Deep Insight Section

Mechanisms of chromosomal instability and carcinogenesis

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Summary

Most carcinomas present some form of genetic instability, either as a shortening or lengthening of PCR markers termed microsatellite instability or as a change in the relative intensity of paternal and maternal alleles termed chromosomal instability. Although chromosomal instability is found in the vast majority of carcinomas, its exact contribution to tumor formation has long been a matter of debate. The mutagenic effects associated with microsatellite instability might active known oncogenes, and therefore are an accepted cause of cellular transformation, but the mere numerical changes associated with chromosomal instability only seem to compromise cellular fitness. Whereas the mitotic origins of aneuploidy are now accepted, the role of spindle microtubules in chromosome breakage and translocations remains disputed. Nonetheless, a comparison of several proposed mechanisms of structural instability reveals a striking convergence towards a key role for merotelic kinetochore attachments, which is further corroborated by the pattern of copy number alterations in clinical tumors. The recent discovery of gene dosage effects and cancer stem cells hint at the deregulation of intertwined regulatory networks and a cellular response to reduced fitness as a possible carcinogenic mechanism related to chromosomal instability. These emerging paradigms of cancer however need to be validated by relevant experimental models.

Keywords

Chromosomal instability, kinetochore, merotelic attachment, aneuploidy, translocation, centromere, telomere, non-homologous end-joining, gene dosage

Abbreviations

MIN: microsatellite instability, CIN: chromosomal instability, W-CIN: whole chromosome CIN, S-CIN: structural CIN, SAC: spindle assembly checkpoint, DSB: double-strand break, HRR: homologous recombination repair, NHEJ: non-homologous end-joining, SCNA: somatic copy number alterations

Introduction

Most tumors show complex patterns of translocations, amplifications, and deletions, which have kept scientist occupied for decades. A specific problem occurs in carcinomas, most of which suffer from a particular kind of genetic defect termed chromosomal instability (CIN); these solid tumors combine gains and losses of entire chromosomes with segmental defects caused by chromosome breaks.

Another, indpendent class of tumors shows a different type of genetic alterations termed miscrosatellite instability (MIN). The terms MIN and CIN were originally devised to describe how a particular pattern of PCR markers - typically di- and trinucleotide repeats - deviates from the control, and in this way give an indication of the phenotype carried by the corresponding tumor sample or cell line. MIN is manifested by a shortening or lengthening of PCR markers compared to the same marker in the control tissue. MIN is a condition contributed to defects in DNA mismatch repair on the basepair level (Røyrvik et al., 2007). Whereas MIN results in a size difference, CIN manifests itself as a change in the relative intensity of PCR markers compared to the same marker in the control tissue. Only certain markers, for which the paternal and maternal alleles have a natural size difference, can be evaluated for intensity changes, and

are termed informative. Differences in the intensity of two alleles signal a copy number of the corresponding genomic region, brought about by the loss or gain of a large chromosome fragment or entire chromosome in the corresponding cancer (Geigl et al., 2008). The frequent observation of aneuploidy in the corresponding tumors suggests that chromosome segregation defects are a possible mechanism leading to CIN.

Available data indicate that CIN is the most common instability phenotype. The relatively small proportion of tumors that show MIN, approximately 15% of detected cases, are distinctly associated with a hereditary component (Charames and Bapat, 2003; Pihan and Doxsey, 2003); the defects in DNA mismatch repair are usually caused by recessive mutations and only become evident in homozygosis (Røyrvik et al., 2007). In addition to several hereditary conditions that cause CIN, this type of instability has been found in approximately 85% of sporadic carcinomas. Whereas the carcinogenic potential of MIN has been recognized early on - defects in mismatch repair might directly lead to mutations in oncogenes and tumor suppressor genes -, the relation between CIN and cancer has remained unclear for a long time. A search for mutations that cause CIN has identified more than a hunderd candidate genes in yeast (Spencer et al., 1990), underlining the complexity of this type of genetic instability. The question as how mere numerical alterations can provoke cellular transformation still is a matter of debate. One suggested mechanism involves the collaboration of MIN and CIN in a tumor progression (Pihan and Doxsey, 2003). According to this theory, CIN can amplify the carcinogenic effects of MIN by generating additional copies of activated oncogenes. Although evidence for widespread MIN and CIN in a single sample is occasionally found (Abdel-Rahman et al., 2001), most CIN tumors seem to accumulate mutations only in a limited number of oncogenes and suppressor genes and do not show systematic MIN. Other studies indicate that numerical alterations arise before sequence alterations (Nowak et al., 2002), and that CIN has an oncogenic effect independent of intragenic mutations (Abdel-Rahman et al., 2005). Thus, mutations in tumