrest via the so-called p53-mediated 'G1 tetraploidy checkpoint', which can then trigger apoptosis.[77,78] However, recent doubts about the existence of this checkpoint came from studies of cancer cell lines treated with mitotic spindle inhibitors. A proportion of these cell populations escape the mitotic arrest and enter into a "G1-like state" with a 4N set of chromosomes. This phenomenon is known as 'mitotic slippage'. If these tetraploid cells have functional p53, they arrest in this G1 phase.[44] However, p53-deficient cells progress through the next S-phase, undergo an abnormal mitosis and become aneuploidy.[79] It was initially considered that p53 might directly monitor the ploidy status of cells, perhaps via DNA content or centrosome number.[77] However, it was later shown that the high concentrations of spindle inhibitors (e.g., cytochalasin B) that were used in these experiments also caused DNA damage and that was the actual cause for inducing a p53 response. When repeated with lower but still effective doses, tetraploid cells formed, but did not arrest in G1 and instead showed normal cell cycle progression despite the p53 presence.[80,81] In sum, these studies concluded that mammalian cells do not possess a "tetraploidy" checkpoint, meaning that an abnormal chromosome number is not the direct trigger of the checkpoint response.[80,81]

The p53-dependent cell-cycle arrest of tetraploid cells shares features with the p53-dependent G1 arrest upon DNA damage. For instance, in both cases cell-cycle arrest coincides with induction of the CDK inhibitor p21 and hypophosphorylated retinoblastoma protein (Rb).[75,82] Furthermore, tetraploid cells that lack either p21, Rb or p53 all fail to arrest in G1 and proceed into an aberrant cell division.[62] Not without significance, p53 and Rb are the most frequent tumor suppressors functionally inactivated in human cancers. Disruption of the cytoskeleton by failed cytokinesis, abnormal spindle geometry in tetraploids,[80] or redistribution of nuclear proportions and lamins by increased DNA content[71] were also proposed as potential p53 activators. Thus, although its precise trigger is unknown, p53 prevents abnormal polyploid cells to occur in vitro as well as in vivo. The polyploid/aneuploid cells that arise from multipolar mitosis are rapidly eliminated in cells that contain p53.[61] Moreover, p53 null mice have 23% 4N cells in the pancreas compared with 7% in wild type mice.[28] Also, disabling p53 by a pancreas-specific SV40 T-antigen produced >45% polyploid pancreatic cells.[28] Thus, it will be important to establish the degree to which the antipolyploidization effect of p53 contributes to cancer suppression.

Agonists and Antagonists of p53 Function in Genome Stability

a. Mice deficient in genes important for telomere function, DNA damage checkpoint activation and DNA repair (both nonhomologous end joining (NHEJ) and homologous recombination) allow proliferation of cells with damaged DNA and a high degree of genomic instability.[83] The majority of these mice present with developmental problems or even embryonic lethality, all attributable to the activation of p53 in response to DNA damage signals, followed by induction of apoptosis. The developmental abnormalities are rescued by codeleting p53, but with a price: mice predisposed to genomic instability and lacking p53 are highly tumor-prone. For instance, the lethality of mutations in genes for DNA ligase IV or XRCC4 can be relieved by a mutation in p53 or ATM, but the double-knock-out mice develop T-cell lymphomas at a very early age.[47,84,85] These latter mouse models prove that p53-mediated apoptosis is an essential tumor suppressor mechanism to eliminate cells that are genomically unstable.[83]
b. Msh2 and its heterodimeric binding partner Msh3 are necessary for removal of nonhomologous tails during recombination.[86] In vivo, combined loss of Msh2 and p53 leads to embryonic lethality of female mice and to synergistically increased tumorigenesis in males on a C57BL6J background.[87] Drug-induced polyploidization studies in MEFs revealed that, while $Msh2^{-/-}$ MEFs showed no increase in the 8N population compared with WT MEFs, the $p53^{-/-}$ $Msh2^{-/-}$ MEFs showed a clear increase in cells with an 8N DNA content, over and above that seen in MEFs deficient in p53 alone. On a larger scale of DNA repair, it is possible that p53 monitors unresolved or aberrant recombination structures and allows cells to mend such DNA structures. However, when the signal from mismatch repair proteins is missing, these cells are not able to complete mitosis. Moreover, when p53 is also missing, cells may aberrantly re-enter S-phase. Thus, polyploid cells might appear from a combined defect in DNA repair and checkpoints.[88]
c. Lats2: The tumour suppressor Lats2, which is localized at the centrosome during a normal cell cycle, interacts with and inhibits the Mdm2 E3 ligase activity and thereby promotes p53 activation in cells with mitotic spindle defects. The Lats2-Mdm2 interaction occurs specifically when centrosome function is disrupted. Moreover, RNAi knockdown of Lats2 in cells that lack p53 function leads to accumulation of polyploid cells after exposure to nocodazole. However, if p53 is activated, proliferation of these cells is prevented. Thus, p53 and Lats2 cooperate via Mdm2 in preventing tetraploidization upon spindle defects.[89]
d. Wnt-1 is a member of a family of cysteine-rich, glycosylated signaling proteins that intervene in diverse developmental processes ranging from the control of cell proliferation, adhesion, cell polarity, to the establishment of cell fates. Alterations of Wnts are associated with carcinogenesis. Wnt-1 transgenic mice crossed into $p53^{-/-}$ nullizygosity develop mammary tumors with increased genomic instability, aneuploidy, amplifications and deletions.[18]
e. p73 is another member of the p53 family that contributes to the maintenance of genomic integrity. Mouse cells that harbor deletions in both p53 and p73 are marked by a higher degree of polyploidy and aneuploidy than the one observed in p53 null cells. Its role is detailed in the next part.

Introduction to p73

20 years after p53 was discovered, two structurally similar genes—p63 and p73—were discovered and placed into the same family. Initially thought to be tumor suppressors like p53, these two proteins proved to have a more complicated and intriguing behavior. Structurally, p63 and p73 have in common with p53 an amino-terminal transactivation domain, a highly conserved central DNA binding domain and a carboxy-terminal tetramerization domain. In overexpression studies, p63 and p73 can function as sequence-specific transcription factors that activate expression of genes containing p53-binding sites (like Bax and p21).[90,91] Moreover, tumor-associated stress signals (i.e., deregulated oncogenes and DNA damage) that activate p53 also induce p73.[91]

However, p53 and p73 are not functionally equivalent in vivo. While p53 plays a clear role in tumor suppression, p73 has long eluded efforts to place it into a defined category of cancer biology. Indeed, p53 null mice are highly tumor-susceptible but show no major developmental defects. In contrast, p73-deficient mice show defects in neuronal development and immune function, with the majority of animals dying within 2 months after birth due to chronic infection.[92] p63-deficient mice show epithelial defects with absence of skin, hair, truncated or absent limbs, craniofacial malformations and perinatal death due to desiccation.[93] Although p73 null mice were originally reported as not tumor prone,[92] a more recent re-analysis of ageing $p53^{+/-}p73^{+/-}$ mice (as well as a small number of $p53^{+/+}p73^{-/-}$ and $p53^{+/+}p73^{+/-}$ mice) did reveal an elevated incidence of microscopic carcinomas, T-lymphomas and sarcomas and increased metastatic ability, compared to $p53^{+/-}$ mice. Moreover, these tumors underwent loss of heterozygosity and loss of the remaining wtp73 allele. Thus, heterozygous loss of p73 increases the p53-dependent tumor phenotype in severity, frequency and breadth of tumor spectrum.[94] The latter result suggests that p73 acts as a tumor suppressor in certain tissues.[94] In the same study, an increase in tumor burden and spectrum was noticed in ageing $p63^{+/-}$ or $p63^{+/-}$ $p53^{+/-}$ mice.

On the other hand, p63 and p73 do not contribute to gamma irradiation-induced p53-mediated T-cell lymphoma suppression in vivo.[95] Moreover, in a different p63 KO mouse strain, Mills et al obtained a different result: no signs of malignancy, but accelerated aging instead.[96]

The story gets even more complicated in the analysis of human tumors. Unlike the clear picture that p53 offers with mostly inactivating mutations or rarely deletions in more than 50% of human cancers, p63 or p73 inactivating mutations are rarely found in human tumors.[97,98] One major reason for the lack of clear interpretability lies in the complex gene loci of p63 and p73, which produces two classes of isoforms with opposing activities. Thus, splice variants of p63/p73 exist that lack the N-terminal transactivation domains (ΔN p63/p73) and may function to interfere with the activity of their full-length counterparts (TA p63/p73).[91,99] Splice variations in the C-terminus adds another layer of isoforms (named α, β, γ, etc.) in the already extensive family.[100] The longest α variant of p63 and p73 contains a sterile-α motif (SAM), a known protein-protein interaction domain.[101]

Thus, multiple primary tumor types and tumor cell lines overexpress these genes and often concomitantly the TA as well as the reportedly oncogenic ΔNp isoforms (see below).[100,102] Numerous studies highlight the oncogenic potential of ΔNp63 in skin, the main isoform grossly overexpressed in human squamous cell carcinoma, including its clinical correlation to poor prognosis.[103] However, other tumor types (mainly some lymphomas and leukemias) show loss or reduced levels of p63 and/or p73.[98]

Further complicating the picture is a potential interference with p73 activity by mutant forms of p53, which might contribute to cancer development in vivo.[22,23,104]

p73 Functions

Although cancer cells deficient for p53 are more resistant to chemotherapy, they are still responsive to drugs, suggesting that other pro-apoptotic pathways are also involved. One of these rescue pathways might be mediated by the activation of p73. Thus, the majority of studies on p73 focused on its pro-apoptotic role. Based mostly on overexpression studies, it is thought that TAp73 has p53-like functions, while ΔNp73 isoforms have an opposing inhibitory role. When ectopically overexpressed, TAp73 can replace p53 in various cancer cell lines and induce apoptosis, cell cycle arrest and DNA repair by activating effectors like Bax, p53AIP1,[105] p21, GADD45, 14-3-3σ and p53R2.[106] However, while common promoters for p53 and p73 are numerous, differentially sensitive genes also exist.[107]

TAp73 participates in apoptosis and growth suppression in p53 null cells in response to DNA damage (chemotherapeutic drugs or γ-irradiation) or oncogenic stress (E2F1, cMyc, E1A).[107] In response to cisplatin, the apoptosis-inducing function of p73 is regulated by the c-Abl kinase and the mismatch-repair system.[108] Moreover, as part of normal T-cell development and selection, E2F1-p73 pathway induces cell death in response to T-cell receptor activation.[109] Consistently, in

radiation-induced mouse T-cell lymphomas, the p73 locus undergoes LOH in 33% of the cases.[110] Flores et al showed transcriptional cooperation between p53 and either p63 or p73 in inducing apoptotic effector genes in E1A-expressing MEFs and primary neuronal cells.[111] In their experiments, adriamycin-induced death was dependent on the copresence of at least two of the family members. While the expression of p21 (cell cycle arrest related protein) was not changed, the expression of Bax, Noxa and PERP was suppressed in $p63^{-/-}p73^{-/-}$ MEFs that were still $p53^{+/+}$.

The Role of p73 in Genomic Stability

Using genetically defined primary MEFs, we recently showed that p73 indeed plays an intriguing and unique role in genomic integrity that is manifested when p53 is lost. Isolated p73 loss does not induce genomic instability but instead results in impaired proliferation, transformation and premature senescence due to compensatory constitutive activation of p53. Combined loss of p73 and p53 completely rescues these defects, but at the expense of markedly exacerbating genomic instability. This leads to a rapid increase in polyploidy and aneuploidy, markedly exceeding that of p53 loss alone. Constitutive deregulation of Cyclin-Cdk and p27/Kip1 activities and excess failure of the G2/M DNA damage checkpoint fuel the increased ploidy abnormalities, while primary mitotic defects do not play a causal role. Thus, interference with p73 function—in the absence of protective mechanisms provided by p53—markedly exacerbates polyploidy and random loss or gain of chromosomes.[112] Of note, in human tumors concomitant inactivation of p53 and p73 often co-exist.[102] Below are highlights of our results:

Combined Loss of p53 and p73 Leads to Excess Polyploidy and Aneuploidy

When assayed on the 3T3 protocol, WT and $p73^{-/-}$ MEFs retained diploid status throughout their lifespan until they senesced at passage 7 and 4, respectively (Fig. 1). On the other hand, $p53^{-/-}$ MEFs (SKO) show a gradual increase in hyperdiploid cells with passaging, while also preserving significant diploidy (Fig. 1).[28,68] In contrast, the majority of freshly isolated $p53^{-/-}p73^{-/-}$ (DKO) MEFs were already hyperdiploid and after only 5 passages, virtually all cells were tetraploid and octaploid (Fig. 1). By FACS quantitation at p5, DKOs showed 88% aneuploidy and only 12% diploidy, in contrast to SKOs with 41% aneuploidy and 59% diploidy. Since each passage corresponds to 3 population doublings, we calculated that at least 5% of DKO cells, but only 2.5% of SKO cells lost their diploid status with each round of cell division. Thus, DKO cells have twice the rate of polyploidization than SKO cells.

By SKY (Spectral Karyotype Analysis) analysis at p7, DKO cells had the lowest diploid populations (only 2% with 2n) (n = 20 chrom. in mouse cells), but the highest polyploidy and aneuploidy (98% with 4n + >4n and 36% with >4n) (Fig. 2A). In contrast, SKOs had an intermediate phenotype, while WT cells were diploid. Most strikingly, extreme cases of DKOs were readily detectable with cells containing >300-400 chromosomes, a phenotype never seen in SKOs of the same passage (Fig. 2B). Notably, freshly isolated thymoctes from a young, healthy DKO mouse already contained a small (6%) but definite subpopulation of triploid normal T-cells, in contrast to its age-matched $p53^{-/-}$ control that had none, supporting polyploidization in vivo and in another tissue (not shown).

Thus, loss pf p73 in normal young thymocytes in vivo may have an impact on genomic stability. Human lymphomas can be triploid or near triploid, see reference 114.[113] Most DKO MEFs also contained unequal chromosome numbers by SKY (aneuploidy), reflected by the broad spread obtained when metaphases were quantitated for individual chromosomes (Fig. 2C). However, chromosomal translocations were rare in SKO and DKO. Thus, while p73 is not sufficient to completely prevent polyploidy upon p53 loss, it clearly acts to prevent further genomic destabilization.

The Ploidy Defect Is Not Due to a Mitotic Defect but a Failure of Premitotic Mechanisms

Polyploidy can be caused by several means that uncouple DNA replication from mitotic completion.[40] We therefore scrutinized the mitotic competence of DKOs, but found no intrinsic mitotic defects. First, concerning centrosome hyperamplification (2 being normal), DKOs had a

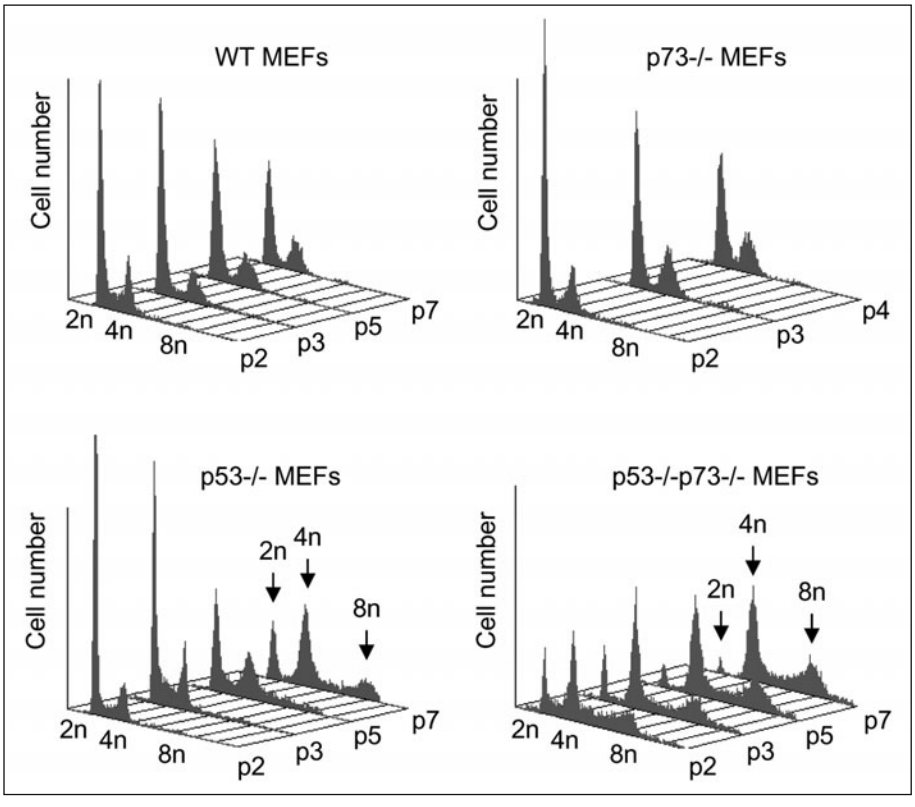

Figure 1. DNA histograms of freshly isolated WT, $p73^{-/-}$, $p53^{-/-}$ and DKO MEFs passaged on the 3T3 protocol. Number of passages in culture is indicated on the right. Ploidy (2n, 4n, 8n) is indicated.

better, rather than a worse phenotype compared to SKOs (Fig. 3A). Second, p7 DKOs were mainly mononucleated and had the lowest number of cells with two or more nuclei (Fig. 3A). Third, DKOs, when forced to undergo chromosomal segregation, showed no mitotic defects. To this end, cells were synchronized by mitotic spindle inhibitor nocodazole (which induced mainly tetraploidy in SKOs and tetraploidy and octaploidy in DKOs) and then released into media containing a G1/S roadblock (imposed by L-mimosine, hydroxyurea (HU) or aphidicolin) (see Fig. 3B). Both genotypes—since they could not go forward—went straight back through a proper mitosis within 4 hrs after release and regained their original ploidy. This was confirmed by direct visualization of chromosome condensation that follows real-time mitotic progression via GFP-tagged histone H2B (Fig. 3B). Thus, p73 loss does not cause defects in the centrosome duplication cycle, mitotic spindle checkpoint, karyokinesis and cytokinesis.

Excess Failure of the G2/M DNA Damage Checkpoint and Constitutive Deregulation of Cyclin-Cdk and p27/Kip1 Fuel Aberrant Ploidy upon p73 Loss

CDK-Cyclins are the driving force of the cell cycle. To determine if and where in the cycle DKOs are defective, we looked for CDK deregulation in cycling DKOs only subject to endogenous DNA damage (endogenous ROS modifies ~ 20,000 bases/day/cell).[114] Compared to SKOs, DKOs indeed have a higher and longer peak of Cyclin E-Cdk2 activity in early S and an elevated

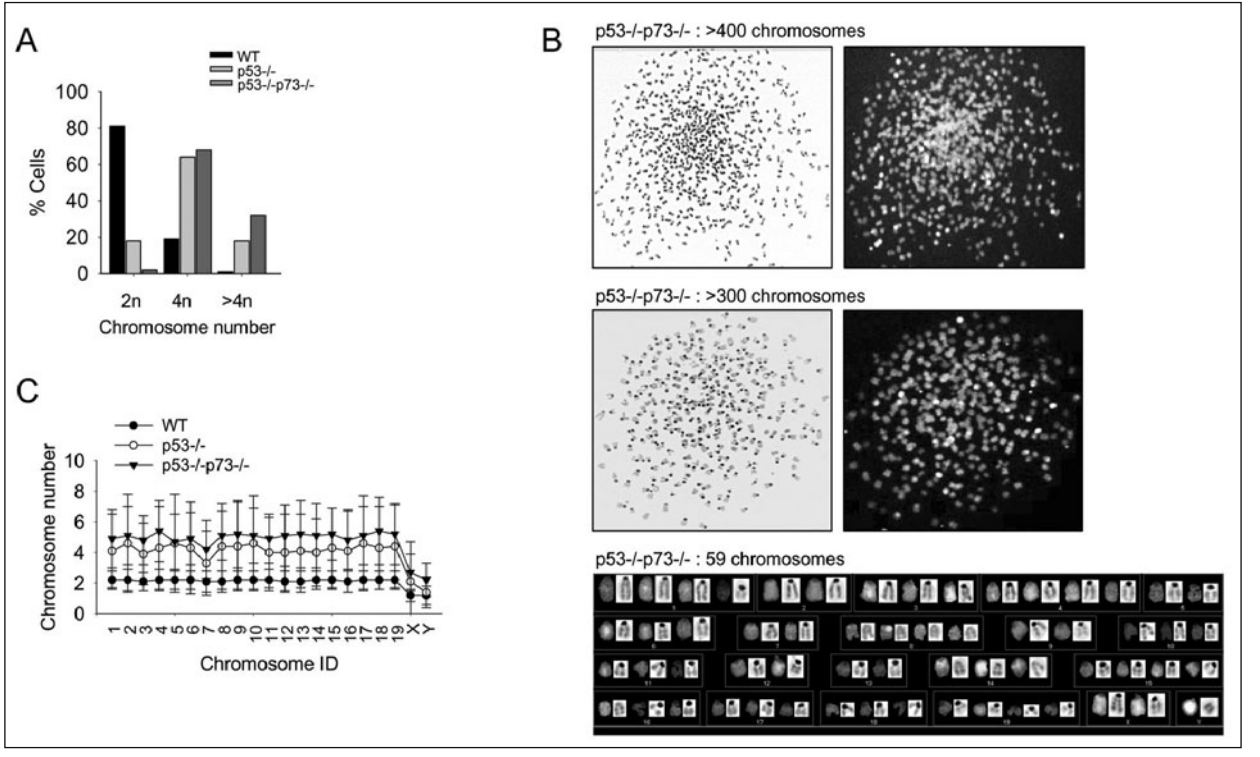

Figure 2. A) Karyotype analysis of >100 random metaphases each from MEFs of the indicated genotypes at passage 7. Note lack of diploidy (2n) and highest degree of polyploidy and aneuploidy (4n plus >4n) in DKO MEFs. B) Polyploidization of DKO cells at passage 7, as shown by Spectral Karyotyping (top). Most cells contain unequal chromosome numbers, indicating aneuploidy (bottom). C) Individual chromosome contents of WT, p53$^{-/-}$ and DKO MEFs at passage 7. Over 100 moderately polyploid metaphases were analyzed for each genotype. Error bars represent the standard deviation.

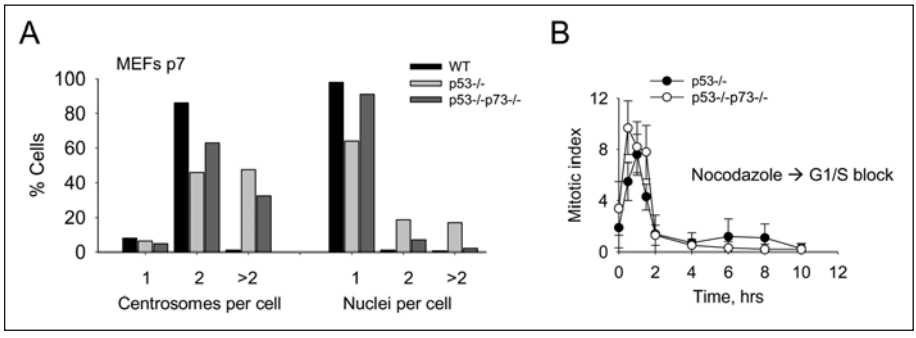

Figure 3. A) Total number of centrosomes (determined by γ-tubulin staining) and nuclei (determined by Hoechst staining) per cell in WT, $p53^{-/-}$ and DKO cells at passage 7. For each genotype, >500 random cells were counted. DKO cells have an improved centrosomal phenotype and are predominantly mononucleated. B) DNA histograms of $p53^{-/-}$ and DKO MEFs at passage 7 treated with nocodazole for 12 hrs, washed out and then released into media containing the G1/S blocker L-mimosine. Both genotypes recover their original ploidy within 4 hours (i.e., 2n for $p5^{-/-}$ MEFs and 4n for DKO cells), indicating that they had passed through a proper mitosis.

Cyclin A-Cdk2 activity in late S (Fig. 4A). Most importantly, DKOs show uncoordinated and premature collapse of Cyclin B-Cdk1 activity, leaving insufficient levels to go through mitosis. We identified that the source of CDK deregulation are not Cdks or Cyclins themselves but constitutively elevated *p27/Kip1* (Fig. 4B). p27 acts as a positive regulator of early phase Cyclin E-Cdk2[115,116] but a negative regulator of late phase Cyclin B1-Cdk1.[117] DKOs have constitutively elevated p27 levels in all phases of the cycle (Fig. 4B) and more p27 bound to Cyclin B-Cdk1 in G2M (not shown). As a consequence, since entry into mitosis depends on sufficient Cyclin B-Cdk1 activity,[118] more DKOs than SKOs are blocked from entering mitosis. Thus, many more DKOs skip mitosis and are reset from G2 back to G1, enabling another round of replication.[40,118] Together with the deregulated S phase, these events contribute to twice the polyploidization rate of p73-deficient DKOs. Of note, deregulated p27Kip1 and Cyclin E drive polyploidization of normal tissues in vivo. E.g., constitutively elevated p27 levels in Skp2 null mice, a component of the SCF ubiquitin-proteolysis system which degrades p27, causes excess polyploidization in many tissues and this is exclusively due to their p27 abnormality, since $p27^{-/-}$ $Skp2^{-/-}$ double knock-out mice rescue this phenotype completely.[119,120] Also, Cyclin $E^{-/-}$ embryos lack normally polyploid megakaryocytes and trophoblasts. Conversely, overexpressed Cyclin E drives non-endoreplicating megakaryoblasts into endomitosis.[121,122] Of note, in our system, retroviral overexpression of Cyclin E and its corresponding Cdk2 in $p53^{-/-}$ cells altered (impaired) the G2 checkpoint to resemble the one of DKO cells (not shown).

The G2M checkpoint is critical because it is the last barrier before mitotic division for cells with wrongly replicated or damaged DNA. Also, polyploid cells are prevented from re-entering mitosis through engagement of the G2M checkpoint.[44,123] Conversely, a defective G2 checkpoint in itself can cause polyploidy and aneuploidy after DNA damage, because cells with inadequate DNA repair in G2 proceed into a catastrophic mitosis, where massive bridging prevents chromosome segregation from which they will exit as polyploid/aneuploid G1 progeny.[44,123] p73-proficient SKOs respond to the DNA damaging G2 inhibitors adriamycin and VM26 with a robust S/G2 block. In contrast, DKOs fail to mount an effective G2M checkpoint and instead continue cell cycle progression, accumulating a large proportion of octaploid cells that enter mitosis (Fig. 5A,B). Conversely, retroviral re-introduction of TAp73α, the major TAp73 isoform in MEFs, re-establishes an efficient S/G2 arrest in VM26- and adriamycin-treated DKOs (Fig. 5C).

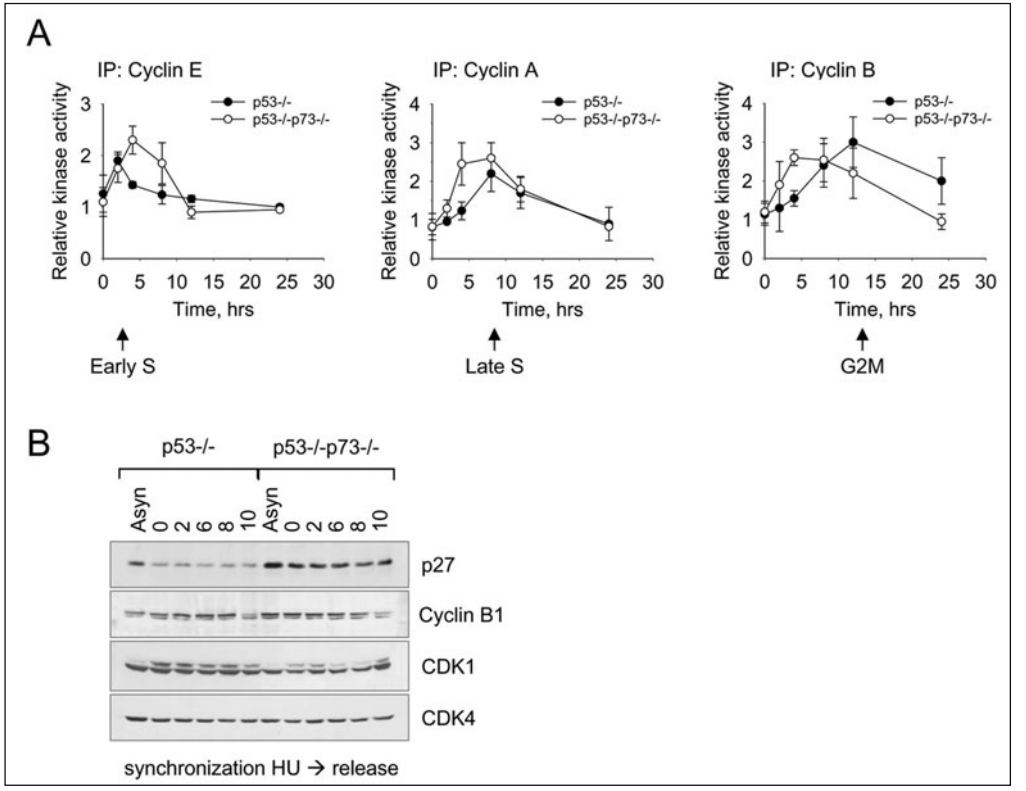

Figure 4. A) In vitro kinase assays of immunoprecipitated Cyclin E-, Cyclin A- and Cyclin B-associated Cdk2 and Cdk1 activity in synchronized $p53^{-/-}$ and DKO MEFs. Cells were analyzed at the indicated times after release from G1/S-phase block with hydroxyurea. Three experiments each, error bars represent standard deviation. B) $p53^{-/-}$ and DKO MEFs were synchronized at the G1/S border by hydroxyurea, Protein lysates prepared at the indicated hours were immunoblotted. Continuously growing (Asyn) MEFs are also shown. DKO cells exhibit constitutively elevated p27 levels.

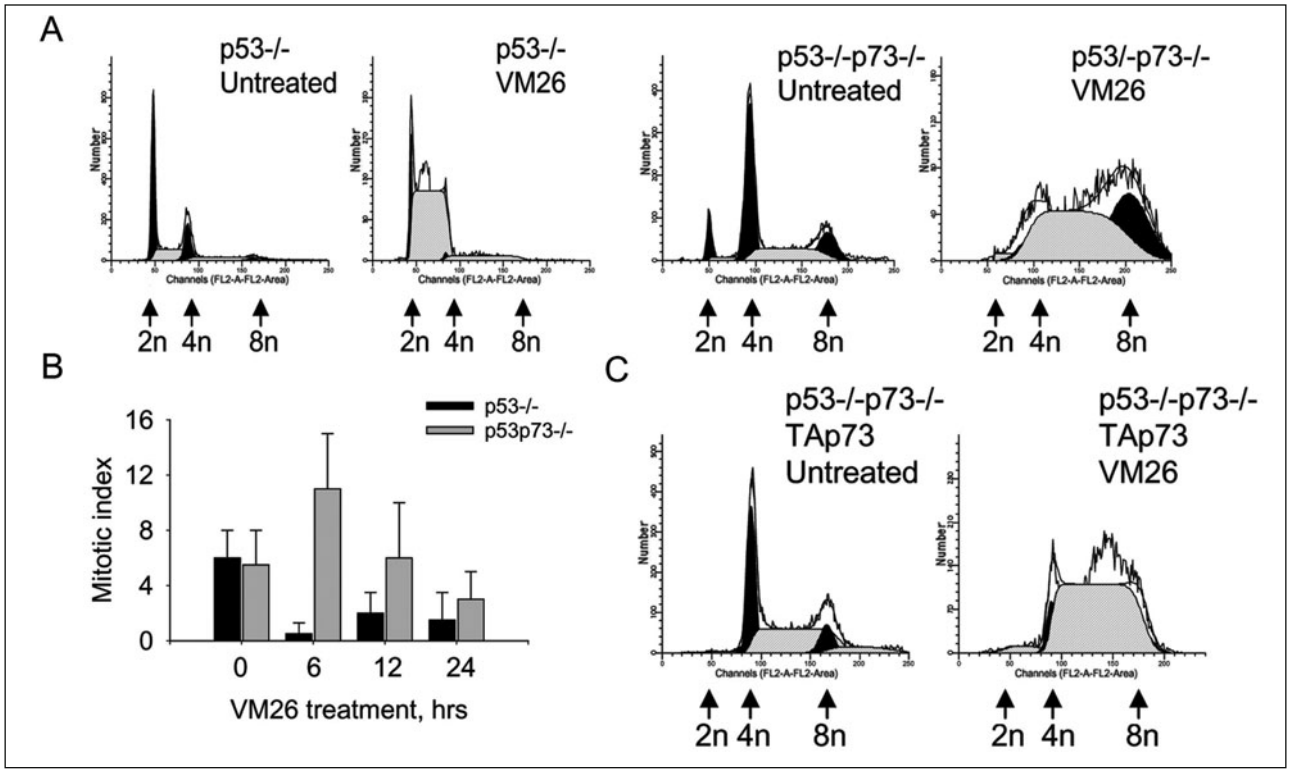

Figure 5. A) DNA contents of $p53^{-/-}$ and DKO MEFs incubated for 20 hrs in the absence or presence of VM26. Note the baseline difference in ploidy between untreated $p53^{-/-}$ and DKO MEFs. B) More DKO than $p53^{-/-}$ cells enter mitosis despite DNA damage. Mitotic indices were determined by real-time quantitative chromosome evaluation of H2B-GFP-expressing $p53^{-/-}$ and DKO cells, treated with VM26 for up to 24 hrs. At least 500 random cells were scored for each time point. Error bars represent standard deviation. C) TAp73 re-establishes an efficient S/G2 arrest and rescues the G2M checkpoint defect of DKO cells. Cell cycle profiles of DKO MEFs expressing ectopic TAp73α in the absence or presence of DNA damage.

The G2M checkpoint is controlled through the ATM/Chk/Cdc25 pathway that blocks Cdk activity.[124] Indeed, while SKO dropped their Cdk1 activity upon VM26, DKOs increased and maintained high levels of Cdk1, enabling DKOs with misreplicated and damaged DNA to exit G2M and enter mitosis (not shown). In contrast, dominant negative mutants of Cdk2 (D145N), Cdk1 (D146N) or Cdc25A (C430S), or pharmacologic Cdk1/2 and Cdc25 inhibitors significantly blocked further polyploidization of DKOs upon DNA damage (data not shown). In sum, this indicates that p73 activates a G2M DNA damage checkpoint.

Conclusion

The exact mechanism by which p73 influences the G2M checkpoint, including its effects on Chk1/Chk2 activity, requires further elucidation. Recent studies identified CDK inhibitors p21Cip1 and p57Kip2 as targets of p73 regulation.[125,126] Although p73 affects expression of these cell cycle regulatory proteins, it is unlikely that variations in the expression levels of a limited number of genes could account for the observed dramatic phenotype resulting from p73 loss. Being a chromatin accessibility factor, p53 contributes to the DNA repair processes by both transcription-dependent and transcription-independent mechanisms.[42,53] Likewise, it is conceivable that transcription-dependent and transcription-independent mechanisms also contribute to the p73-loss-induced phenotype.

Indeed, comparing cell cycles of primary cells, p73-deficient DKO MEFs exhibit a significant increase of DNA replication over $p53^{-/-}$ MEFs, associated with a constitutively deregulated S-phase Cdk2 activity. Recent studies underscore a critical role of the Rb tumor suppressor in maintaining chromatin structure and in DNA-damage checkpoint signaling in S-phase.[127-129] Notably, Rb-null rodent cells are polyploid even in the presence of wild-type p53.[127,129] The mechanism of this Rb activity remains unknown, although it appears to be E2F-independent and therefore transcription-independent.[129] Because a constitutively elevated Cdk2 activity would result in rapid Rb inactivation with dire consequences for the genomic stability of cells, a potential role of Rb in generating the p73-deficient phenotype needs to be addressed by future genetic and biochemical studies. In our study, reintroduction of TAp73α—but not of ΔNp73α—re-established an efficient G2M arrest upon adriamycin and VM26 in DKO MEFs. However, both TA and ΔN isoforms in isolation had minimal effects on polyploidization of DKO cells when passaged on the 3T3 protocol (data not shown), suggesting that a combination of isoforms in the right proportion might be necessary to completely rescue the DKO phenotype. The possible involvement of each of the p73 isoforms in maintenance of genomic stability and tumor suppression requires further investigation. The importance of p73 for genomic stability in the context of human tumors can only be addressed in correlational studies between expression of different p73 isoforms in human tumors and their degree of polyploidy/aneuploidy. Finally, generation of isoform-specific p73 knock-in mice should give insights to the most stringent question: what is the contribution of each isoform to tumor suppression/development? Answering this question will also contribute to the ultimate goal of studying p73: modulation of p73 levels in human tumors in order to activate p73-dependent apoptosis and cell cycle checkpoints.

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Chapter 6

Centrosomes, Polyploidy and Cancer

Anette Duensing and Stefan Duensing*

Abstract

Cancer cells are frequently characterized by ploidy changes including tetra-, poly- or aneuploidy. At the same time, malignant cells often contain supernumerary centrosomes. Aneuploidy and centrosome alterations are both hallmarks of tumor aggressiveness and increase with malignant progression. It has been proposed that aneuploidy results from a sequence of events in which failed mitoses produce tetra-/polyploid cells that enter a subsequent cell division with an increased number of centrosomes and hence with an increased risk for multipolar spindle formation and chromosome missegregation. Although this model attempts to integrate several common findings in cancer cells, it has been difficult to prove. Findings that centrosome aberrations can arise in diploid cells and the uncertain proliferative potential of polyploid cells suggest that alternative routes to chromosomal instability may exist. We discuss here recent results on centrosome biogenesis and the possible link between ploidy changes, centrosome aberrations and cancer.

Introduction

Genomic instability is a hallmark of cancer and tumor cells frequently contain grossly altered genomes with a tetra-, polyploid or aneuploid chromosomal content.[1] In addition to ploidy changes, the vast majority of cancers harbor tumor cells with abnormal centrosome numbers.[2,3] Centrosomes function as major microtubule organizing centers in animal and human cells and contribute to the organization of the mitotic spindle.[4] Aberrant multipolar mitoses have been long recognized as hallmarks of cancer. Whether centrosome aberrations are a cause or consequence of genomic instability, however, is still under debate; it is very likely that both are correct.[5-7]

The incidence of centrosome aberrations and aneuploidy in many advanced stage malignancies has led to the general belief that abnormal centrosome numbers are a consequence of tetra-/polyploidy after a failed mitosis. Aneuploidy would be a result of a two-step process in which the accumulated centrosomes in tetraploid cells increase the risk of chromosome missegregation when cells re-enter the cell division cycle.[6,8] This model attempts to integrate several key findings in tumor cells; however, the question whether cells that have failed cell division once can re-enter mitosis and produce viable and genomically unstable daughter cells has been difficult to prove. This is exemplified by the finding that a fusion of two diploid cells to induce tetraploidy does not necessarily provoke aneuploidy[9-11] and that inactivation of p53 is needed to prompt such outcome.[12] These results leave the possibility that polyploidy and aneuploidy may develop independently, at least at early stages of neoplastic progression when p53 function is often normal. Moreover, tumors can contain numerous cells with aberrant centrosome numbers without signs of ongoing genomic instability[13] whereas, on the contrary, genomically unstable tumors can contain normal centrosome numbers.[14] Centrosome accumulation after failed mitosis is not the only pathway that

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can lead to aberrant centrosome numbers and oncogenic stimuli that rapidly disrupt the centrosome duplication cycle in otherwise normal diploid cells have been identified, for example the human papillomavirus Type 16 (HPV-16) E7 oncoprotein.[15] In addition, there are reports showing that the frequency of centrosome-induced spindle abnormalities in metaphase cells may not be mirrored by a similar increase of such alterations in ana- or telophase, suggesting that many cells undergoing multipolar mitosis are unlikely to ultimately produce daughter cells.[16]

This chapter describes distinct mechanisms leading to centrosome amplification and discusses their potential impact on genome integrity. Mechanisms leading to ploidy alterations and possible consequences with respect to centrosome-mediated chromosomal instability are highlighted.

The Centrosome Duplication Cycle

Centrosomes function as major microtubule-organizing centers in most animal and human cells. During mitosis, centrosomes contribute to the organization of the mitotic spindle. Centrosomes have been implicated in various other cellular processes, many of which involve cell polarization.[17] It is noteworthy that centrioles, the core forming units of centrosomes, have important functions in the formation of sensory and motile cilia by forming basal bodies.[18]

Centrosomes typically contain two centrioles, short microtubule cylinders that are embedded in pericentriolar material (PCM). Nondividing cells contain a single centrosome which duplicates prior to mitosis in synchrony with the cell division cycle.[19] The morphological changes that occur during this process are well characterized; the molecular basis of centrosome duplication, however, is much less well understood. One molecular player that has recently been identified is separase. This protein is involved in the earliest steps of centrosome duplication, the movement of the two centrioles from a perpendicular arrangement to a near parallel position during G1 phase of the cell division cycle (centriole disengagement).[20] During the subsequent S phase, single daughter centrioles form in close proximity to the pre-existing centrioles (mother centrioles). This process involves regulation by Polo-like kinase 4 (PLK4) and HsSAS-6.[21,22] In addition, cyclin-dependent kinase 2 (CDK2) has been implicated in the regulation of centriole duplication. However, CDK2-deficient cells have no apparent centrosome anomalies[23] but it is possible that compensatory CDKs maintain normal centrosome duplication. Nonetheless, CDK2 is indispensable for oncogene-induced centrosome overduplication.[23]The precise role of CDKs and whether they function directly at the centrosome awaits further clarification. At the end of G2 phase, each maternal centriole has nucleated a single daughter centriole and the two centriole pairs separate in late G2. The two centriole pairs now start to move to the opposite spindle poles, which is accompanied by a massive increase of their microtubule-nucleating capacity. The centrosomes then participate in mitotic spindle formation and its three-dimensional orientation. Although mitotic spindles can form without centrosomes,[24] there is evidence that the presence of centrosomes is important for proper completion of cell division and the generation of viable progeny.[25]

In the nematode *Caenorhabditis elegans*, a cascade of events that controls centrosome duplication has been characterized.[26-28] The process is initiated by CDK2-mediated recruitment of the SPD-2 protein to centrioles. SPD-2 is then involved in the recruitment of the ZYG-1 kinase, which in turn recruits two additional proteins, SAS-5 and SAS-6. The latter two have been implicated in the formation of the central tube, the first step of pro-centriole formation.[29] Next, the SAS-4 protein mediates the assembly of microtubules onto the central tube (*C. elegans* centrioles consist of singlet microtubules and not of triplets as in mammalian cells). Although it is conceivable that higher organisms have a more complex network of proteins that regulate centriole biogenesis, recent results suggest a surprisingly high level of conservation.[21-22]

Aberrant Centrosome Numbers in Cancer Cells

Numerical and/or structural centrosome abnormalities have been detected in virtually all human cancers.[30] Many cancer cells contain more than the normal one or two centrosomes and such aberrations are easily detected using antibodies against the pericentriolar material such as γ-tubulin.[31] Structural centrosomal aberrations are typically recognized by an increase in centrosome size and

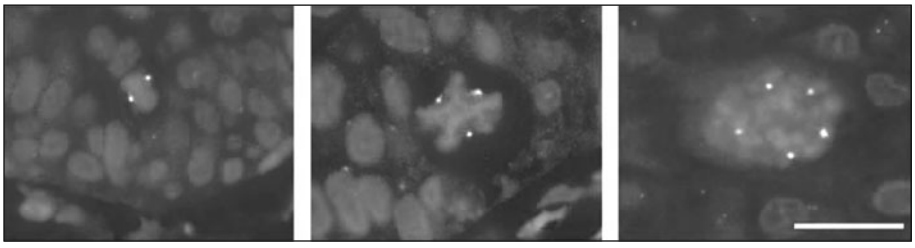

Figure 1. Multipolar mitoses are hallmarks of cancer. Bipolar mitotic spindle (left) in comparision to multipolar metaphases (middle, right) detected by immunofluorescence microscopic analysis of control tissue (left) or high-risk HPV-associated neoplasms (middle, right) for the PCM marker γ-tubulin (green). Note the increase of chromosomal material associated with both multipolar mitoses, a finding that commonly indicates a polyploidy/aneuploid chromosomal content. Chromosomes stained with DAPI. Scale bar indicates 50 μm.

an irregular shape and/or fragmentation of centrioles.[32] Together with centrosomal aberrations, abnormal multipolar mitoses are considered hallmarks of malignant tumors (Fig. 1) and were suggested as a potential source of genomically unstable tumor cells more than 100 years ago.[5] Several studies suggest that the frequency of centrosome aberrations in tumors correlates with increased aneuploidy and certain clinical characteristics that reflect tumor aggressiveness.[33-35]

Multiple Pathways Can Lead to Aberrant Centrosome Numbers: Studies Using Human Papillomavirus (HPV) Oncoproteins

Tumor viruses are elegant tools to explore basic mechanisms of cellular transformation and chromosomal instability because of the limited number of oncogenic proteins that they encode. Human papillomaviruses (HPVs) are the cause of cervical cancer and have also been implicated in the pathogenesis of squamous cell carcinomas of other anatomic locations such anal or oral carcinomas.[36] High-risk HPV types such as HPV-16 encode two major transforming oncogenes, E6 and E7. These oncoproteins function during the viral life cycle to promote efficient replication of the viral genomes.[37] Remarkably, they do so by subverting host cell tumor suppressor pathways that normally restrict DNA replication and that are also altered in the vast majority of nonvirus-associated malignancies.[38] Whereas a major function of the high-risk HPV E6 oncoprotein is to target the p53 tumor suppressor, the high-risk HPV E7 oncoprotein binds and inactivates the pRB tumor suppressor as well as the pRB family members p107 and p130 and interacts with a number of other host cell proteins.[39]

Both HPV-16 E6 and E7 can stimulate abnormal centrosome numbers in primary human cells when expressed under stable conditions.[15] In striking contrast, transient overexpression of HPV-16 E6 or E7 revealed that only the HPV-16 E7 oncoprotein can rapidly induce abnormal centrosome numbers when expressed for approximately 48 h. In contrast, HPV-16 E6 had no effect on centrosome numbers under transient conditions. Further analyses showed that the unique property of HPV-16 E7 to stimulate aberrant centrosome numbers within a short period of time was associated with an excessive formation of daughter centrioles.[40] Moreover, HPV-16 E7 was able to provoke numerical centrosome aberrations in morphologically normal, diploid cells and hence as a potential cause of cell division errors.[40] Recently it was found that HPV-16 E7 stimulates centriole overduplication through a pathway that involves the concurrent formation of more than one daughter centriole at a single maternal centriole (Fig. 2).[41] Interestingly, such phenotype is normally limited to multiciliated epithelial cells that produce numerous basal bodies during ciliogenesis.[42]

The HPV-16 E6 oncoprotein was found to stimulate abnormal centrosome numbers mostly in parallel with significant nuclear atypia including multinucleation and micronuclei.[40] There was a correlation between the degree of nuclear alterations and centrosome aberrations, indicating

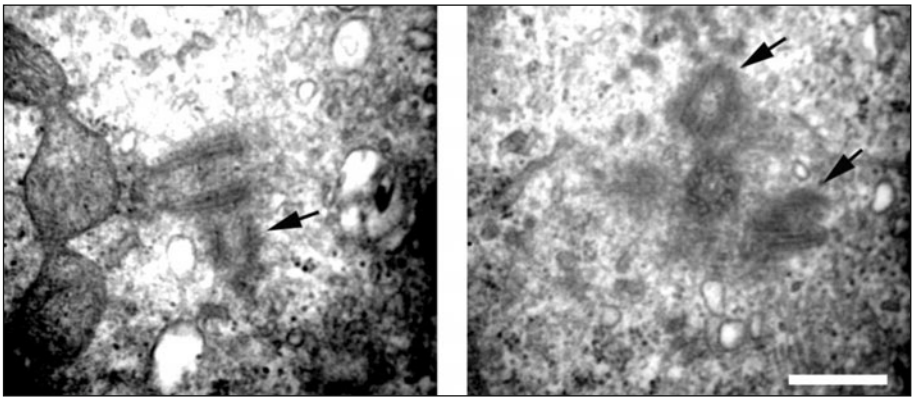

Figure 2. Centriole overduplication through formation of more than one daughter per maternal centriole. Electron micrograph of a normal mother-daughter centriole pair (left) and a mother centriole that nucleates the concurrent formation of two daughter centrioles (arrows). Such phenotype has been identified, for example, in cells expressing the HPV-16 E7 oncoprotein. Scale bar indicates 500 nm.

that these changes developed in parallel. Many of these cells were terminally growth arrested and therefore unable to produce viable progeny (see also below). It is hence likely that centrosome aberrations in HPV-16 E6-expressing cells develop as a consequence of genomic instability reflected by the gross nuclear changes and not as a potential cause as in HPV-16 E7-expressing cells. Moreover, the finding that nuclei of HPV-16 E6 expressing cells were frequently enlarged suggested that these cells were also polyploid. Based on these findings, we have proposed that distinct mechanisms can lead to aberrant centrosome numbers in tumor cells as outlined below. Importantly, the impact on genome integrity may vary depending on the mechanism and the cellular background of abnormal centrosome formation.[43]

Mechanisms of Centrosome Amplification in Tumor Cells

Primary Centrosome Overduplication

Primary centrosome overduplication should be considered when excessive numbers of immature centrioles are induced within a single cell division cycle. The discovery of proteins that specifically label mature centrioles such as ninein[44] or Cep170[45] has been instrumental in proving the existence of this mechanism. In addition, at least a fraction of such changes should occur in morphologically normal diploid cells. Whether oncogenic stimuli that rapidly induce supernumerary centrioles always trigger a concurrent formation of more than one daughter centriole in the presence of a single maternal centriole as recently reported for HPV-16 E7[41] remains to be determined. A similar phenotype has been reported in cells overexpressing Polo-like-kinase 4 (PLK4) and HsSAS-6.[21,22]Given the rapid induction of abnormal centrosome numbers in cells that have not yet acquired a highly abnormal phenotype, it is likely that primary centrosome overduplication increases the risk for cell division errors in subsequent mitoses.

Recently, loss of the CDK inhibitor $p21^{Cip1}$ was found to stimulate centriole overduplication in murine myeloblasts.[46] This finding, together with the results obtained with HPV-16 E7, raise the possibility that an impaired $p21^{Cip1}$-cyclin-CDK2-pRB signaling axis is a frequent stimulus for centriole overduplication.

In breast cancer, centrosome amplification in the absence of genomic instability has been reported. Intriguingly, aberrant centrosome numbers were detected independently of p53 inactivation and it is thus possible that certain oncogenic insults involved in breast carcinogenesis function as a trigger for primary centrosome overduplication.[47]

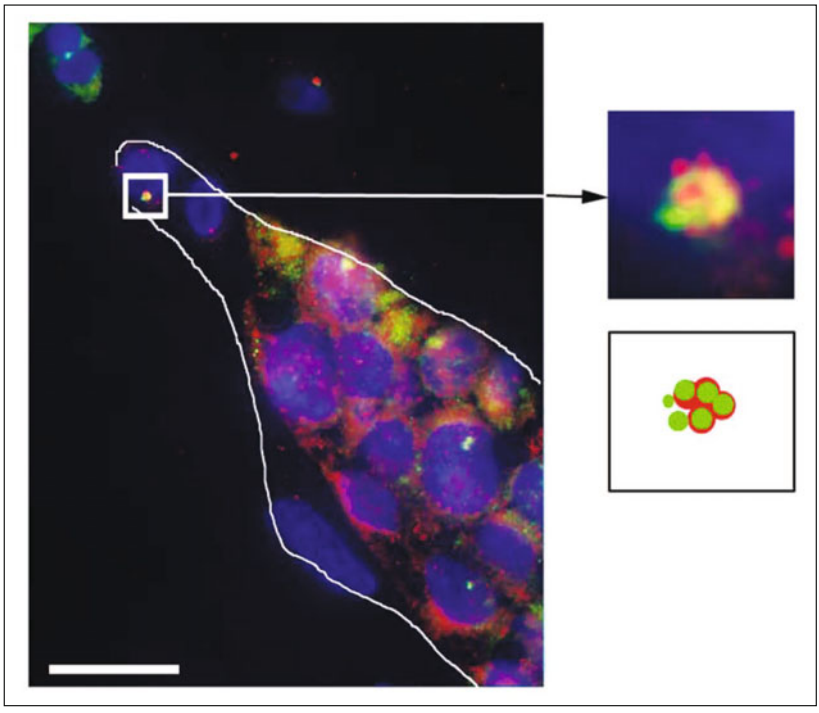

Figure 3. Centrosome accumulation in a malignant tumor. Immunofluorescence analysis of a high-risk HPV-associated squamous cell carcinoma for the PCM marker γ-tubulin (green) and a marker for mature maternal centrioles, Cep170 (red). Note the presence of multiple centrosomes that colocalize with Cep170, indicating an accumulation of mature mother centrioles (in the case of primary centrosome overduplication, only a single centrosome would be Cep170 positive since the supernumerary centrioles would be immature). Nuclei stained with DAPI. Scale bar indicates 25 μm.

Permanent Centrosome Accumulation

In contrast to a primary centrosome duplication error, centrosome accumulation is characterized by the generation of supernumerary centrosomes that does not involve uncontrolled centrosome synthesis but cellular insults that lead to an impaired segregation of centrosomes into daughter cells (for example, cytokinesis defects). The very nature of this mechanism makes it unlikely that centrosomes contribute to cell division errors. Although only long-term live cell imaging can provide an ultimate proof, there is circumstantial evidence that cells with centrosome accumulation have lost the ability to generate daughter cells. Human keratinocytes stably expressing the HPV-16 E6 oncoprotein were found to contain abnormal numbers of centrosomes together with a highly altered nuclear morphology, in particular multinucleation and micronuclei. Approximately one-third of these cells were positive when tested for senescence-associated β-galactosidase activity, indicating a permanent cell cycle arrest.[40] Interestingly, more than 40% of cells were still expressing the proliferation marker Ki67, indicating active DNA replication despite the presence of multiple nuclei.[40] It is likely that such cells undergo repeated rounds of DNA replication without producing daughter cells. HPV-16 E6 inactivates p53[48] and it is noteworthy that p53-deficient cells can also become multinucleated, in particular at later passage numbers.[49] Despite the fact that p53 can also more directly interfere with centrosome homeostasis,[50] the high frequency of p53 inactivation in human cancers suggests that centrosome accumulation may be the prevailing mechanism for supernumerary centrosomes in tumors (Fig. 3).

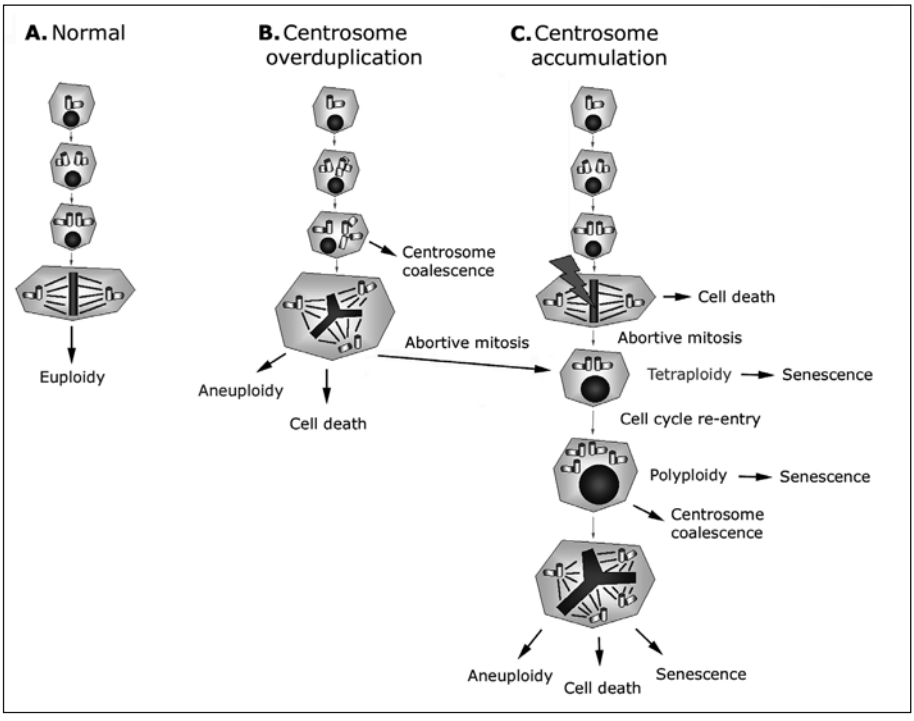

Figure 4. Ploidy changes and centrosome aberrations are in tumor cells. Normal centrosome duplication and bipolar mitotic spindle formation (A) in comparison to centrosome overduplication (B) and centrosome accumulation (C). A defect of the centriole duplication cycle itself (for example induced by the HPV-16 E7 oncoprotein) can lead to an overduplication of centrioles in otherwise normal diploid cells (B). In a subsequent mitosis, a multipolar mitotic spindle may form from which various outcomes can be envisioned. If viable daughter cells are produced, they may become aneuploid. However, the chromosomal alterations may also be detrimental, forcing cells into apoptosis. Another possibility would be that the spindle defects cause cells to adapt and re-enter a G1-like state with a tetraploid chromosomal content. It needs to be pointed out that centrosome aberrations per se have not been implicated in mitotic spindle checkpoint activation. However, prolonged activation of the mitotic spindle checkpoint (bolt of lightening) may lead to abortive mitosis and cells with a tetraploid chromosomal content and accumulation of supernumerary centrosomes (C). If such cells are able to re-enter the cell division cycle (promoted, for example, by p53 deficiency), they would not only replicate their DNA and become polyploid but also duplicate their centrosomes leading to an increased risk for multipolar mitoses. The outcome, again, would be aneuploidy (if daughter cells are viable) or cell death. Another possibility would be a permanent growth arrest and senescence. Note that centrosome coalescence can potentially alleviate centrosome-mediated cell division errors by formation of an essentially bipolar spindle despite the presence of multiple centrosomes.

More direct evidence for centrosome accumulation in cells unable to complete cell division stems from experiments in which $p21^{Cip1}$- or p53-deficient cells were exposed to DNA damage and then followed through mitosis by time-lapse videomicroscopy. Although such cells did enter mitosis, they were found to be unable to complete cytokinesis. Such cells were frequently binucleated and/or contained chromatin bridges,[51] similar to cells expressing the HPV-16 E6 oncoprotein.[52] Furthermore, these results highlight that identical oncogenic insults i.e., loss of $p21^{Cip1}$, may stimulate distinct pathways leading to aberrant centrosome numbers suggesting that

primary centrosome overduplication and centrosome accumulation may co-exist in cell populations and maybe even in the same cell.

Transient Centrosome Accumulation

The idea that cells with a diploid karyotype can become genomically unstable through an abnormal multipolar mitosis has raised concerns regarding cell viability since major chromosomal gains and/or losses in diploid cells may be detrimental. It has hence been proposed that cell division errors as a cause of genomic instability are more likely to occur in cells that enter mitosis with a tetraploid chromosome content. Tetraploid cells can arise through multiple mechanisms including endoreduplication of DNA, abortive mitosis, rereplication of DNA (i.e., re-initiation of DNA replication before completion of S phase) or cell fusion (see also below). It is conceivable that all these conditions lead to cells that contain more than the normal number of centrosomes (Fig. 4). One can imagine that re-entry into the cell division cycle of such cells is very likely to be associated with increased numbers of spindle poles. Whether cells are able to re-enter the cell division cycle may depend mainly on the p53 status but it is probable that other checkpoint proteins are also involved.[53] Possible consequences include multi- or tetrapolar spindle formation or spindles where two or more centrosomes function together as one spindle pole (coalescence).[54] It is noteworthy that centrosome coalescence could lead to more subtle changes of spindle function resulting in chromosome segregation defects that cells are able to cope with even when they are diploid. Examples of oncogenic stimuli that may lead to a transient centrosome accumulation, in particular in the absence of p53, are overexpression of Aurora-A or Polo-like kinase 1 (PLK1).[8] In addition, various other oncogenic insults such as inactivation of BRCA1, BRCA2 or SKP2[55-57]have been shown to cause centrosome amplification together with increased ploidy.

Aberrant Centrosome Numbers as a Consequence of Polyploidy—Implications for Genomic Instability in Cancer

Since ploidy alterations and numerical centrosome anomalies frequently coincide in tumor cells, the following paragraphs discuss different pathways to ploidy alterations, how they may affect centrosome numbers and the potential outcome regarding chromosomal instability.

Endoreduplication

Polyploidy per se is not pathological and several cell types in humans, including megakaryocytes or trophoblast cells, normally contain a polyploid chromosomal content. In addition, regenerative processes can increase the proportion of polyploid cells in certain tissues, for example in the liver. It is believed that nonmalignant polyploid cells have undergone rounds of endoreduplication of their genomes. During this process, DNA replication is not followed by a productive cell division. Megakaryocytes enter mitosis and form a mitotic spindle but do not complete cytokinesis.[58] Such cells can contain dozens of centrosomes and centrioles,[59] which underscores the notion that polyploidization can cause accumulation of multiple centrosomes. Daughter cells are usually not generated and it is, therefore, unlikely that endoreduplication increases the risk for chromosomal instability.

Abortive Mitoses

Cell cycle checkpoints are commonly disrupted in cancer cells, which may permit progression of cells beyond the G2/M checkpoint and into mitosis despite the presence of altered DNA or other cellular abnormalities. The mitotic spindle checkpoint is the major checkpoint that is active during mitosis and it monitors proper attachment of spindle microtubules to the kinetochores of mitotic chromosomes. The checkpoint becomes activated when spindle microtubules are not properly attached to kinetochores and delay anaphase onset. There is increasing evidence that other cellular insults including genotoxic stress can also delay or block anaphase entry.[60] The meta- to anaphase transition may hence function as a last line of defense to prevent the propagation of altered chromosomes. It is important to consider, however, what the fate of cells that have been arrested during mitosis may be. Besides undergoing apoptosis directly from mitosis (mitotic

catastrophe), it is possible that cells undergo adaptation during which they enter a G1-like state and decondense their chromosomes with a tetraploid chromosomal content.[61] It has been suggested that in this situation the cell fate critically depends on the p53 status. Based on the fact that cells with impaired p53 function can re-enter the cell cycle after prolonged spindle disruption, a tetraploidy checkpoint has been proposed.[62] Whether this p53-dependent postmitotic arrest is in fact triggered by increased ploidy or by other changes associated with spindle disruption and/or cytoskeletal aberrations remains controversial.[10,11]

Regardless of whether a ploidy checkpoint exists, there is solid evidence that p53-deficient cells are prone to initiate multiple rounds of DNA replication.[63] It has recently been reported that p53-deficient tetraploid cells become aneuploid and such cells were found to be able to form tumors in vivo.[12] The precise role of tetraploidy is difficult to ascertain from these experiments but it has been proposed that altered ploidy in yeast profoundly affects cell homeostasis and genome integrity.[64] As expected, the majority of tumor cells derived from tetraploid p53-deficient tumor cells contained supernumerary centrosomes.[12] It is conceivable that abortive mitoses generally lead to centrosome accumulation and that the genetic background of a cell determines whether it arrests permanently or re-enters mitosis. In the latter case, centrosome aberrations would increase the risk for multipolar spindles and chromosome missegregation. A detailed analysis of centrosome-mediated chromosomal instability after tetraploidization, however, has not yet been performed.

Cell Fusion

It has been proposed that cells can fuse under various conditions and that cell fusion may be a particularly common event in malignant tumors.[65] Such a mechanism would lead to tetraploid or polyploid cells and such cells would likely contain supernumerary centrosomes. Fusion events between differentiated normal cells have been shown to result in multinucleated cells that are unable to proliferate. Fusion events between malignant cells have been proposed to result in cells that are capable of producing viable progeny, a prerequisite for centrosome-mediated cell division errors and chromosomal instability. However, the survival rate of cell fusion products (hybrids) is low, suggesting that cell fusion is followed by massive cell death or permanent growth arrest in the vast majority of cells.[65] Whether cell fusion exists in primary human tumors and the contribution of fused tumor cells to chromosomal instability remains elusive. If such a mechanism can be substantiated and cells are capable of producing viable daughter cells, it is likely that supernumerary centrosomes may contribute to cell division errors and chromosomal instability.

DNA Rereplication

Another mechanism that can lead to tetra- or polyploidy is DNA rereplication i.e., an initiation of DNA replication before the previous S phase is completed.[66] It is conceivable that this can lead to aberrant DNA structures and it was found that rereplication of DNA causes an activation of DNA damage checkpoints. A prolonged DNA damage checkpoint activation, however, may allow centrosome overduplication in G2 phase.[67] In addition, overexpression of cyclin E, which is detected in many cancers, may trigger rereplication of DNA by inactivation of geminin, a major inhibitor of licensing of DNA replication.[66] At the same time, cyclin E has been implicated in centrosome overduplication.[68] Hence, cells undergoing DNA rereplication may be prone to centrosome-mediated cell division errors, in particular when loss of p53 impedes antiproliferative responses.

Aberrant Centrosome Numbers as a Cause of Polyploidy

The question whether centrosome aberrations can be a cause of polyploidy has not been addressed in detail. Previous studies suggest that a "centrosome checkpoint" which would block the progression of multipolar metaphases[69,70] into anaphase does not exist. Nonetheless, there are reports showing a discrepancy between the frequency of multipolarity in metaphase cells in comparison to anaphase cells,[40,71] which raises the question whether other yet to be identified mechanisms can hinder progression from multipolar metaphase into later stages of mitosis. A failure of such cells to complete cytokinesis would result in increased ploidy and centrosome accumulation (Fig. 4).

As discussed above, the further cell fate would likely be determined by the genetic background, specifically the p53 status.

Conclusion

Altered ploidy and centrosome aberrations are hallmarks of malignant growth and frequently arise concomitantly in tumor cells. This coincidence has led to the idea that tetra- or polyploid cells represent "seeds" of chromosomal instability. However, there are clearly examples where the formation of supernumerary centrosomes does not require an altered ploidy and that tetraploid cells do not necessarily become aneuploid. A key task for the future will be to ascertain the frequency at which cells that have failed mitosis once will re-enter mitosis and give rise to viable progeny. Moreover, the role of centrosome-mediated cell division errors in chromosomal instability requires further investigation since many studies have shown significant underrepresentation of multipolar ana- or telophase cells when compared to metaphases. In future studies, it will be important to distinguish between centrosome aberrations that have the potential to trigger cell division errors and those that merely are a side effect of cellular insults. Such studies will be critical for the use of centrosome aberrations as surrogate biomarkers of chromosomal instability and tumor progression.

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Chapter 7

Polyploidy:
Mechanisms and Cancer Promotion in Hematopoietic and Other Cells

Hao G. Nguyen and Katya Ravid*

Abstract

Polyploidy, the state of having greater than a diploid content of DNA (e.g., tetraploid, octaploid, etc) has been recognized in a large variety of both, plant and animal cells. Human and murine megakaryocytes, hepatocytes, arterial smooth muscle cells and cardiac myocytes, all develop a certain degree of polyploidy during their normal lifespan. In addition, polyploid cells may be found in some tissues under conditions of stress, including uterine smooth muscle during pregnancy, aortic vascular smooth muscle cells during aging and hypertension, beta-cells in diabetic human or mouse thyroid cells in hyperthyroidism and cells in seminal vesicles with aging. Polyploid cells are also found in malignant tissues in which they are believed to contribute to the development of cells with intermediate DNA content values (e.g., 3n, 4.5n, etc.) (reviewed in refs. 1,2). With the use of micro-array, researchers have demonstrated that genetically identical yeast strains (*Saccharomyces cerevisiae)* with differences only in ploidy status (from haploid to tetraploid) display a substantial difference in gene expression, including of the G1 cyclins.[3] This finding has suggested that DNA content per se might affect cellular functions.

Overview: Characteristics of Polyploidy and Its Induction Under Different Conditions

Currently, the relationships between polyploidy and aneuploidy has not been studied extensively considering the prominent role of genetic instability in tumorigenesis.[4] An understanding of the biochemical, gene expression and signaling pathways that drive normal and abnormal polyploidization could lead to useful insights with respect to novel anticancer therapeutic approaches. The occurrence of polyploidy in normal and transformed cells poses a number of questions. Is polyploidy a protective mechanism upon stress, as suggested,[2,5,6] or rather a maladaptive response? What mechanisms or signaling pathways are employed by normal developing polyploid cells (e.g., megakaryocytes) to safeguard them from becoming aneuploid?

In megakaryocytes, polyploidization up to 128N can be attained, if the cells undergo repeated endomitotic cell cycles, characterized by a well coordinated entry of cells into a normal early mitotic phase, which includes prophase, metaphase and early anaphase. However, these cells skip late anaphase and cytokinesis (this truncated mitosis is referred to as *polyploidy* via *endomitosis*, reviewed in ref. 2). In contrast, polyploidy may result from another type of truncated mitosis, referred to as *polyploidy* via *abortive mitosis* to describe the generally uncoordinated events that are driven by spindle checkpoint defects or by chemical treatments. These events are often associated with pathological

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conditions (reviewed in refs. 1,2,4 and see Illustration 1). It has been shown, in both tissue culture and in transgenic mice, that polyploidy via endomitosis in megakaryocytes is tightly regulated by a series of signaling pathways and gene expressions, including signaling through thrombopoietin (TPO), binding to its receptors c-Mpl[1,2] and is associated with elevated cyclin D3 expression and a rapid reentry into S-phase.[7-9] There is also evidence that these cells possess a gene expression profile that is different from their diploid counterparts, including low expression of the tumor suppressor gene p53[10] in conjunction with high expression of the cell cycle inhibitor p21 to allow a short-lived progression through G1 phase.[11,12]

Numerous studies have shown that normal diploid cells of other lineages can be induced to undergo polyploidization via endomitosis as a consequence of stress (e.g., hypertension and senescence (reviewed in ref. 2). In addition, polyploid hepatocytes have been shown to increase in number dramatically upon oxidative stress or after partial hepatoectomy.[13-15] Endothelial cells and fibroblasts have been shown in tissue biopsies and in cell culture to become polyploid upon aging and during tissue repair.[6,16] Hypertension can induce vascular smooth muscle cells and cardiac myocytes to become polyploid.[17,18] In these cases, polyploidy is believe to be a protective mechanism, which acts to prevent cellular proliferation in the vasculature or to increase DNA content in order to compensate for mutations introduced by genotoxic agents.[2,19] On the other hand, tetraploidy (cells with a double diploid DNA content) may reflect tissue damage as in Barret's esophagus,[20] in which there is dysplasia of the

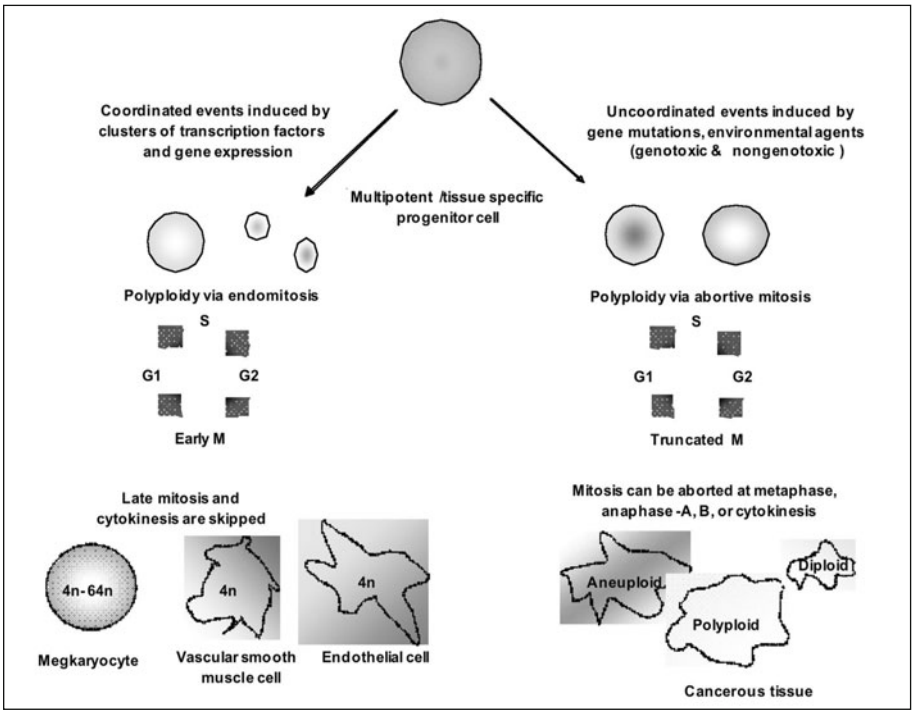

Figure 1. Pathways to polyploidy. Left panel) Polyploidy via Endomitosis—a shortened mitosis without anaphase-B and cytokinesis, followed by reentry into G1 phase of cell-cycle. This well-controlled truncated mitosis is a part of megakaryocytes development.[2,4] Right panel) Polyploidy via Abortive Mitosis—an abrupt termination of mitosis at metaphase, anaphase A, anaphase B or cytokinesis, followed by reentry into the cell-cycle with a tetraploid DNA content. These cells can have a single or multiple nuclei, depending on the timing of the defective events. This phenomenon is often associated with pathological conditions, including cancer (reviewed in refs. 2,4).

esophageal epithelium following repeated exposure to acid reflux. The most pronounced dysplastic changes include the appearance of tetraploid cells and predict for esophageal cancer.[20]

Tetraploidy can be induced in a variety of ways, including aberrant expression of proteins regulating the G2/M phase (Cyclin-B1, Aurora-A, Forkhead transcription factor M3),[21,22] mitotic spindle checkpoint proteins (BUBR1, Mad2 Aurora-B, Survivin)[23,24] leading to abortive cytokinesis. Tetraploidy can also be induced by chemical agents and/or irradiation and be associated with tumorigenesis[25-27] (see Fig. 1). This latter type of polyploidy is thought to be a by-product of uncoordinated events during mitosis in which a defect in mitotic spindle checkpoint arrest allows for a "mitosis slippage", resulting in cells with truncated mitosis, sometime at anaphase A, other times at anaphase B, or at cytokinesis.[28] The resulting tetraploid cells can be cell cycle arrested, undergo apoptosis or continue to the next division, to produce aneuploid daughter cells.[29-32]

Prevalence of Polyploidy/Aneuploidy in Different Cancers

Polyploidy often precedes aneuploidy during the events of tumorigenesis that are associated with high incidence of malignancy and poor prognosis.[33-35] It is generally accepted that aneuploidy in cancer cells is the rule and not the exception. Most heterogeneous tumor tissues (colorectal cancer, lung, breast, prostate, renal cell carcinoma, bladder cancer, thyroid cancer, some types of leukemia, glioblastoma and melanoma and rare childhood tumors) contain large populations of aneuploid cells in conjunction with a relatively smaller percentage of polyploid cells.[36-45] Among hematological malignancies, a shift in ploidy is often observed in acute lymphoblastic leukemia (ALL). In addition to a high frequency of translocations, deletions and fusion of chromosomes (70% of adults and 80% of children), a common cytogenetic abnormality in childhood ALL is the occurrence of massive hyper-diploid (defined as having greater than 50-65 chromosomes, a condition observed in 20-30% of the cases). Polyploidy has also been described in choroid plexus carcinoma, a rare form of childhood brain tumor, in which freshly isolated tumor cells were found to have up to 200-400 chromosomes.[46] Moreover, there is a recent report that primary keratinocytes infected with human papillomavirus (HPV) Type 16 E6, E7 become polyploid, possibly by abortive

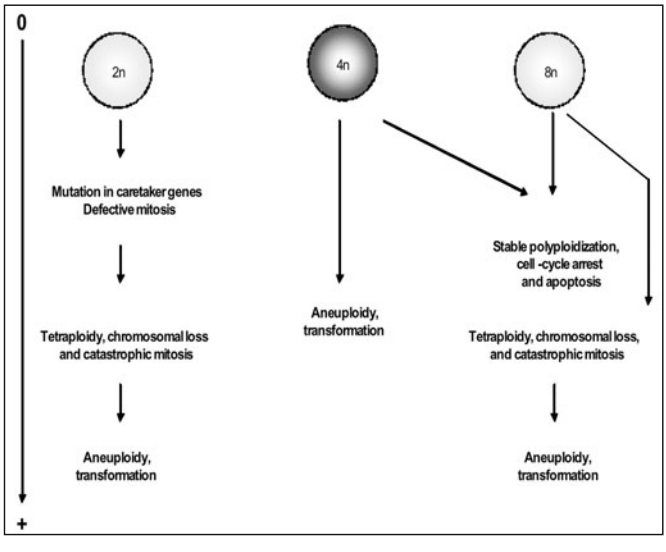

Figure 2. Possible models for polyploidy acquisition and tumorigenesis. Shown are the potential events that a cell with diploid, tetraploid or octaploid DNA content may acquire to reach malignant transformation as a function of time. This model predicts that cells with a tetraploid content are more prone to cancer development with a shortest latency period when compared to their counterparts. This model is based on previous works as reviewed in reference 4.

mitosis.[47] In most solid cancers, the modal chromosome number is near triploid, or near tetraploid. In some instances, the appearance of polyploid cells from a normal diploid cell background may be mediated by the tetraploidy/polyploidy checkpoints. This checkpoint ensures that cells with greater than 2N DNA content do not progress past G1 after exiting from mitosis.[29] There are several mechanisms that can be envisioned as causes of a ploidy shift, including doubling of a hyper-haploid cell (defined as having a total of 30-40 chromosomes), a single event of aberrant mitosis, or normal polyploidization with subsequent loss and/or gain of chromosomes.[48] Because of the high rate of chromosome loss in cycling polyploid cells,[31,49,50] it is very possible that aneuploidy develops from a tetraploid/polyploid population of cells during tumorigenesis (see Fig. 2).

Cancer Theories: Potential Involvement of Aneuploidy in Cancer Promotion

Malignant cancer cells are generally defined as cells that: (1) escape programmed cell death; (2) enter a proliferative state without mitogenic signals; (3) are unresponsive to antiproliferative signals; (4) escape programmed senescence; 5) metastasize and thrive in different tissues, including recruitment of new blood vessel formation; and (6) can eventually kill the host organism.[51] However, the transforming events that allow cancer cells to develop are not fully understood. Nonetheless, a number of theories focused on multi-step gene mutation,[52] genomic instability[53] and aneuploidy,[54] offer at least partial explanations for tumorigenesis and the development of cancers.

The Somatic Gene Mutation Theory

Cancer cells are characterized by a variety of genomic defects, such as inactivation of DNA repair genes, over-expression of growth promoting oncogenes, possession of extra or missing chromosomes, an abnormal number of centrosomes and aberrant mitosis and cytokinesis.[23] The Somatic Gene Mutation theory (SMT) has emerged as the basis for much of current cancer research and rests upon three principal assumptions. (1) Cancer is a genetic disease caused by mutations in cancer related genes, such as p53, Rb and Ras. (2) Mutations of tumor suppressor genes and oncogenes allow cells to disregard inhibitory growth signals and permit them to grow uncontrollably. (3) For a cell to become malignant, several damaging gene mutations are required, or both alleles of those genes must be affected (two hits hypothesis).[55,56] First, this theory implies that cancers are derived from individual cell clones that have accumulated mutations sequentially over time (i.e., tumors are monoclonal in nature). Second, this theory suggests that normal cells destined to become cancerous must have faster than normal rates of mutation to acquire these genetic changes (i.e., a fast rate of 10^{-3} mutations as oppose to a normal rate of 10^{-7} to 10^{-8} mutation per nucleotide per cell division). Numerous tumor suppressor genes and oncogenes have been identified and mutations of these genes have been shown to lead to neoplastic transformation in transgenic mice.[51] Yet, the somatic gene mutation theory fails to explain why cells within the same invasive tumor do not uniformly share the same mutations of relatively important genes, i.e., Ras and p53. Such cells also may share substantial differences in chromosome numbers, although they are thought to originate from clonal expansion.[57] The heterogeneous nature of tumor cells (both in the rate and type of gene mutations and ploidy status) has prompted scientists to look for additional or alternative unifying principles to explain tumorigenesis.

The Mutator Phenotype Hypothesis

Loeb and colleagues proposed the *Mutator Phenotype Hypothesis* to explain why cancer cells have a much faster rate of random mutations and how this phenotype may account for the genetic changes observed in cancer.[58,59] This theory postulates that once normal cells acquire mutations of genes that control the fidelity of DNA replication and repair, they develop an explosive increase in random mutations (Mutator Phenotype). Some of these mutations may permit cells to have selective advantages to expand and achieve clonal dominance.[60,61] This theory implies that genetic instability/aneuploidy is a consequence of these random mutations.

The Genomic Instability Theory

Lengauer and Vogelstein observed that a very high degree of genomic instability, characterized by the gain or loss of portions of chromosomes or entire chromosomes is present in the early stages (preneoplastic) of colon cancer development. Based on this finding, they proposed the *Genomic Instability Theory* of cancer in 1997. This theory argued that, at least in colon cancer, chromosomal losses or gains are the early events that lead to the loss of tumor suppressor genes and/or gain of oncogenes, which are widely believed to drive malignant transformation.[34,53,62-64] One of the main assertions of this theory is that as cells acquire mutations in master genes (or genes required for cell division and segregation of chromosomes), subsequent divisions are prone to result in more mistakes, leading to an instability in chromosome number, a critical early event in tumorigenesis. While emphasizing the importance of genetic instability as early events, this theory still holds that mutations in cancer related genes are a prerequisite for transformation. This theory explains a number of characteristic of tumor cells, including aneuploidy and fast rates of mutation. Compelling evidence in support of this theory was recently reported by Hanks et al (2004),[65] in relation to individuals with a rare genetic disorder, mosaic variegated aneuploidy, in which more than 25% of the cells in the body may be found to be aneuploid. This phenotype is characterized by mutation in both alleles of the chromosome segregation gene, BUB1B. Affected individuals frequently develop childhood cancers such as rhabdomyosarcoma and leukemia. This report is the first to suggest that aneuploidy may have a direct causal role in the development of cancer in human.

The Aneuploidy Theory of Cancer

The Mutator Phenotype and Early Genetic Instability Theories cannot explain malignancies caused by nongenotoxic carcinogens, which are not mutagens but can act as aneugens (chemical agents that disrupt the mitotic spindle and cause chromosome mis-segregation) and are associated with tumorigenesis. For instance, asbestos, a nonmutagenic carcinogen, has been shown to bind to the mitotic spindle, causing chromosome mis-segregation and genetic instability.[49] Asbestos has not been reported in the literature to cause specific cancer related gene mutations. In light of this, Duesberg and colleagues proposed the Aneuploidy Theory in 1999.[54,66] The first assumption of this theory is that cancer is not a disease of gene mutations per se but a disease of gene dosage (i.e., having 3, 5, or zero copy/copies of a normal set of genes via random aneuploidization). The second assumption is that carcinogens or spontaneous cell-cycle accidents are more effective inducers of aneuploidy than specific mutations. Hence, according to this theory cancer development does not necessarily require mutations in cancer related genes at the DNA level but an imbalance in the dosage of thousands of normal genes caused by chromosomal gains or losses. Therefore, cells may become transformed before mutations of tumor suppressor genes and/or oncogenes occur.

Regardless of which theory of cancer evolution best explains individual types of cancer, they each identify aneuploidy and genetic instability as having a causal role in tumorigenesis.

Regulators of Mitosis and Mechanisms Leading to Aneuploidy

There are a variety of ways in which cells may become aneuploid,[34] including: (1) Telomere dysfunction, which has been linked to aneuploidy in cancer. Studies have shown that telomere shortening in telomerase knockout mice after succeeding generations is associated with tumorigenesis. Cells with truncated telomeres are more prone to chromosome translocation and fusion, (reviewed in ref. 67). (2) Defective mitotic spindle checkpoint. During the transition from metaphase into anaphase, cells evolve surveillance mechanisms to ensure proper attachment of mitotic spindles to kinetochore/centromere before the segregation of chromosomes begins. Important protein components of this spindle checkpoint include: BUB1, 2, 3, Mad-1,2,3 and the chromosome passenger protein Aurora-B. Seminal studies in diverse species, ranging from yeasts to humans, have concluded that defects in this checkpoint allow the cell to progress through metaphase/anaphase with unequal attachment of the spindle/kinetochore, giving rise to aneuploid daughter cells (reviewed in refs. 68,69). (3) Defective Mitotic spindle assembly. The aberrant duplication of centrosomes at early mitosis (often due to mutation of genes involved in centrosome matura-

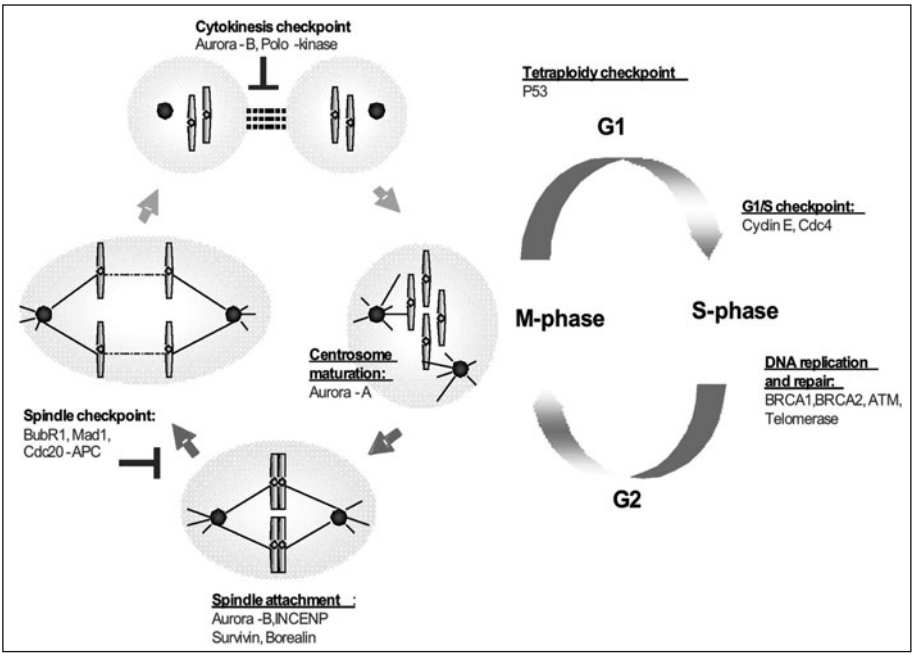

Figure 3. Pathways to aneuploidy. Shown are crucial events (underlined text) of the cell cycle that have been implicated in the generation of aneuploidy. The deregulated genes (blue text) have been shown to underlie the mechanism leading to aneuploidy. Defective regulation of mitotic genes seems to be the main routes to aneuploidy. This illustration was adapted from two review articles.[34,72] A color version of this figure is available at www.landesbioscience.com/curie.

tion and duplication, including Aurora-A, or via chemical agents) has been demonstrated to cause polyploid or aneuploid daughter cells. The resulting functional defects of mitotic spindle assembly lead to lagging chromosomes as they segregate during a precise time frame at the transition into anaphase.[31] (4) Abnormal chromosomal rearrangement, breakage and fusion, has been demonstrated to be a source of aneuploidy.[70] (5) Abortive cytokinesis, if occurred in diploid cells can lead to the formation of polyploid cells and has been suggested to cause aneuploidy via tetraploid intermediates (see Fig. 3).[71]

Stem Cells and Cancer Development

With the findings of stem cells in breast cancers, Wilm's tumors, hematological malignancy and neuroblastomas[73,74] (referred as tumor stem cell, TSC), there has been increased interest in understanding the role of tumor stem cells in tumorigenesis. Given the scarcity of stem cells in tumors, their existence in the heterogeneous tumor tissue has been demonstrated experimentally only recently, although hypothesized decades ago.[73] Tumor stem cells are phenotypically similar to normal stem cells in their abilities to self-renew and to differentiate into multiple tissues or cell lineages within the same tissue. However, they differ from normal stem cells with respect to the balance of self-renewal and differentiation. Normal stem cells generally give rise to progenitors cells, which commit into a specific cell type with a limited life span. Under normal physiological conditions, the self-renewal capability of normal stem cells is inhibited by cell cycle inhibitors, such as p21 and p18 and is tightly and reversibly regulated by the need for differentiation or tissue regeneration (reviewed in ref. 73). In p21 $^{-/-}$ mice, hematopoeitic stem cells (HSC) tend to cycle faster than wild-type cells, while the proliferation of marrow progenitor cells is repressed,

resulting in a larger pool of HSC and a smaller pool of lineage committed progenitors.[12] Hence, as observed in other systems, the proliferation of normal stem cells requires a balance between differentiation and self-renewal, depending on their stage of development.[73] In contrast, tumor stem cells are believed to have irreversible defects in cyclin inhibitors, coupled with disruptions in feedback mechanisms to control differentiation or apoptosis.[67,75] Tumor stem cells in leukemia are thought to originate directly from hematopoeitic stem cells or marrow progenitors, depending on the developmental stage at which genetic changes occur.[43] It is reasonable to hypothesize that some cancers may evolve from tissue specific progenitor stem cells because of their self-renewal, tissue evasion and ineffective senescent properties. The propensity for environmental agents, reactive oxygen species and hormones to cause genetic and epigenetic changes in stem cell is greater than that in their short-lived, differentiated counterparts. Although not yet proven, studies[74,76,77] have suggested that loss of heterozygosity of cancer related genes in mammary stem cells may contribute to genetic instability in progeny cells and subsequent breast cancer development. With the exception of polyploidy resulting from stem cell fusion,[78] a role of polyploidy and aneuploidy in the development of tumor stem cells has not been reported. However, given the important role of polyploidy and aneuploidy in tumorigenesis, an analysis of the degree of changes in ploidy in tumor stem cells would be worthwhile.

Chromosome Passenger Proteins and Their Role in Ploidy Promotion

Accurate segregation of chromosomes following each cell division requires a perfect synchrony of regulated protein proteolysis, phosphorylation and dephosphorylation, the localization and recruitment of a chromosome passenger complex and the physical interaction between the centromere and the mitotic spindles at the metaphase to anaphase transition. At the same time, the surveillance mechanism orchestrated by Mad1 and BubR1 ensures that the separation of chromosomes does not progress if these processes become asynchronous. In mammalian cells, the protein complex consisting of Aurora B kinase, Survivin, INCENP and Borealin, (also referred as the Chromosome Passenger Complex (CPC)) displays a distinct localization pattern throughout mitosis, suggesting that it has an important function in regulating mitosis. During prophase, this complex associates with condensed chromosomes and then concentrates at the inner centromere during prometaphase. At the onset of anaphase, the complex relocates to the central spindle. As the central spindle elongates at cytokinesis, the chromosome passenger proteins coalesce at the midbody, the site of the cleavage furrow. It is hypothesized that during telophase, this complex must be degraded for cells to exit mitosis normally. Various studies have demonstrated that altered subcellular localization patterns are associated with mitotic arrest, mis-segregation of chromosomes, abortive cytokinesis and polyploidy (reviewed in ref. 79). Moreover, the progeny of cells with such defects have been shown to be tumorigenic in xenograft mouse models.[72,80]

INCENP (Properties and Effects of Its Deregulated Expression on the Cell Cycle)

Inner Centromeric Protein (INCENP) was the first protein identified in the chromosome passenger protein complex. The C-terminus (IN-Box) of this protein is conserved from yeast to humans. INCENP binds to Aurora-B through the IN-Box sequences and stimulates its kinase activity during mitosis.[81] Deletion analysis of this protein has revealed that an N-terminal region (amino acid 1-68) is important for targeting INCENP to the centromere/kinetochore and midzone at anaphase.[82] INCENP is an essential gene, given that its targeted deletion in mice leads to polyploidization of embryonic cells and induces early embryonic lethality (32-64 cell stage).[83] RNAi mediated down regulation of endogenous INCENP has been shown to produce severe mitotic mis-segregation of chromosomes in *C. elegans* and Drosophila.[84,85] Overexpression of the dominant negative form of INCENP in mammalian cells showed similar defects in addition to the appearance of abnormal number of centrosomes. Thus, tight regulation of INCENP is clearly essential for cell division.[86] In vitro studies have shown that aberrant levels of INCENP disrupt the chromosome passenger complex and cause Aurora-B and Survivin to mislocalize in prometaphase.[84,87,88] Aberrant expression of INCENP also induces chromosome mis-segregation

and abortive cytokinesis in yeast, fruit flies and mammalian cells.[88,89] It has been shown in yeast that dephosphorylation of INCENP by Cdc14 is required for the transfer of the chromosome passenger complex to the central spindle at anaphase. Point mutations that generate a nonphosphorylated INCENP resulted in daughter cells with chromosomal loss, likely due to lagging chromosomes.[90] Interestingly, chromosomal alignment remained intact while the nonphosphorylated INCENP localized prematurely at the centromere prior to anaphase onset.[91] This study implies that the function of Aurora-B as a guardian of spindle attachment and alignment does not depend solely on the localization of INCENP. Hence, the protein level, kinase activities and sub-cellular localization of the Chromosome Passenger Complex proteins appear to be equally important in preventing polyploid and aneuploid phenotypes.

Borealin (Properties and Effects of Its Deregulated Expression on the Cell Cycle)

Borealin (alternatively called Dasra) was recently cloned and characterized as a new member of the chromosome passenger complex in vertebrate.[92,93] Borealin displays a typical pattern of subcellular localization to the centromere, central spindle and midbody during mitosis. Depletion of Survivin or INCENP by RNAi has been shown to disrupt this specific localization of Borealin. Similar to other chromosome passenger proteins, RNAi mediated knock down of endogenous Borealin also causes spindle defects, chromosome mis-segregation and pronounced disruption of spindle assembly.[93] Interestingly, Borealin appears to act prior to the onset of anaphase. Borealin is a direct substrate of Aurora-B and is required to target the CPPs to the centromere but not to the midzone during anaphase.[92] Given the similarity in the expression pattern and functions of Borealin to those of other CPPs, it will be important to elucidate the functional links between Borealin, Survivin, INCENP and Aurora-B in normal and cancerous cells.

Survivin (Properties and Effects of Its Deregulated Expression on the Cell Cycle)

Survivin, a 16 KDa protein as a monomer and 32 KDa as a dimer, is the smallest member in the Inhibitor of Apoptosis Protein (IAP) family and contains a BIR domain, which is characteristic of this family of proteins. Unlike other members of the IAP family, Survivin does not have ubiquitin ligase activity (E3) and is the only member protein that forms a homodimer in solution.[94-96] Interestingly, it is also a component of the chromosome passenger complex that associates with Aurora-B and it follows a similar pattern of expression and localization during mitosis. Its expression has been found to peak at G2/M and its degradation occurs in a cell-cycle dependent manner.[97] In differentiated tissue, Survivin expression is virtually absent, in contrast to its high expression in actively proliferating lineages, including CD34+ hematopoietic stem and progenitor cells (when stimulated by the combination of Thrombopoietin (TPO), Stem Cell Factor (SCF) and Flt3 ligand (FL)),[98] vascular endothelial cells,[99] vascular smooth muscle cells,[100] thymus T- and B-cells[101] and particularly in tumor cells (reviewed in ref. 102). Survivin can be found in three splice variants that differ in size (Survivin 2B, Delta Ex3 and 3B) as a result from translation of an alternate exon 2B, skipping of exon 3 and/or a frameshift with premature stop codon.[103] However, these splice variants still retain two features in common: the dimer interphase and the BIR domain at the N-terminus.[102] Published studies have suggested that survivin can form homodimers or heterodimers with its splice variants.[102,104] These homodimers/heterodimers are hypothesized to have distinct functions in regulating apoptosis or cellular proliferation, depending on the type of dimer and its subcellular localization.[104] The Survivin 2B variant is cytosolic, while the Delta Ex3 variant is localized mainly in the nucleus. The Delta Ex3 variant contains a nuclear localization sequence (NLS, R/K-rich region ^{81}RRKNLRKLRRK^{91}).[102] Survivin 2B expression is lost at later stages of malignancy, while normal Survivin and its Delta Ex3 variant maintain a high expression profile, suggesting a differential role in tumor development.[105,106] In vitro studies have demonstrated that survivin's localization to the central spindle and midbody at telophase is dependent on phosphorylation at Thr^{117} by Aurora-B and mutation of this site leads to disruption of its association with INCENP,[107] suggesting that phosphorylation of Thr^{117} is important for Survivin's role as a chromosome passenger protein. Homozygous deletion of Survivin in mice results in embryonic lethality at day 4.5, characterized by the presence of catastrophic mitosis (cell

death during mitosis), giant multinucleate cells, in addition to a large population of polyploid cells.[108] Forced overexpression of Survivin has been shown to inhibit IL-3 induced apoptosis in B-lymphocytes[109] and in UV-induced apoptosis in primary keratinocytes.[110] In addition, published studies have suggested that overexpression of Survivin shortens G1 phase arrest and accelerates S phase, potentially through activation of Cdk2/Cyclin-E complex.[111,112] The important role of Survivin in regulating endomitosis in polyploidizing megakaryocytes and vascular smooth muscle cells has been implicated in work reported from our laboratory.[113] In addition, it has been shown that during the endomitotic cell cycle of vascular smooth muscle cells, Survivin does not colocalize with Aurora-B or INCENP, as typically observed at the centromere and at the central spindle/midbody during cytokinesis of normally dividing cells. Interestingly, defects in sister chromatid separation and reversal of cytokinesis has also been observed in this generally normal and well coordinated endomitotic events.[113] Overexpression of Survivin has also been shown to reduce polyploidization in cultured primary vascular smooth cells.[113] Hence, the atypical localization pattern of Survivin appears to account for polyploidization in this lineage. Based on these studies, the function of Survivin in regulating endomitosis appears to be important. In accordance with our earlier report, survivin was not detected in endomitotic murine megakaryocytes, although these authors questioned the quality of the antibody used. In low ploidy human megakaryocytes, survivin was described as being properly localized in endomitotic megakaryocytes.[114] Several possibilities could account for the discrepancy in the reports on Survivin expression/localization in human and mouse megakaryocytes: a. According to Baccini et al, a group that has extensively studied megakaryocytes biology (e.g., refs. 115,116), human megakaryocytes grown in vitro present a defect in their polyploidization and hence, the authors caution others from using them as a model system for the study of endomitosis.[115] In vivo, most polyploid human megakaryocytes are 16N and 32N, as is the case in mice and rats. However, while the mouse cultures mimic the in vivo profile, the human cultures present less than 10% of the cells as polyploid, with the vast majority having a ploidy level not greater than 8N.[115,116] Hence, in the recent study that used this culture system,[114] survivin localization might have been primarily followed in proliferating or very low-ploidy megakaryocytes (as also pointed out by the authors); b. In the literature there is a recognized controversy about the specificity of available antibodies to survivin. For instance, W. Earnshaw's lab described conflicting survivin localization in mitosis with two published antibodies and noted that researchers need to also confirm data on protein localization with ectopically expressed GFP-labeled protein.[88] c. Although there is one survivin gene, there are three splice forms of mRNA yielding three different proteins, of which only the longer one (142 amino acids) displays typical properties attributed to survivin.[103] Some of the available antibodies might be detecting one of the nonfunctional splice variants.

With regard to its antiapoptotic properties, Survivin has been shown to bind to Smac/Diablo, a caspase activator and/or to procaspase 9 via the hepatitis B X-interacting protein (HPXIP) complex to mediate this effect.[117,118] A study by Song et al (2004)[104] demonstrated that a single amino acid change (Asp53→Ala53) converts Survivin from an antiapoptotic to proapoptotic regulator, suggesting that it has a dual role in controlling cell death at mitosis. Studies of Survivin function as both a Chromosome Passenger Protein and as an anti/pro apoptotic factor has been a subject of much interest. Recent work has described a new type of cell death, termed "mitosis catastrophe", often observed in cells with defective mitosis spindle assembly checkpoint, chromosome mis-segregation and abortive cytokinesis (reviewed in ref. 119). Although "mitosis catastrophe" is believed to be triggered by aberrant events during mitosis and not signals originating in G1 or S-phase, this type of programmed cell death still converges on the action of caspases, as suggested by several studies.[110,120,121] It is tempting to hypothesize that Survivin is a critical regulatory protein that determines the life and death of a cell undergoing division. Survivin may ensure the survival of cells with correct chromosome segregation by directly inhibiting caspases through its anti-apoptosis and/or chromosome checkpoint properties. On the other hand, Survivin, through its pro-apoptotic properties, may also ensure that cells undergo apoptosis if mitotic events are defective.

Aurora-B (Properties and Effects of Its Deregulated Expression on the Cell Cycle)

The Aurora/Ipl1 (Increase-in-Ploidy protein-1) protein kinases have been shown to orchestrate vital mitotic events, including G2/M transition, centrosome duplication, chromosome condensation, bi-polar spindle-kinetochore attachment, chromosome segregation and cytokinesis. Their roles are evolutionarily conserved in yeast, nematodes and mammalian cells (reviewed in refs. 72,122). While lower organisms have only one form of Aurora kinase (Ipl-1), mammalian cells have three types, Aurora-A, Aurora-B and Aurora-C, whose function and localization are distinct in space and time during cell division. The function of Aurora-C in mammalian cells has not been studied extensively. Aurora-A localizes to the centrosomes during early anaphase and is required for mitotic entry.[123] Aurora-B, (also called AIM-1, Stk-5) regulates the formation of a stable bi-polar spindle-kinetochore attachment in mitosis. It colocalizes with Survivin, Inner Centromere Protein (INCENP) and a recently discovered protein named Borealin or hDasra B to form the chromosome passenger complex, needed for chromosome segregation and cytokinesis.[21,92,93] Aurora-B is regulated at the mRNA level, at the protein level and at the level of its kinase activities (reviewed in refs. 72,122,124,125). INCENP has been shown to stimulate the kinase activity of Aurora-B[84,126,127] and there are conflicting reports on the regulation of Aurora-B by Survivin.[126] In a cell-free system, Survivin seems to enhance the kinase activity of Aurora-B (via Histone-H3-Ser10 phosphorylation),[128] provided that its kinase activity is first reduced in cells with siRNA-mediated Survivin knock down. How Aurora-B activity/function is terminated at the end of mitosis is an additional intriguing question.[23] Studies pursued in this thesis demonstrate that Aurora-B is regulated by protein degradation through the A-box and KEN box sequences.[129] Most importantly, overexpression of a nondegradable A-Box mutant leads to aneuploid/polyploidy, suggesting that Aurora-B's proteolysis plays an important role in the regulation of Aurora-B and chromosome stability at each cell division.[129] A recent study[130] identified a very short sequence in the C-terminus of Aurora-B (326-331) as responsible for its function and subcellular localization. Taken together, these studies demonstrate that Aurora-B's stability is regulated through its N-terminus, whereas the C-terminus contains the sequences required for its function and subcellular localization.

The most extensively studied function of Aurora-B is its involvement in mitotic spindle attachment. In order for chromosomes to separate equally, a synchronized alignment of sister chromatids at metaphase coupled with stable bi-polar attachments between the mitotic spindle and the kinetochore must take place. During this dynamic process, there are various ways in which the kinetochore-microtubule can form unstable attachments. This includes the case of kinetochore attaching to the spindle from both poles (merotellic) or when both sister kinetochores are attached to the same spindle pole (syntellic). If these unstable attachments are not corrected in time as the cell enters anaphase, lagging chromosomes and unequal separation of chromosomes occur in the daughter cells. Reduction of endogenous Aurora-B by genetic (iRNA) or pharmacological agents (ZM447439 and Hesperadin) results in merotellic and/or syntellic attachment and subsequent disruption of chromosome segregation.[21] Experiments using microinjection of anti-Aurora-B antibodies reveals that inhibition of Aurora-B in mitotic Xenopus tissue culture cells abrogates the spindle checkpoint and causes an early exit from mitosis with no evidence of anaphase or cytokinesis, concomitantly with the appearance of chromosome misalignment and polyploid cells.[131] How Aurora-B promotes stable bi-polar attachment and prevents unstable merotellic and/or syntellic attachment continues to be under investigation. The current model, derived from various studies with both yeast and mammalian cells, proposes that Aurora-B, through its kinase activity and interaction with various proteins (such as the Mitotic Centromere Associated Kinesin, MCAK), actively facilitates the depolymerization of microtubules associated with unstable attachments. Evidence for this model in mammalian cells includes the finding that Aurora-B directly interacts with MCAK to promote microtubule depolymerization.[132,133] In addition, its interaction with protein phosphatase I (PPI) keeps depolymerization in check, once stable attachments are achieved.[134,135] In budding yeast, Aurora-B is believed to function as a sensor for the pulling force and tension generated by the spindle-kinetochore complex. Yeast mutants, unable to generate spindle tensions accumulate merotellic and/or syntellic spindle attachments.[136-139] Hence, as a sensor of

the pulling force, Aurora-B may sense unequal forces and promote their elimination.[139] Recently, the yeast Aurora-B homolog, Ipl1 has been shown to interact with the Damp1 complexes, which interact directly with the kinetochore and microtubule to regulate bipolar attachment of mitotic spindle.[140] Specific mutation (S to A) of all four Ipl1 phosphorylation sites in the Dam1p protein causes cell death, suggesting an essential role for Ipl1/Dam1p phosphorylation.[140] Because of its vital role in correcting chromosome-spindle attachment, deregulated expression of Aurora-B/Ipl1 can be expected to impair chromosome segregation and mitotic progression.

Another function of Aurora-B, that has been described, concerns its role in the spindle checkpoint. To ensure viable and functional progeny after each cell division, cells have evolved several cell cycle checkpoints to allow adequate time for repair prior to progression to subsequent stages of cell division. One of the most important and final checkpoints of the cell cycle is the spindle assembly checkpoint during the transition into anaphase. Defective mitotic spindle assembly or detachment of the kinetochore directly triggers Bub1, Mad1 and other spindle checkpoint proteins to bind and inhibit the activity of the Cdc20-APC/c E3 ligase (a component of proteasome mediated degradation, as described below and also regarded as the effector of the spindle checkpoint), leading to a transient arrest of the cells at metaphase. Several studies have demonstrated that Aurora-B participates in the recruitment/association of Mps1, Bub1, CENP-E, Bub3, Mad1 and Mad2 to kinetochores.[141,142] Studies described in this thesis show that Aurora-B directly associates with the Cdc20-APC/c complex.[129] Moreover, other studies indicate that depletion of endogenous Aurora-B impairs the cells' ability to localize Cdc20, Cdc27 and Cdc23 (subcomponents of the APC/c) to unattached kinetochores such that cells fail to activate the spindle checkpoint in response to microtubule destabilization.[139,142-144] These functional studies have demonstrated that Aurora-B is an indispensable member of the spindle assembly checkpoint, acting upstream of Bub1 and Mad1 and indicate that deregulation of Aurora-B disrupts this protein composition to prevent the spindle checkpoint.[145]

During telophase, Aurora-B also has a role in ensuring the completion of cytokinesis.[146-149] Drosophila cells lacking Aurora-B protein do not undergo cytokinesis and undergo polyploidization.[150] Drug-mediated inhibition of this kinase in proliferating mammalian cells can also induce polyploidy[21] and/or cell death by "catastrophic mitosis".[151] In bone marrow megakaryocytes (platelet precursors), which undergo endomitotic cell cycles and polyploidization during normal development, Aurora-B has been shown to be absent from the midzone.[152] Of note, a very recent study reported results similar to ours with respect to proper Aurora-B expression/localization during early mitosis and lack of it at late anaphase in murine megakaryocytes.[114] In few human megakaryocytes examined,[114] Aurora-B was detected at late anaphase. In this study, Aurora-B kinase activity was measured in polyploid, nonsynchronized megakaryocytes and it was concluded that Aurora-B is not limiting for polyploidy in this lineage. Similar to our conclusion, this study also suggested that further study of midzone organization and composition is needed. Aurora kinases have been found to be overexpressed in a variety of malignant cancers (as listed in: http://cgap.nci.nih.gov) and this overexpression is suspected to contribute to chromosome instability.[153] Studies by our group[152] have shown that in vivo overexpression of Aurora-B transgene in megakaryocytes increases the proliferative potential of these cells, but does not by itself induce malignant transformation.[152]

Chromosome Passenger Proteins and Cancer (Emphasis on the Role of Aurora-B)

The link between overexpression of the Aurora kinases in mammalian cells and carcinogenesis is believed to be causal and to be dependent on perhaps, the disruption of normal centrosome or centromere function, spindle checkpoint regulation and cytokinesis.[30,80,153-155] Overexpression of the Chromosome Passenger Proteins (CPP), including Aurora-A, Aurora-B, Survivin and INCENP has been observed in ovarian, breast and prostate cancers and shown to correlate with aneuploidy.[72,81,156-159] In addition, chromosomes containing the CPP are often affected in aneuploid cells.[160,161] The mechanisms that explain how overexpression of CPP proteins, individually or together, promotes aneuploidy remain an important, unanswered question. Only recently,

have studies been carried out to determine whether ectopic expression of the CPP drives cellular transformation by means of increasing proliferation, by centrosome amplification, or by inducing chromosome instability. Overexpression of Aurora-A has been shown to potentiate HRAS (Harvey sarcoma virus oncogenes) induced transformation in-vitro, whereas reduced endogenous Aurora-A expression by short hair-pin RNA (shRNA) decreased transformation.[162] It has been suggested that overexpression of Aurora-A results in cells with increased number of centrosomes (3-4) and consequently impairs their ability to segregate chromosomes equally.[50] Similarly, correlative data showing overexpression of Aurora-B kinase in solid tumors and tumor cell lines has been reported.[80,157,160,163-165] However, given the tight regulation of Aurora-B at the protein level,[152] only a handful of studies have been able to demonstrate overexpression of Aurora-B induces oncogenic transformation. These studies include those using xenograft models of localized tumor formation in mice injected with cells overexpressing Aurora-B (80 and Nguyen Hao and Katya Ravid unpublished data). In these studies, oncogenic transformation appears to be mediated by aneuploidy and to be a consequence of Aurora-B overexpression. Inhibition of the Aurora kinases, in general, blocks progression of the cell cycle and induces cell death by "catastrophic mitosis". Several studies have exploited this type of cell death using Aurora kinase inhibitors (VX-680 for Aurora-A, Hesperadin and ZM447439 for Aurora-B (refs. 21,166-168 and reviewed in ref. 151)) to suppress tumor growth in vivo. Reduction of endogenous Aurora-B expression by such means has been shown to diminish the growth of thyroid anaplastic carcinoma tumor cells.[163] However, it is possible that inhibition of Aurora kinases is unable to completely prevent tumor growth, since reduced expression of these kinases also leads to aneuploidy (as reviewed in refs. 88,119,122,124,125,155,158,169,170) and such inhibition would be expected to prevent the activation of the spindle checkpoint, causing cells to exit mitosis prematurely.

Overexpression of Survivin in a wide range of tumor tissues, including leukemia (ALL, AML), colo-rectal cancers, astrocytic tumors and breast cancer has been consistently reported in the literature.[102,159,171] Moreover, overexpression of Survivin in cancer tissues is closely correlated with poor prognosis.[172-176] The role of Survivin in cancer promotion has been studied using a transgenic mouse model.[110] Grossman et al[110] have shown that exogenous expression of Survivin (driven by the keratinocyte specific promoter (K14)) inhibits UVB induced apoptosis and thus inhibition is more pronounced when the expression of p53 is reduced. Hence, this study suggests that Survivin functions as an inhibitor of apoptosis and thereby contributes to transformation. However, given Survivin's role as a chromosome passenger protein, the consequences of its overexpression on chromosome stability have not been fully explored.

Conclusion

It is not clear yet if polyploidy is a protective mechanism upon stress, or rather a maladaptive response. Progress has been made, however, on mechanisms and signaling pathways that are employed by normal developing polyploid cells (e.g., megakaryocytes) to safeguard them from becoming aneuploid, as well as on the mechanisms leading to aneuploidy and its relation to cancer development.

Acknowledegments

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Chapter 8

Polyploidization of Liver Cells

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Abstract

Eukaryotic organisms usually contain a diploid complement of chromosomes. However, there are a number of exceptions. Organisms containing an increase in DNA content by whole number multiples of the entire set of chromosomes are defined as polyploid. Cells that contain more than two sets of chromosomes were first observed in plants about a century ago and it is now recognized that polyploidy cells form in many eukaryotes under a wide variety of circumstance. Although it is less common in mammals, some tissues, including the liver, show a high percentage of polyploid cells. Thus, during postnatal growth, the liver parenchyma undergoes dramatic changes characterized by gradual polyploidization during which hepatocytes of several ploidy classes emerge as a result of modified cell-division cycles. This process generates the successive appearance of tetraploid and octoploid cell classes with one or two nuclei (mononucleated or binucleated). Liver cells polyploidy is generally considered to indicate terminal differentiation and senescence and to lead both to the progressive loss of cell pluripotency and a markedly decreased replication capacity. In adults, liver polyploidization is differentially regulated upon loss of liver mass and liver damage. Interestingly, partial hepatectomy induces marked cell proliferation followed by an increase in liver ploidy. In contrast, during hepatocarcinoma (HCC), growth shifts to a nonpolyploidizing pattern and expansion of the diploid hepatocytes population is observed in neoplastic nodules. Here we review the current state of understanding about how polyploidization is regulated during normal and pathological liver growth and detail by which mechanisms hepatocytes become polyploid.

Introduction

The liver is an essential organ with a high regenerative capacity and complex functions.[1-3] This organ has a central role in metabolic homeostasis, as it is responsible for the metabolism, synthesis, storage and redistribution of nutrients, carbohydrates, fats and vitamins. Nutrients entering the liver are transformed into secreted proteins (albumin, most coagulation factors and several plasma carrier proteins), carbohydrates stored in the liver as glycogen (the main glucose reserve used for stabilization of glucose levels in the blood) and lipids sent as lipoproteins into the other tissues. Importantly, the liver is also the main detoxifying organ of the body, which removes wastes and xenobiotics by metabolic conversion and biliary excretion. The main cell type of the liver that carries out most of these functions is the parenchymal cells, or hepatocytes, which constitute approximately 60% of all cells in the liver and 90% of liver cell mass. The other 40% comprise the nonparenchymal cells, which include endothelial cells, kupffer cells, lymphocytes and stellate cells. Hepatocytes within the liver lobule differ in their enzyme content and subcellular structure according to their location (for reviews see ref. 4). In fact, based on the blood vessels location and the blood flow direction, the individual liver lobule can be subdivided into an upstream "periportal" and a downstream "perivenous"

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(pericentral) region. Amino-acid metabolism, gluconeogenesis, lipid oxidation, energy metabolism and glycogen synthesis from lactate take place in the upstream, periportal hepatocytes. On the other hand, glycolysis, lipogenesis, cytochrome P450-dependent detoxification and glycogen synthesis from glucose are located in the downstream, pericentral hepatocytes.

Polyploidy is a characteristic feature of mammalian hepatocytes.[5-8] During postnatal growth, the liver parenchyma undergoes dramatic changes characterized by gradual polyploidization during which hepatocytes of several ploidy classes emerge as a result of modified cell-division cycles. This process generates the successive appearance of tetraploid and octoploid cell classes with one or two nuclei.[8-10] The hepatocyte ploidy level practically reaches the plateau at maturity. Interestingly, a second wave of ploidy elevation has been also observed at senescence in different species.[11,12] The biological significance of hepatic polyploidy is not clear, but the presence of advanced polyploidy is generally considered to indicate terminal differentiation,[13,14] with decreased proliferative capacity.[15,16] In this chapter, we discuss how polyploidization is regulated during normal and pathological liver growth and try to understand by which mechanisms hepatocytes become polyploid.

Polyploidization During Normal (Developmental) Liver Growth

Hepatic development is an extended process that continues thought early postnatal life. Through E14, most hepatoblasts are bipotent with the ability to differentiate into hepatocytes as well as biliary cells but by E15 most hepatoblasts are committed to the hepatocyte lineage.[17,18] During the remaining period of gestation and the first four postnatal weeks, hepatoblasts acquire functions of the differentiated hepatocytes and metabolic zonation.[4,17,18] In parallel with this process of hepatocyte differentiation, there is a progressive decline in cellular proliferation. The DNA synthesis rate is elevated in rats two hours after birth with 18% of the hepatocytes incorporating ^{3}H-Thymidine.[19] Three weeks after birth, ~9% of the hepatocytes show evidence for DNA synthesis; within six weeks DNA synthesis is detected in only few hepatocytes, ~0.05% which is similar to a normal adult liver.

The onset of polyploidy is clearly correlated with the end of the proliferative state in the liver. Several techniques have been reported for ploidy determination using isolated hepatocytes (ex vivo studies). Hepatocyte ploidy has been investigated in the past essentially through karyometry[20] and cytophotometry.[21,22] More recently, several groups have used flow cytometry (FACS).[9,14,23] However, FACS cannot resolve nuclearity (counting binucleated cells) and a second step is required to count the number of nuclei per cell (microscopy approach).[24,25] Recently, reports have taken advantage of fluorescence imaging to directly assess in vivo nuclearity and to measure DNA content in order to determine the liver ploidy/binuclearity profile[26,27] (Fig. 1A,B). In fact, the results of ex vivo and in vivo studies are in line with each other. For example, in the newborn rat liver, all hepatocytes are diploid (Fig. 1C). From the first three weeks postnatal, the proportion of diploid cells starts to fall significantly, with the successive appearance of binucleated 2 × 2n and mononucleated 4n hepatocytes (Fig. 1C). Binucleated 2 × 2n cells are first detected after weaning (day 21) and then their proportion rise rapidly to reach ~30 % of the total hepatocytes population at day 30. The mononucleated 4n hepatocytes population is only present at significant levels 25 days after birth. At day 30, a sharp increase is observed in the number of mononucleated 4n hepatocytes and this hepatocytes contingent is in the majority at day 40, reaching ~45% of the total population (Fig. 1C). Octoploid (binucleated 2 × 4n and mononucleated 8n) hepatocytes accumulate in significant numbers during the second and third months, in parallel with a decline in the relative number of tetraploid hepatocytes.

The degree of polyploidization varies in different mammals (Table 1).[28,29] In humans, the number of polyploid cells averages 30% to 40% in the adult liver.[11-27] A negative correlation exists between the mitotic index in the liver and the level of hepatocyte polyploidization found in different species.[30,31] For example, a mouse liver has a much lower mitotic index than a rat liver and accordingly the higher level of hepatocyte polyploidization was found in the mouse liver. Polyploidization is minimal in a guinea pig liver which has the highest mitotic index of hepatocytes amongst rodents.[29] Increased cell size is the most obvious and consistent consequence of an increase in ploidy. Different studies have demonstrated in both human and mouse liver cells that the volume of hepatocytes is approximately twice with doubling DNA content.[8,25,32,33] Moreover, there is no significant difference in the volume

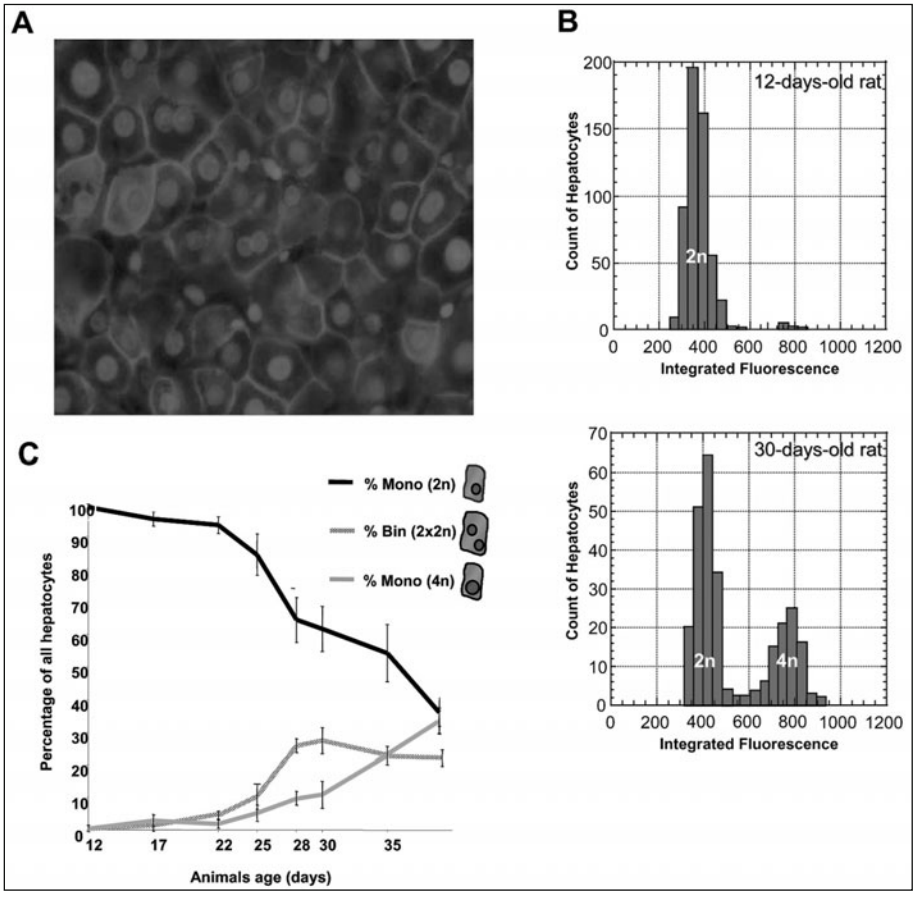

Figure 1. In situ analysis of hepatocyte polyploidy during liver growth. A) Imaging of a liver section after double staining with Hoechst (nuclear labelling) and β-catenin (plasma membrane labelling) enable a distinction between mononucleated 2n, 4n and binucleated 2 × 2n hepatocytes. B) Representative histogram of the DNA content distribution of mononucleated hepatocytes from 12-days-old and 30-days-old rats. DNA content is evaluated by recording the Hoechst integrated fluorescence in each nucleus. Integrated fluorescence is expressed in arbitrary units. The first peak is representative of hepatocytes with 2n DNA content. The second peak is positioned at twice the value of the first peak and is representative of hepatocytes with 4n DNA content. C) Polyploidization during postnatal liver growth: percentages of mononucleated 2n (black line), binucleated 2 × 2n (dark line) and mononucleated 4n (grey line) hepatocytes. The average percentage of each population is shown on the curve.

of binucleated 2 × 2n and mononucleated 4n hepatocytes and between binucleated 2 × 4n and mononucleated 8n hepatocytes.[25] The relationship between DNA content and cell volume is in fact conserved in evolutionarily distant eukaryotes. For example, the volume of budding yeast cells increases linearly with each extra complement of chromosomes.[34] How are polyploid hepatocytes distributed in adult hepatic lobules? Different studies have suggested the existence of ploidy zonation within the hepatic lobules, periportal hepatocytes exhibiting less ploidy and perivenous hepatocytes greater ploidy.[13,35,36] However, recent discrepant results have been reported suggesting that similar proportions of binucleated hepatocytes are present in both periportal and perivenous areas.[37]

Table 1. Distribution (%) of the different hepatocytes ploidy classes

	Hepatocyte Ploidy Classes					
	2n	**2 × 2n**	**4n**	**2 × 4n**	**8n**	**2 × 8n**
Bos taurus	97,3	2,0	0,7			
Equus caballus	96,7	2,3	1,0			
Ovis aries	92,7	4,3	3,0			
Homo sapiens	75,8	14.3	9,6	0,3		
Canis lupus	87,3	6,0	6,7			
Gorilla gorilla	81,3	12,21	5,6	0,6	0,3	
Cavia porcellus	68,7	28,0	3,3			
Talpa europaea	63,7	31,0	5,3			
Sorex araneus	15,0	84,7	0,3			
Rattus rattus	4,0	16,3	67,7	8,7	3,0	0,3
Rattus norvegicus	5,3	2,7	76,0	10,0	6,0	
Mus musculus	5,7	7,3	60,0	17,0	7,7	2,3

The molecular events that cause polyploidy remain elusive. The onset of polyploidy is clearly associated with weaning and independent feeding.[29] Liver polyploidy would be influenced by dietary restriction (DR). The effect of the DR on liver polyploidy has been analyzed by measuring the volume of hepatic nuclei. When dietary restriction is imposed on one group by reducing their food intake to 60% of ad libitum food intake, onset of polyploidization is delayed in food-deprived rats.[38] The same results are obtained if mice are fed with a low protein diet.[39] The endocrine regulation of the growth and polyploidization of liver nuclei has been extensively studied in the past. Forty years ago, morphometric studies revealed that hormones alter mitotic activity and hepatocyte ploidy, the regulation being predominantly carried out by growth and thyroid hormones with modulators effects of sex steroids hormones.[40-44] When summarizing the role for pituitary-mediated hormones, it was suggested that the action of the thyroid hormone is largely mediated through an effect on the release of the anterior pituitary growth hormone (GH).[30] However, more recently two studies have investigated the effect of T_3 in thyroidectomized rats on the ploidy of liver nuclei. A complete cessation of 4C nuclei formation was reported in these rats; treatment of thyroidectomized rats with a single dose of T_3 was sufficient to abruptly increase the percentage of 4C nuclei.[45,46] By contrast, hGH injection has no effect on the 4C nuclei fraction in the hypothyroid rats.[46] These results suggest that the processes of hepatocyte polyploidization are under endocrine control, with thyroid hormones playing the essential regulatory role.

Mechanism of Binucleation and Polyploidization

One fascinating question is how a diploid organism can give rise to polyploid cells in some tissues. In the liver, the cellular mechanisms that govern the passage from mononucleated 2n to binucleated 2 × 2n and/or mononucleated 4n hepatocytes have long been unknown. One explanation for liver polyploidization is that a mononucleated 2n hepatocyte gives rise directly to mononucleated 4n cells through endoreplication. During this physiological process, DNA replication is uncoupled from cell division: the cell undergoes several rounds of DNA replication without mitosis, leading to the genesis of terminally differentiated nondividing autopolyploids cells.[47] This process has already been described in plants, *Drosophila* and mammals, notably in megakaryocyte and trophoblast cells.[47-50] In the liver, such a mechanism has also been described but only in pathological murine models exhibiting an absence or deregulated expression of genes such as p21, S-phase kinase associated protein 2 (Skp2) and excision repair cross-complementing protein 1 (ERCC1). In these mice, endoreplication takes place and induces premature liver poly-

ploidization with an increase in the number of mononucleated polyploid fractions.[51-53] However, this process doesn't take into account the formation of binucleated cells.

An alternative mechanism could be cell fusion, where two mononucleated 2n cells can fuse together and produce one binucleated 2 × 2n hepatocyte. Hereafter, binucleated 2 × 2n hepatocytes may divide, leading to the genesis of two daughter mononucleated 4n hepatocytes. A mononucleated 4n hepatocyte then embarks upon a new round of binucleation/polyploidization with the formation of 2 × 4n and 8n cells. In some cell types (skeletal muscle cells and osteoclasts), cell fusion is a normal developmental programmed step, which leads to the production of terminally differentiated cells.[54,55] In the liver, there is no strong evidences that such phenomenon can occur between two hepatocytes, even if an old study suggests that cultured primary hepatocytes with one or two nuclei can fuse themselves spontaneously and that the frequency of this phenomenon increases with culture time.[56] However the relevance of this phenomenon is controversial and could be attributed to the cell culture conditions, as it has been described for other kind of cell types.[57]

Finally, it was suggested a long time ago that a process of abortive cell cycle could produce binucleated hepatocytes.[20] Generally correlated with pathological proliferation, this particular event is due to a wide variety of defects in different aspects of cell division such as DNA replication, dissolving sister-chromatid cohesion, mitotic spindle function and cytokinesis. Recently, two studies have clearly demonstrated that binuclated hepatocytes have a pivotal role for the establishment of liver cell polyploidization during postnatal development (Fig. 2). In the first study, Guidotti and collabora-

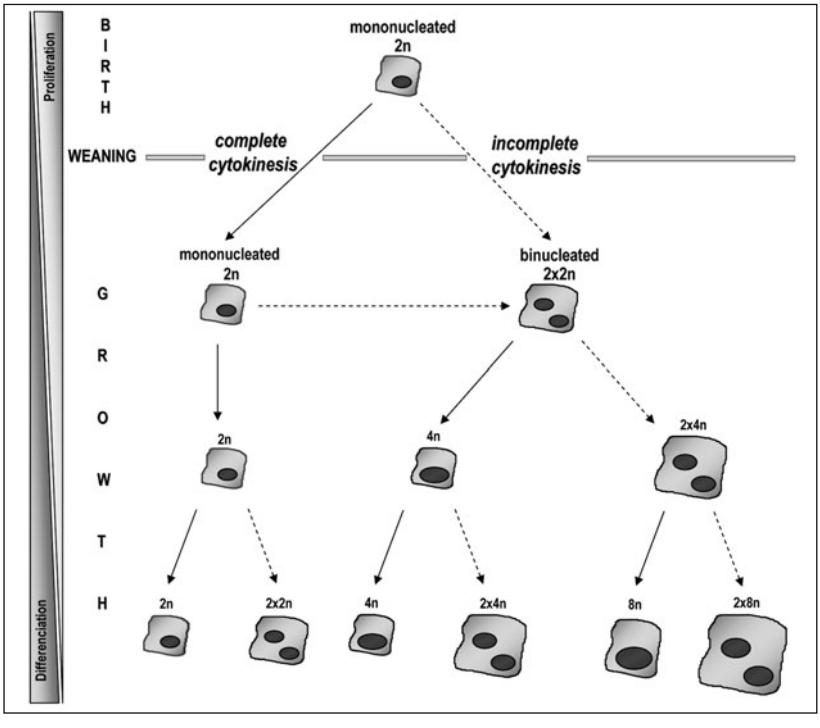

Figure 2. Lineage of hepatocytes of different ploidy during postnatal liver growth. Hepatocytes of newborns are exclusively diploid (mononucleated 2n). At the weaning period, the mononucleated 2n hepatocyte can engage either into a normal cell division cycle (whole arrow) and gives rise to two mononucleated 2n hepatocytes, or follow an adaptive cell cycle with incomplete cytokinesis (arrow in dotted line) and give rise to one binucleated 2 × 2n hepatocyte. During liver growth, progressive polyploidization appears and tetraploid and octoploid cells classes with one or two nuclei are formed.

tors showed that the formation of binucleated hepatocytes was the consequence of a modified cell cycle.[26] Indeed, using a live-cell microscopy approach, they clearly demonstrated that binucleated 2 × 2n hepatocytes derive from mononucleated 2n hepatocytes that have not completed cytokinesis. In another study, the molecular process of the incomplete cytokinesis was deciphered.[37] This specific division program is triggered by weaning. Indeed, in suckling rats, all late telophase hepatocytes

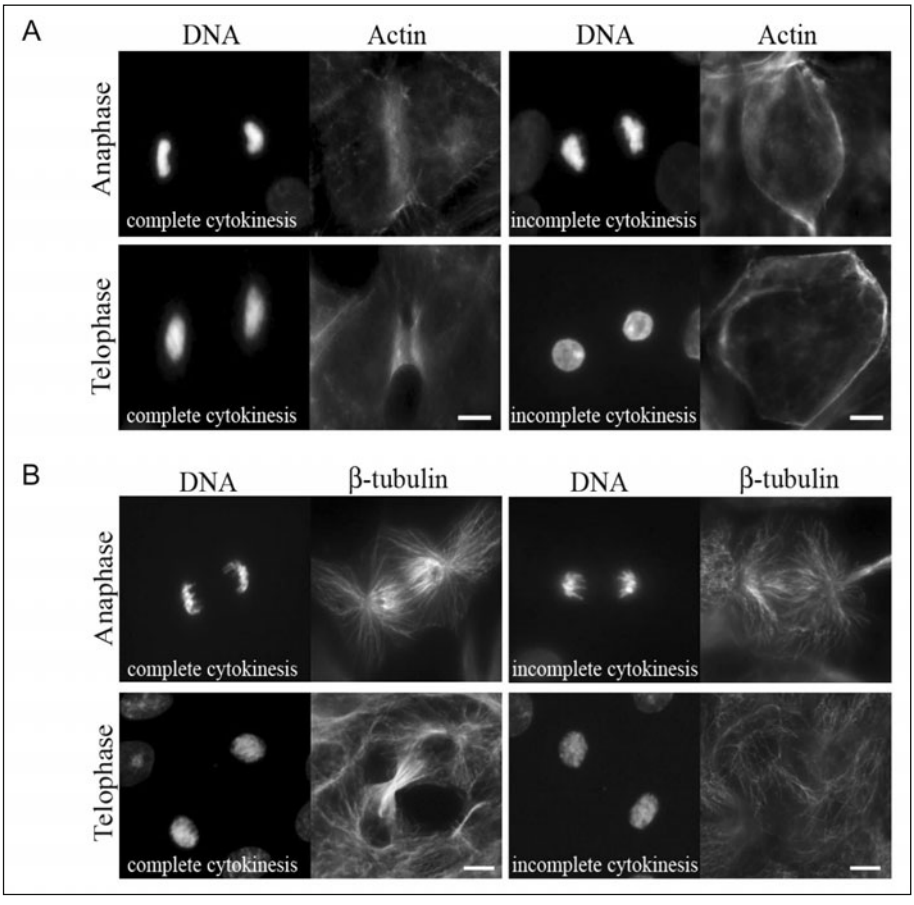

Figure 3. Physiological incomplete mode of cytokinesis in the liver. (Adapted from Margall-Ducos G et al. J Cell Sci 2007; 120:3633-3639)[37]. A) Actin cytoskeleton rearrangement doesn't occur during an incomplete cytokinesis process. When hepatocytes complete cytokinesis, the presence of an actin belt parallel to the cleavage plane is observed in anaphase with its ingression during telophase (left panels). By contrast, during incomplete cytokinesis, the actin belt is always absent, actin cytoskeleton not being able to reorganize at the cleavage plane (right panels). Consequently, during telophase, there is no ingression. Hepatocytes are stained with Alexa Fluor488 phalloidin (actin) and nuclei with Hoechst (DNA). Scale bars represent 5 μm. B) Organization of the microtubules network during incomplete cytokinesis. Staining for b tubulin reveals that the microtubules network is correctly organized in anaphase when hepatocytes complete cytokinesis, microtubules being compressed in the midzone during telophase as consequence of furrow ingression (left panels). When hepatocytes do not complete cytokinesis, the cells present disrupted microtubules network due to the absence of anchorage to the equatorial cortex (right panels). Scale bars represent 5 μm. Reproduced with permission of the Company of Biologists.

present a cell shape characteristic of cleavage furrow ingression.[58] In fed rats, although some late telophase hepatocytes are engaged in a normal cytokinesis process (60%), others present a round shape, indicating an absence of ingression (40%). Following living cells divisions after weaning, the authors established that anaphase cell elongation, a crucial step in the cytokinesis process, is clearly impaired in hepatocytes presenting an incomplete cytokinesis. In fact, the actin cytoskeleton is not reorganized to the cleavage plane during anaphase-telophase transition (Fig. 3A). Moreover, during an incomplete cytokinesis process there is an absence of astral microtubules anchorage to the equatorial cortex inducing a total destabilization of microtubules network (Fig. 3B). Signals transmitted by astral microtubules are not delivered to the equatorial cortex. In this condition, the RhoA pathway, the orchestrator of cytokinesis, is not activated. These findings reveal a new developmental cell division program in the liver which prevents cleavage-plane specification leading to the genesis of binucleated hepatocytes.

In contrast to a transformed cell that does not complete cytokinesis,[59] a binucleated 2 × 2n hepatocyte is able to proceed through a new cell cycle.[26] This cell progresses through the S phase (Fig. 4A) and during mitosis cytokinesis may or may not be completed, leading to the genesis of two

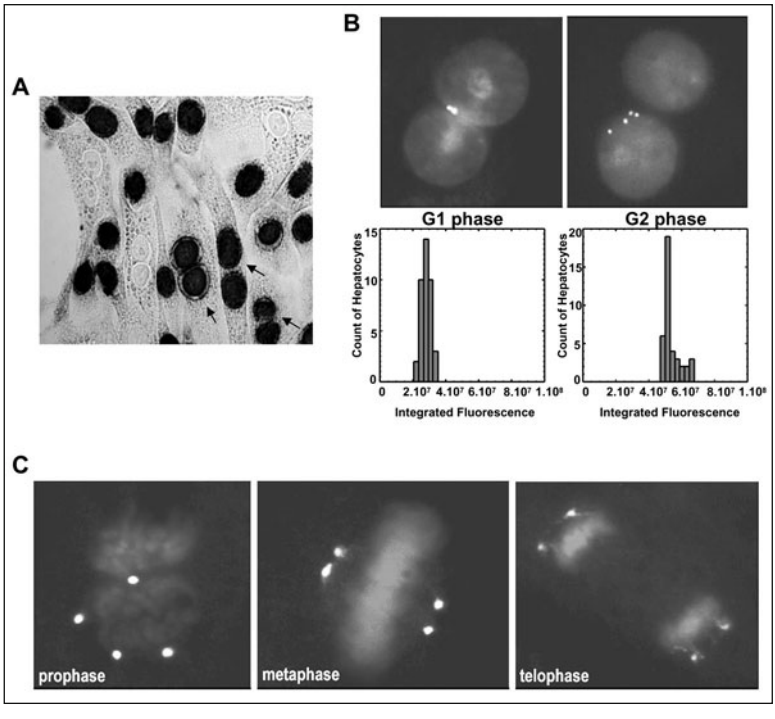

Figure 4. Centrosomes traffic during the division of binucleated 2 × 2n hepatocytes. (Adapted from Guidotti JE et al. J Biol Chem 2003 23; 278(21):19095-101)[26]. A) Binucleated hepatocytes progress through the S phase. Analysis of BrdU incorporation on primary cultures of hepatocytes reveals that DNA replication occurs in binucleated as well as mononucleated hepatocytes. B) A binucleated hepatocyte duplicates its centrosomes. A binucleated hepatocyte displays two centrosomes during G1 phase (γ-tubulin labelling) and four centrosomes during G2 phase. DNA content is evaluated by recording Hoechst fluorescence in each nucleus. The peak DNA content of hepatocyte (2 × 4n) with four centrosomes is positioned at twice the value of one of the hepatocyte (2 × 2n) with two centrosomes. C) Specific migration of centrosomes during mitosis of binucleated hepatocytes. During prophase, the four centrosomes move apart and at metaphase cluster in pairs at opposite poles of the cell. At telophase, centrosome clustering is maintained. Reproduced with permission of the Company of Biologists.

mononucleated 4n hepatocytes or one binucleated 2 × 4n hepatocyte (Fig. 2). Note that animal cells face a specific problem when they become tetraploid: they acquire an extra centrosome that could potentially compromise the assembly of a bipolar spindle during metaphase, contributing to the accuracy of chromosome segregation. Hyperamplification of a centrosome has been observed in many tumor tissues and cell lines and is linked with both aneuploidy and tumorigenesis.[60-65] In order to prevent proliferation or survival of tetraploids cells, the cell has evolved several mechanisms: G1 cell cycle arrest, apoptosis, adaptive silencing of extra centrosomes and specific clustering of these extra centrosomes.[59] Interestingly, binucleated hepatocytes during the S phase correctly duplicate their centrosomes (Fig. 4B). During mitosis these cells formed a unique bipolar spindle, leading to the alignment of all chromosomes on one metaphase plate. This event is driven by a specific clustering of supernumerary centrosomes, two by two at the cellular pole (Fig. 4C). This centrosome clustering is essential to give rise solely to viable polyploid progeny and prevent the genesis of aneuploid cells.[59] In conclusion, the hepatocyte constitutes a particularly interesting model of a ploidy process leading first to binucleated 2 × 2n cells, which then evolve into mononucleated 4n or binucleated 2 × 4n cell. Furthermore, it is quiet fascinating that hepatocytes can adapt to the presence of extra centrosomes. Understanding the mechanism that controls centrosome clustering would be important for cancer biology in order to prevent aberrant mitosis.

Polyploidy in Regenerating Liver and During Pathological States

The adult liver retains a high proliferative capacity. It responds to tissue injuries such as partial hepatectomy, toxin and drug-induced liver disease as well as the administration of a specific growth factor by priming quiescent hepatocytes.[66] During liver regeneration after partial hepatectomy, quiescent hepatocytes undergo one or two rounds of replication to restore the liver mass by a process of compensatory hyperplasia. Many studies have shown that during this process hepatic polyploidy is modified.[6,9,14,20,29] Regenerative liver growth differs markedly from developmental liver growth with the most striking difference being the rapid disappearance of binucleated hepatocytes (Fig. 5). The hepatocellular growth pattern is thus switched to a nonbinucleating mode of growth; nuclei with diploid or tetraploid DNA is converted to tetraploid and octoploid ones respectively.[14] Interestingly, the diploid hepatocytes seem to have a higher tendency that the polyploid ones to undergo several rounds of division.[14] Moreover, after partial hepatectomy, polyploid hepatocytes exhibit senescence-type changes with increased lipofuscin accumulation, β-galactosidase activity (a marker of cell senescence[67]) and accumulation of p21.[14] Different studies have also shown, in adult rodents, that induction of DNA synthesis by different chemicals is associated with disparate changes in liver ploidy and nuclearity profile. Thus, liver growth induced by the mitogen lead nitrate, unlike liver regeneration induced by PH, is associated with cellular polyploidy mainly resulting from an increase in binuclearity.[68] By contrast, an increase in mononucleated octoploid hepatocytes has been described following administration of hepatic mitogens as 1,4-dichlorobenzene[24] and sodium phenobarbitone (PB)[69] and peroxisome proliferators as WY-14 643 and methylclofenapate.[70] All these results suggest that different chemicals may selectively induce DNA synthesis in hepatocytes of one particular ploidy or nuclearity class.

Hepatic polyploidy can also be modified by metabolic overload that induces liver lesions (Fig. 5). It has been reported that Long-Evans Cinnamon (LEC) rats spontaneously develop a necrotizing hepatic injury.[71,72] These rats are deficient in the P-type copper ATPase gene (atp7b), the gene responsible for human Wilson's disease (WD). LEC rats accumulate excess copper in the liver but have decreased levels of serum ceruloplasmin activities, a clinical presentation similar to human WD.[73] Moreover, LEC rats also accumulate as much iron as copper.[74] In this animal's model, hepatocytes present large polyploid nuclei and a delay in mitotic progression has been also observed.[75,76] Similarly, in normal mice the injection of iron-dextran induces liver polyploidization; this effect is inhibited by the oral intake of iron chelator.[77] Oxidative damage to the liver is also associated with a pronounced increase in the population of polyploid hepatocytes. Gorla and collaborators demonstrated that subsequent to radiation, hepatocytes exhibit evidence for oxidative injury with the deletion of intracellular antioxidants (as glutathione and catalase) and for an

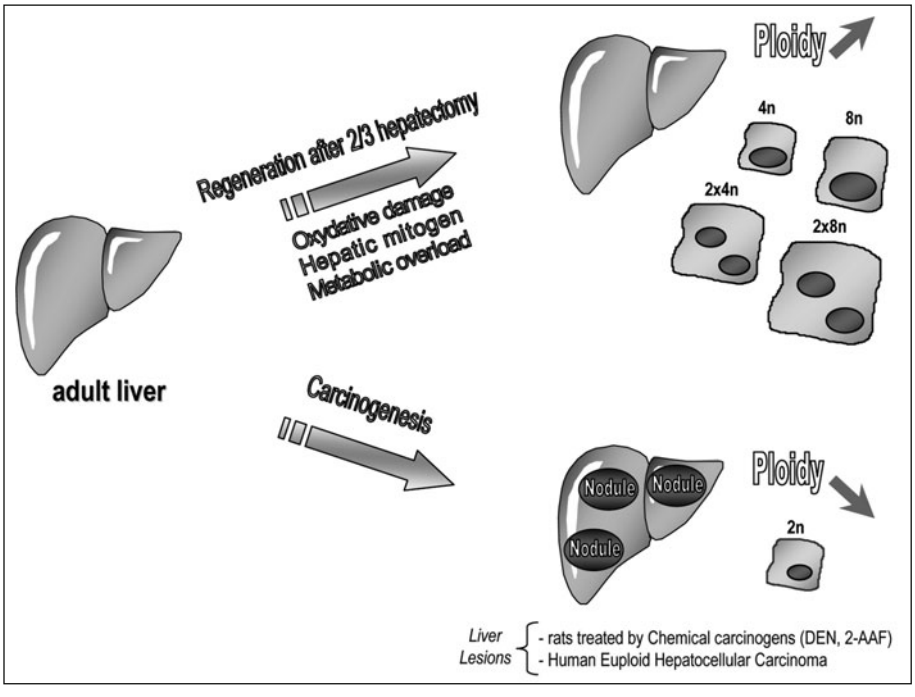

Figure 5. Ploidy modification during regenerative and pathological proliferation. In adults, liver polyploidization is regulated differently upon loss of liver mass and liver damage. Liver regeneration induced by partial hepatectomy leads to the disappearance of binucleated hepatocytes and the formation of mononucleated tetraploid and octoploid hepatocytes. Induction of DNA synthesis by chemicals, oxidative damage or metabolic overload is selectively associated with a pronounced increase in the population of polyploid hepatocytes of one particular ploidy or nuclearity class. Liver lesions induced in the rat by chemical carcinogens (diethylnitrosamine (DEN) and 2-acetyl-aminofluorene (2-AAF)) lead to an overall reduction in liver ploidy and an expansion of the diploid cell population. Increases in diploid mononucleated hepatocytes have been reported in human euploid hepatocellular carcinoma (HCC).

increase of polyploidy.[78,79] In the same line, another study demonstrated that the overexpression of antioxidant enzymes (glutathione peroxidase, Cu, Zn-superoxide dismutase) in transgenic mice decreases hepatocyte ploidy during liver regeneration.[80] All these results argue with the fact that an extensive correlation exists between the generation of polyploid hepatocytes and a variety of cellular stress as it has been demonstrated in other tissues.[59]

Finally and in contrast of what we described above, hepatocellular carcinoma has a lower polyploid fraction compared to an age-matched normal liver(Fig. 5).[81] Thus, liver lesions induced in the rat by chemical carcinogens (diethylnitrosamine (DEN) and 2-acetyl-aminofluorene (2-AAF)) lead to an overall reduction in liver ploidy and an expansion of the diploid cell population which prevails at the different stages of hepatocyte transformation: foci, nodule and hepatocarcinoma.[15,82-86] Studies in humans have also shown a shift towards diploid cell growth during hepatocarcinogenesis. An increase in diploid mononucleated hepatocytes with a decrease in polyploid hepatocytes (including binucleated fraction) have been reported in human euploid hepatocellular carcinoma (HCC).[87,88] Diploid cells are also detected in macronodules (dysplasia, high grade) in cirrhotic livers, suggesting an early shift to diploid cell expansion during hepatocarcinogenesis.[89] Therefore, the selective proliferation of mononucleated 2n hepatocytes could be one of the early events of the liver transformation process. Since a diploid genome would be less protected against recessive

mutations than a polyploid genome, a predominance of diploid hepatocytes may predispose to further progression of the lesions toward increasing malignancy.[84]

Conclusion

The onset of polyploidy in the liver has been described for quite some time. Polyploidization is emerging as an important restriction mechanism for hepatocellular growth. However, the biological significance of this original physiological process remains unclear. Presently, little is known about the function and fate of polyploid hepatocytes. Different hypotheses have been put forward: (i) it may protect cells from genotoxic damage by increasing their gene copy number; (ii) it allows the liver to adapt to aging-related cellular loss and still preserve function; and (iii) it may affect the expression profile of specific genes. Further work concerning this fascinating process will offer insights into hepatic pathophysiology.

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Chapter 9

Analysis of Cellular DNA Content by Flow and Laser Scanning Cytometry

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Abstract

This chapter covers several aspects of methodology of DNA content analysis in individual cells that is most commonly used for assessment of DNA ploidy and for enumeration of cells in particular phases of the cell cycle. Briefly presented are general principles of instrumentation and cell analysis by flow- and laser scanning- cytometry. Described are major methods designed to stain DNA with fluorochromes in live cells, in detergent-permeabilized cells, in cells fixed prior to DNA staining as well as in nuclei of cells isolated from paraffin-embedded tissues. Briefly addressed are approaches to estimate cellular DNA content in conjunction with cellular immunophenotype. Discussed are factors that affect accuracy of DNA content measurement such as: (i) differences in chromatin structure of the analyzed cells that restrict DNA accessibility to fluorochromes, (ii) stoichiometry of interaction between fluorochromes and DNA in chromatin and (iii) chemical mass action law defining dependency of fluorochrome binding to DNA in relation to fluorochrome concentration and number of potential binding sites in a sample. Described also are controls used to ensure accuracy of DNA ploidy determination, the principles in ploidy assessment and possible pitfalls in analysis.

Introduction

DNA content is the most frequently measured entity of the cell. Analysis of DNA content reveals cell ploidy, provides information on cell position in the cell cycle and also allows one to estimate frequency of apoptotic cells that are characterized by fractional DNA content. Distribution of cells within the major phases of the cell cycle is based on differences in DNA content between the cells in prereplicative phase ($G_{0/1}$) versus the cells that actually replicate DNA (S phase) versus the postreplicative plus mitotic (G_2 + M) phase cells (Fig. 1). It is generally accepted that DNA content measured by cytometry (DNA ploidy) is defined as DNA index (DI) and for normal (non tumor, euploid) cells in $G_{0/1}$ phase of the cell cycle DI = 1.0. Cells in G_2/M phase of the cell cycle have DI = 2.0 and the S-phase normal cells are characterized by 1.0 < DI < 2.0. Because extensive DNA fragmentation preferential to internucleosomal DNA sections takes place during apoptosis, the low molecular (mono- and oligo- nucleosomal) DNA fragments are extracted during cell preparation for staining and such apoptotic cells can be identified as the cells with fractional DNA content (DI < 1.0). They are often being defined as "sub-G_1" or "sub-diploid" cell population (Fig. 2).[1-4]

Flow- or laser scanning- cytometry (LSC) are the methodologies of choice for cellular DNA content analysis. They provide the means to estimate DNA content in individual cells in large cell populations rapidly and accurately (Figs. 3,4). Historical progression of development of cytometric

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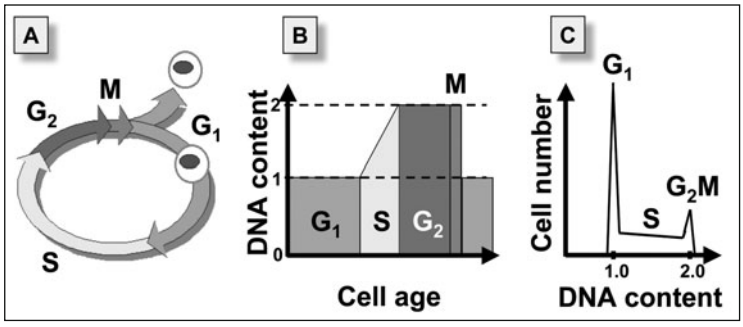

Figure 1. Schematic representation of cellular DNA content changes during cell cycle progression. DNA replication during cell cycle is discontinuous, occurring exclusively during S phase (A), which results that the postreplicative G_2-phase cell has twice higher cellular DNA content compared to the G_1 cell (B). After completion of mitosis (M) the cell divides (undergoes cytokinesis) generating two daughter cells (G_1) each having half DNA content of the mother cell (M). Based on differences in DNA content therefore one can distinguish G_1 from S from G_2M cells. When DNA content is measured in a population consisting of a large number of cells the data can be shown in a form of the cellular DNA content frequency histogram with a characteristic G_1- and G_2M- phase peaks at DNA content DI = 1.0 and DI = 2.0, respectively and S-phase cells are distributed in between the peaks (C).

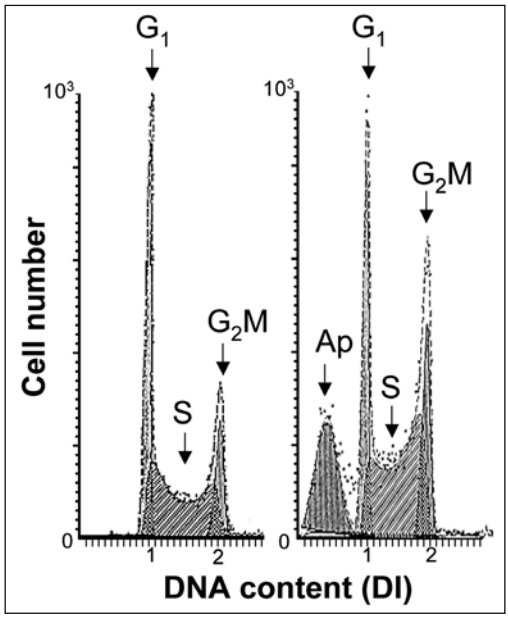

Figure 2. Location of apoptotic cells (Ap) on DNA content histograms. The presence of apoptotic cells manifests as the "sub-G_1" ("sub-diploid") peak on DNA content histograms. This is due to the fact that activation of endonucleases during apoptosis leads to DNA cleavage preferentially at internucleosomal ("linker") sections[1] and the fragmented mono- and oligo-nucleosomal DNA is extracted from cells during their processing and staining.[2,3] It should be noted that apoptotic cells not always have so distinctly lower DNA content and can be well identified. In some instances, particularly if cells in G_2M- and/or S-phase undergo apoptosis, DNA content of apoptotic cells may overlap with that of G_1 cells.[5]

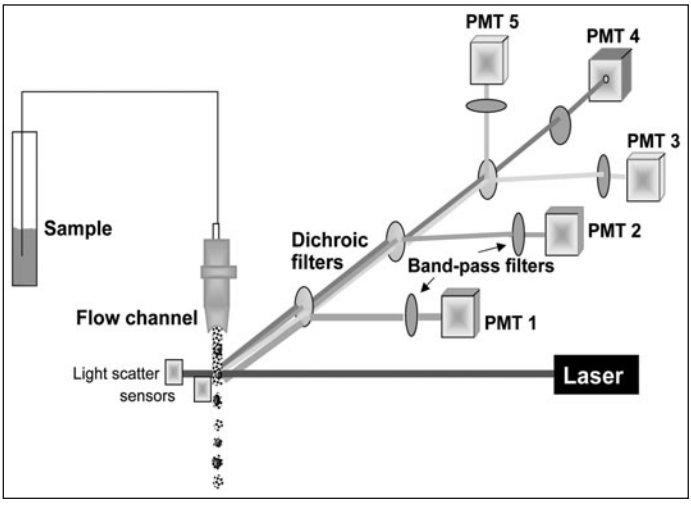

Figure 3. Schematic representation of flow cytometry. Suspension of fluorochrome-stained cells is transported through the cytometer fluidic system in which the individual cells transect the path of laser's beam. Their emission is collected by set of dichroic optical filters which reflect light at a specific wavelength towards the photomultipliers (PMTs) and transmit light at longer wavelength. The band-pass filters located in front of PMTs allow light to pass only at a specific, relatively narrow wavelength range. Intensity of fluorescence emission at these wavelength ranges, integrated over whole cell, is measured by individual PMTs. The light scatter signal generated by the cell when it passes through the laser beam is additionally measured, often at forward and 90° angle ("side scatter"), by separate sensors. The scatter signals provide information about cell size and some morphological features. More than 1,000 cells can be measured per second with an accuracy of fluorescence measurement approaching 1% and sensitivity approaching 200 molecules of fluorescein/cell. Many models of flow cytometers have not one but two or three lasers as excitation source, emitting at UV, blue, green and/or red wavelength. This allows one to select a desired fluorochrome from variety of the available ones. A color version of this image is available at www.landesbioscience.com/curie.

methods and their application for cell cycle and DNA ploidy analysis have been recently reviewed.[5] These methods rely on cells being labeled with a fluorochrome that is expected to stain DNA stoichiometrically and thus to accurately report DNA content. The intensity of DNA-associated fluorescence integrated over the individual cell or cellular nucleus is measured by photomultipliers. The latter offer a wide dynamic range of detection and measurement of fluorescence intensity, much wider compared with the alternative approach of fluorescence measurement, namely the fluorescence image analysis (FIA). A large number of DNA fluorochromes can be used for DNA content analysis and a great variety of techniques have been published during the past three decades.[5] The techniques differ primarily by the mode of cell permeabilization (detergent versus prefixation), choice of the DNA-specific fluorochrome, composition of the stain solution and applicability to different cell preparations.

The results of cellular DNA content measurements are generally presented in the form of frequency histograms (Figs. 2,5). Discrimination of cells in particular phases of the cell cycle based on differences in their DNA content (deconvolution of the histograms) is helped by computer analysis. The software used for this purpose allows one to estimate the percentage of cells in major phases of the cell cycle (G_1, versus S versus G_2/M) as well as the frequency of apoptotic cells with fractional ("sub-G_1") DNA content.[6,7] This software is often included with the purchase of the flow cytometer but is also commercially available (Phoenix Flow Systems, San Diego, CA; Verity Software, Topsham, ME).

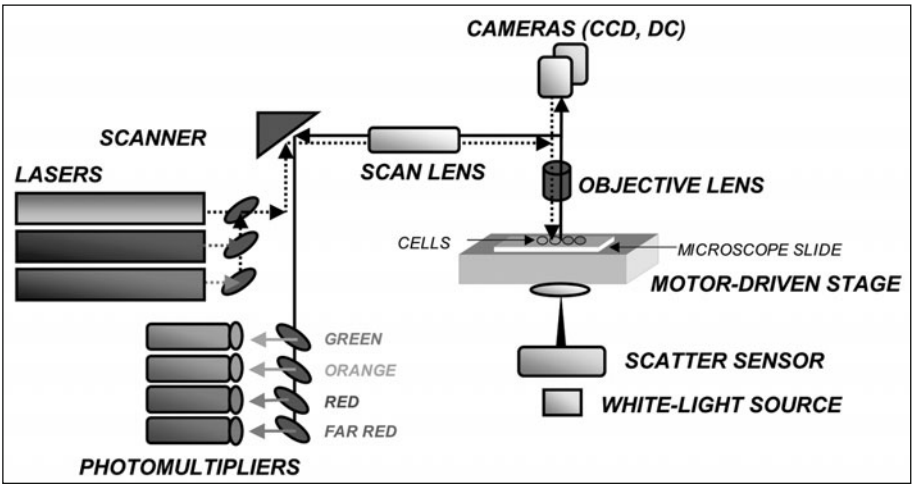

Figure 4. Schematic representation of the laser scanning cytometer (LSC). The microscope is the key part of LSC and it provides structural and optical components. The emission beams from lasers are directed onto computer controlled oscillating mirror, which reflects them through the epi-illumination port of the microscope and images through the objective lens onto the slide. The mirror oscillations cause the laser beam to sweep the area of microscope slide under the lens. The slide is located on the computer-controlled motorized microscope stage which moves perpendicular to the laser beam scan at 0.5 μm steps per each scan. The cell-emitted fluorescence is collected by the objective lens and directed to the scanning mirror. Upon reflection it passes through a series of dichroic mirrors and emission filters to reach one of the PMTs, which records the fluorescence intensity at a specific wavelength range. Laser light scattered by the cell is imaged by the condenser lens and its intensity is recorded by sensors. A white-light source provides transmitted illumination to visualize the objects though an eyepiece or cameras. Up to 100 cells can be analyzed per second with accuracy and sensitivity comparable to that of flow cytometry (Fig. 3). A color version of the figure is available at www.landesbioscience.com/curie.

Supravital Cell Staining

Cellular DNA content can be fluorochrome-stained either in unfixed, usually still live cells, or in the cells following their fixation. Staining of live cells (supravital staining) requires use of a fluorochrome that penetrates the plasma membrane and stoichiometrically stains DNA. Unfortunately, the choice of such fluorochromes is limited. Hoechst 33342 is one of such dyes and when used in combination with the membrane potential sensing dye DiOC5(3) offers relatively good resolution in measurement DNA content of live cells.[8] The dye is excited at UV wavelength (350 nm) and fluoresces in blue (460 nm). Inclusion of DiOC5(3) serves to suppress efflux of Hoechst 33342 from the cell by the active P-glycoprotein pump which otherwise breaks up equilibrium of the binding/staining reaction. Similar effect can be achieved by using the efflux blocker such as verapamil.[9]Another fluorochrome that is being used to supravitally stain DNA is DRAQ5.[10] Its emission can be detected in far-red wavelength (maximal at 670 nm) while the excitation (maximal at 640 nm) is at wide range of the spectrum, stretching down to 488 nm.

The protocols designed to supravitally stain DNA are simple. Generally, inclusion of the fluorochrome into the culture medium for 30-60 min during cell culture is followed by subjecting cells to cytometric analysis without a need for rinsing or centrifugation. However there are cell type (cell line) differences in the rate of the fluorochrome uptake. Occasionally, therefore, several concentrations of the fluorochrome and various time of incubation, different than in the protocols e.g., provided by the vendors of the reagents, have to be tested to optimize staining conditions for

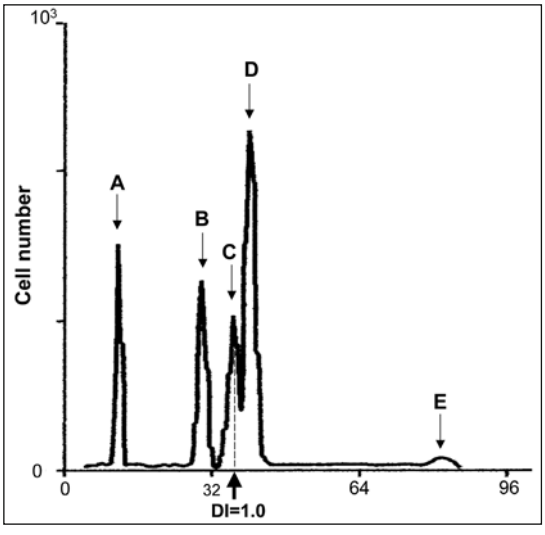

Figure 5. DNA content analysis of human breast cancer biopsy specimen according to the protocol developed by Vindelov et al.[13,14] Cellular DNA content was measured in a sample obtained from a fine needle aspirate of a surgical biopsy of human breast cancer and stained with PI. Chicken erythrocytes and trout erythrocytes were included as internal standard. The peaks from left to right represent chicken (A) and trout (B) erythrocytes, diploid normal nuclei (C; DI = 1.0), hyperdiploid (DI > 1.0) population of tumor $G_{0/1}$ phase cells (D) and G_2 population of tumor cells (E). Under proper conditions of DNA staining, the ratio of the mean DNA content of diploid human cells to chicken erythrocytes is 2.857, the ratio to trout erythrocytes is 1.258 and the ratio of mean DNA content in trout vs chicken erythrocytes is 2.28.[13] Another landmark of linearity in DNA content analysis is the ratio of G_2 to G_1 peaks, which is expected to be 2.0. Modified after Vindelov and Christensen.[13]

a particular cell type. Furthermore, the resolution of DNA content analysis in cells supravitally stained is never as good as that of fixed or detergent-permeabilized cells. The application of protocols designed to supravitally stain DNA is primarily for cell sorting, where the cells selected based on differences in their DNA content can be further subcultured for the purpose of analyzing their growth characteristics, sensitivity to drugs, cloning or expanding their number. It should be noted, however, that exposure of Hoechst 33342-stained cells to UV light during sorting may damage their DNA and be cytotoxic.

DNA Staining after Disruption of Plasma Membrane

Treatment of live cells with detergents causes rupture of the plasma membrane or leads to nuclear isolation which makes DNA accessible to fluorochromes. This approach has been initially used to permeabilize cells to acridine orange, the metachromatic dye that differentially stains DNA and RNA.[11] Exposure of cells to hypotonic salt solution also leads to their lysis and DNA within the nuclei isolated this way is accessible and can be stained with a variety of fluorochromes.[12] Further improvement in the accuracy of DNA content analysis is obtained after controlled proteolysis of detergent-lysed cells. This approach was perfected by Vindeløv and his collaborators who developed a highly accurate method of cellular DNA content measurement, particularly useful for analysis of DNA ploidy in human tumor samples.[13,14] These authors also introduced internal DNA content standards such as nuclei of chicken- and/trout- erythocytes, as intrinsic part of the staining protocol. Their methodology designed for needle biopsy of normal and tumor tissue

is now used worldwide. Figure 5 illustrates DNA content measurement of the specimen of the fine-needle aspirate of human breast cancer specimen by this method.[14]

The accuracy of DNA content measurement for DNA ploidy or cell cycle phase estimate is much greater when isolated nuclei rather than whole cells are analyzed. This is due to the fact that some cytoplasmic constituents may be auto-fluorescent, or contain DNA (e.g., in mitochondria) or nonspecifically stain with DNA-fluorochromes. This background cytoplasmic stainability, thus, lowers accuracy of nuclear DNA determination. Furthermore, the proteolytic step in the Vindeløw's procedure removes some nuclear proteins that are known to restrict the accessibility of DNA to fluorochromes,[15] which additionally leads to improved stoichiometry of DNA staining.[15]

It should be noted, however, that the lysis of plasma membrane of mitotic cells lacking nuclear envelope, that occurs in the detergent or hypotonic-treatment based methods, leads to dispersion of individual chromosomes or chromosome aggregates which are then suspended free in the solution. These methods therefore may not detect mitotic cells, particularly when the cell suspensions are mechanically agitated, pipetted or vortexed. Furthermore, individual or aggregated chromosomes may be erroneously identified as apoptotic cells with fractional DNA content ("sub-G_1" cells). In addition, lysis of apoptotic cells that have fragmented nuclei releases several nuclear chromatin fragments from a single cell. Because each fragment is classified in the cytometer as individual event ("cell") the frequency of "sub-G_1" objects after cell lysis may be much higher than actual frequency of apoptotic cells in a given cell population. This generally precludes application of cell-lysis based methods for analysis of the frequency of apoptotic cells, particularly when mitotic cells are in large proportion (e.g., after arrest in the cell cycle by the mitotic poisons).[16]

DNA Staining in Fixed Cells

Preference for analysis of fixed cells as opposed to the detergent-permeabilized cells is dictated by the need to store or transport samples, for example clinical specimens that cannot be immediately processed. Their storage, unless done at low temperature in cryopreservative media, leads to cell deterioration (autolysis). Fixed cells on the other hand can be stored for months or even years. For analysis of DNA content precipitating fixatives (alcohols, acetone) are preferred over cross-linking agents (e.g., formaldehyde, glutaraldehyde). This is because cross-linking of chromatin constituents impairs stoichiometry of DNA staining with intercalating fluorochromes and thus decreases accuracy of DNA content measurement.[15] It should be noted, however, that highly fragmented DNA such as present in apoptotic cells leaks out from the ethanol-fixed cells during their hydration and staining, but is preserved and remains within the cell upon fixation by formaldehyde. Fixation in ethanol, therefore, rather than in formaldehyde, has to be used to detect apoptotic ("sub-G_1") apoptotic cells. While absolute alcohols or acetone, or a mixture of absolute ethanol and acetone can serve as fixatives (in some instances they may be preferred e.g., for immunocytochemical detection of some antigens concurrently with DNA content) they induce more extensive cell aggregation compared e.g., with 70% or 80% ethanol, which is most commonly used when analysis is limited to DNA content alone.

A variety of DNA fluorochromes may be used to stain DNA in the fixed cells. The most commonly used are 4′,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI) and 7-amino-actinomycin D (7-AAD). Staining with dyes that react with both DNA and RNA, such as PI requires incubation with RNase. The cells may be pre-incubated with RNase and subsequently stained with PI, or RNase (usually at concentration within a range between 10 and 100 µg/ml) is included into a solution containing PI in PBS. In the latter case the cells suspended in that solution are maintained for about 30 min or longer at 37°C or room temperature to allow RNase to digest RNA, before measurement by cytometry. It is of importance that the RNase used is free of DNase activity. If such is not available, one may heat the solution of RNase at 95-100°C for 5 min to destroy DNase- while still preserving RNase- activity. PI is excited in blue light, which is conveniently provided by the 488-nm line of the argon ion laser available on most flow cytometers, while DAPI requires UV or near UV excitation.

Analysis of DNA in Paraffin-Embedded Samples

The method of isolating cell nuclei from paraffin-embedded tissues was developed by Hedley and his colleagues to retrieve archival samples for flow cytometric analysis.[17,18] This methodology enables for retrospective studies to determine the prognostic significance of DNA ploidy or cell cycle distribution (usually frequency of S-phase cells) in tumor progression. The method can also be applied for prospective studies when fresh material is unavailable. One advantage of this methodology is that it offers a possibility to examine by microscopy the tissue sections and thus select the adjacent tumor area of interest to be processed by flow cytometry. The paraffin blocks can then be trimmed to exclude areas of noninvolved tissue in order to diminish the proportion of stromal cells, or of necrotic and hemorrhagic areas to decrease the quantity of debris, as well as to select areas of noninvolved tissue to be used as internal DNA content standard (see further). The accuracy of DNA content analysis of nuclei from paraffin blocks is generally inferior compared to the methods that rely on either ethanol fixation or detergent or hypotonic treatment of fresh tissues. This is due to the fact that the cells embedded in paraffin frequently are usually prefixed in formaldehyde. As mentioned, by cross-linking DNA and proteins formaldehyde fixation impairs stoichiometry of DNA. Because crosslinking by formaldehyde is to some extent reversible, long incubation of the rehydrated nuclei in aqueous solutions, after their isolation from the paraffin blocks, improves resolution of DNA analysis. In nuclei isolated from paraffin blocks DAPI is the preferable fluorochrome since it the least affected, in terms of stoichiometry of DNA staining, by the chromatin structure and thus by protein-DNA crosslinking.[15]

Another factor that lowers accuracy of DNA content analysis and thus identification of aneuploid cells or discrimination of cells in different phases of the cycle in samples of nuclei isolated from paraffin blocks is the presence of debris. Most debris is due to the presence of transected nuclei with incomplete DNA content. Because probability of transecting a nucleus is proportional to thickness of the section and to nuclear size, preparation of thicker sections (≥50 nm) for nuclei isolation is advisable, particularly for tumors with large nuclei such as tetraploid and larger stemlines.

Concurrent Analysis of Cell Surface Antigen and DNA Content

It is often desirable to know the DNA content distribution (histogram) of the particular cell subpopulation identified by its surface immunophenotype. The most common approach, in such a case, is to perform standard immunocytochemical labeling of live cells with the fluorochrome- (most frequently FITC or Alexa Fluor 488) conjugated Ab, which is then followed by short fixing the cells in 0.5-1.0% methanol-free formaldehyde ("paraformaldehyde") in PBS. Because formaldehyde fixation does not adequetely permeabilize the cells it is critical to subsequently have detergent (e.g., Triton X-100) in the staining solution to make DNA accessible to DNA-fluorochrome such as PI or DAPI. Post-fixation in alcohol (methanol or ethanol) following formaldehyde also permeabilizes cells. A gentle fixation with formaldehyde (0.25%) followed by permeabilization in Tween 20 detergent is another procedure designed to preserve both external and internal antigens that can be detected immunocytochemically concurrently with analysis of DNA content.[19] Cellular green (FITC or Alexa Fluor 488) and red (PI) or blue (DAPI) fluorescence is then measured by flow cytometry. During analysis, the cell subpopulation of interest is gated based on its immunophenotype (green fluorescence) and DNA content of this selected subpopulation is then plotted in form of a frequency histogram It is also possible to to combine analysis of DNA content with both cell surface phenotype markers and telomere length.[20]

A simpler approach to concurrently measure DNA content and cell surface immunofluorescence is to combine the supravital staining of DNA with Hoechst 33342 with surface immunophenotyping.[21] For this combination, however, the cytometer with the two- or more- lasers, including one emitting UV light is required. Furthermore, as mentioned, in some cell types it is difficult to obtain high resolution of DNA content analysis after supravital staining of DNA either with Hoechst 33342 or DRAQ5.

Accuracy of DNA Content Measurement

The accuracy of DNA content measurement is reflected by variation in fluorescence intensity between individual cells with identical DNA content, such as G_0/G_1 cells. This variation is being assessed by the value of coefficient of variation (CV) of the mean value of DNA content of the G_0/G_1 cell population. The CV of the DNA-associated mean fluorescence of G_1 cells is thus considered an index of the accuracy of the DNA measurements. High accuracy is required in particular in assessing DNA ploidy to distinguish between DNA diploid and aneuploid cells, which may differ minimally in DNA content. Accurate DNA content measurement is also critical in analysis of cell cycle distributions. There is no formal consensus regarding the acceptable maximal CV value of the mean DNA content of the G_0/G_1 cell population i.e., the acceptable error in cellular DNA content estimate. Most researchers, however, would consider the accuracy to be poor and results unacceptable if CV values of normal, nontumor cells exceed 6%, optimal resolution is achieved when CV is <3%. An exception is analysis of the DNA content of cell nuclei isolated from paraffin blocks, where by the nature of the sample (formaldehyde fixation) good accuracy is difficult, to achieve.

A number of factors can contribute to poor accuracy in DNA content analysis. Most common is inappropriate sample flow and optical adjustment of the flow cytometer. Proper maintenance of the instrument and its careful adjustment prior to analysis, e.g., using fluorescent calibrated standard beads, to maximize the electronic signal intensity and minimize variability of the measurement of the beads, are required to achieve accurate DNA measurements. Problems in sample preparation, either resulting in mechanical damage to the cells or involving incorrect composition of buffers and staining solutions, are another reason of poor resolution in DNA analysis. An excessively large number of cells (DNA) in the sample which leads to significant depletion of the free, unbound fluorochrome in the solution and alters the staining equilibrium (see below), may be still another source of the problems that prevent accurate DNA content analysis. Adjusting samples to achieve a proper fluorochrome to DNA content (cell number) ratio improves the results.

It should be noted that despite good accuracy of DNA content measurements (in terms of proper instrument adjustments and sample staining) the CV of G_1 cell populations may still remains high. This may occur when significant numbers of dead or dying cells are present in the sample, or when the cells were treated with DNA-interacting drugs. Also, in tumors that are polyclonal or have developed drug resistance by gene amplification (e.g., presence of minute chromosomes) the G_0/G_1cell populations may have intrinsically variable DNA content and therefore high CV values of the G_0/G_1cell populations.

Accessibility of DNA in Chromatin to Fluorochromes

The accessibility of DNA to fluorochromes is restricted by chromosomal proteins, predominantly by histones and varies between different cell types. The maximal restriction is seen in cells undergoing terminal differentiation such as during spermatogenesis or erythropoiesis, when DNA stainability (per unit of DNA) is significantly lower compared with other cell types.[15,22] This obviously creates difficulties in assessment of DNA ploidy in such differentiating cells. The degree of reduction varies for individual fluorochromes and DAPI is the least influenced by chromatin structure whereas binding of 7-AAD, an intercalating but more bulky fluorochrome, is affected to a much larger degree. In practical terms, therefore, one may expect intercellular variation in DNA stainability when mixed cell types are measured in the same sample. This can be manifested on DNA content frequency histograms as the presence of pseudo-aneuploid populations, or widening of the G_1 peak (increased CV value). For example under certain conditions of staining monocytes show higher DNA stainability with PI compared to lymphocytes or granulocytes and form a typical pseudo-hyperdiploid peak on DNA frequency histograms. As mentioned, subjecting cells to the detergent methods and in particular the combination of detergent and proteolytic treatment such as in the Vindeløv's procedure,[13,14] increases accessibility of DNA and thereby improves the stoichiometric relationship between DNA content and fluorescence intensity.

There are several ways to estimate stoichiometry of DNA staining. Thus, fluorescence intensity of the cell populations represented by the G_2/M peaks on DNA histograms is expected to be DI = 2.0 as compared to DI = 1.0 for $G_{0/1}$ cells and deviation from this value indicates on problems in DNA quantification.[23] Normal hepatocytes grow at different DNA ploidy levels and therefore may also serve as markers of linearity in DNA measurement. Inclusion of internal standards such as chicken or trout erythrocytes provides still another marker of the stoichiometry of DNA measurement and is highly recommended when DNA ploidy is estimated.[13,14] To demonstrate stoichiometry of DNA staining one has to use linear and not exponential scale for plotting intensity of DNA-associated fluorescence (x-coordinate) and include the origin (point zero) of this coordinate, on the DNA content frequency histogram.

In some instances, however, stoichiometry in DNA staining with fluorochromes cannot be attained. This can be seen when cells were treated with antitumor drugs that modify DNA and/or chromatin structure. Intercalating drugs that interact with DNA fluorochromes by fluorescence resonance energy transfer (FRET), or drugs damaging DNA structure, or crosslinking chromatin, all can alter staining properties of in situ DNA, often in unpredictable ways. As mentioned, the possibility of stoichiometric measurement of DNA content may also be hampered when cells differing markedly in chromatin structure are being compared.

Fluorochrome Binding to DNA—Mass Action Law

Staining of cellular DNA is being done at equilibrium between the ligand (fluorochrome) and the ligand-binding sites in the DNA within the cells sample and thus it follows the chemical law of mass action. Stable level of staining is achieved when there is large excess of the ligand per binding site so a small variation in cell number per sample (binding sites) has no significant effect on the equilibrium. Because it is difficult to have an identical cell number in each sample, the variation is inevitable. One can calculate however approximate concentration of the fluorochrome and relate to the cell number to find out the range within which a decrease in free ligand concentration may not significantly affect DNA stainablility.There are 3×10^9 DNA base pairs per cell (diploid cell in G_1). Most intercalators such DNA-binding fluorochromes, reacting with free (naked) DNA at saturation bind every second base pair. Thus, potentially, in a single diploid cell there are ~1.5×10^9 binding sites. However, because a large portion of nuclear DNA within the cell is inaccessible to the intercalators,[15] only a fraction of the potential binding sites (10-70%, depending on the fluorochrome) can actually bind the ligand (fluorochrome). Thus, there are between 1.5 to 10.0×10^8 sites that actually bind the ligand in a single cell and therefore 1.5 to 10.0×10^{14} binding sites in 10^6 diploid cells, which is approximately a size of average sample subjected to staining. Assuming average MW of most DNA fluorochromes to be about 300, one can estimate (from Avogadro number) that at a concentration 100 μM (30 μg/ml) there are 6×10^{16} molecules of the ligand in 1 ml of the stain solution. Considering the above there is nearly 100-fold excess of the ligand per binding site when 10^6 cells are stained in 1 ml volume at 100 μM dye concentration. One would expect that under these conditions a change in cell number from 1 to 2 million (which alters a concentration of the free, unbound ligand by 1%) should not be reflected by greater than 1% change in stainability of DNA. However, at lower dye concentration (e.g., below 20 μM) or when cell number is drastically changed (e.g., from 1 to 5 million) the change in DNA stainability becomes noticeable. Needless to say, if cells have higher DNA content, (tetraploid, arrested in G_2/M) the equilibrium is shifted even more towards lesser concentration of free dye, which leads to further decrease in DNA stainability. The above estimates have to taken into an account when samples with different cell number are stained to compare DNA ploidy.

Assessment of DNA Ploidy

As mentioned, DNA content measurement by cytometry serves to estimate frequency of cells in particular phases ($G_{0/1}$ versus S versus G_2M) of the cell cycle as well as to assess DNA ploidy. In most situations DNA ploidy is being assessed in hematological or solid tumors; the evidence of aneuploidy by itself is a definitive marker of a presence of the tumor. Often is also considered to

be an prognostic indicator of tumor progression and outcome of the treatment. To assess DNA ploidy of the tumor sample one has to compare DNA content of the $G_{0/1}$ cells population of the presumed tumor cells with that of normal (control) cells. Towards this end most frequently the peak value of the integrated fluorescence (peak channel) of $G_{0/1}$ population of normal cells is being considered to be DI = 1.0 and DNA ploidy of the tumor cells is expressed as a ratio of the peak value (channel) of fluorescence intensity of these cells with respect to that of the normal $G_{0/1}$ cells. It is also common to express DI of the tumor as a ratio of modal rather than the peak value of fluorescence intensity representing DNA content of $G_{0/1}$ population tumor cells to modal value of $G_{0/1}$ population of normal cells. Some authors still prefer to use the mean values of fluorescence intensity of $G_{0/1}$ population rather than the peak or modal values to obtain this ratio. In essence, when DNA measurement is done correctly and accurately, either of these appropaches is expected to yield similar estimate of DI of aneuploid cells.

Normal lymphocytes, including lymphocytes from the same patient whose tumor is being analyzed, or fibroblasts, are often used as standard of DI = 1.0. For comparison with the tumor it is necessary to use normal cells both as external and internal control standards. When used as external control they have to be subjected to identical processing and staining procedure and measured by cytometry under identical laser and detector settings as tumor sample. The external control cells should be measured prior to- and also after- measurement of tumor sample. This double-measurement of control cells allows one to detect the possible shift in fluorescence readout e.g., due to misadjustment in instrument settings in the course of the sequential measurements. In addition to external control, normal cells should also be admixed (e.g., in 1:1 proportion) with the tumor sample cells and used then as internal control in another set of measurements. Often, normal stromal- or tumor infiltrating cells are already present in the tumor sample and they can be used as an internal control of DNA ploidy. In fact, when DNA ploidy is assessed based on measurement of nuclei isolated from paraffin blocks, the internal control provided by the presence of stromal and infiltrating normal cells that provide standard for DI = 1.0 is the only way to assess DNA ploidy of the tumor. This is due to the fact that DNA stainability after formaldehyde fixation and paraffin embedding is markedly altered making external standards useless.

Chicken and trout erythrocytes have been proposed as internal standards for analysis of DNA content by cytometry (Fig. 5).[13,14] Their use is helpful to control and maintain consistency of the staining and measurement procedures. However, one has to be cautious using them as absolute standard for DNA content analysis. Trout, like other fish species, are known to vary in their DNA ploidy level (most species are tetraploid) and it is therefore important to know ploidy of these cells when used as a standard.

Despite the difficulties and potential pitfalls outlined in this chapter, cytometry, including flow cytometry and LSC, is the methodology of choice in analysis of DNA content for DNA ploidy assessment. This methodology is complemented by fluorescence in situ hybridization (FISH) analysis, which provides a possibility to identify individual chromosomes or chromosome components contributing to aneuploidy. It should be noted that LSC, by offering rapid and semi-automatic enumeration of the fluorescent foci within individual nucleus in addition to DNA content measurement can also be used for FISH analysis.[26]

Conclusion

As outlined in this chapter variety of methods are available to estimate cellular DNA content by flow- or image assisted- cytometry. They offer wide choice of analytical capabilities to assess DNA ploidy and cell cycle distributions. The possibility of use of diverse fluorochromes differing in absorption and emission properties as well as in mode of binding to DNA provide the means to optimize DNA content measurement for different cell types. The principles of DNA staining and data analysis described in this chapter will be of assistance for the beginners who initiate DNA content analysis by cytometry. It may also provide useful information for advanced researchers who contemplate change in the methodology to further improve accuracy in assessment of DNA ploidy and cell cycle distribution.

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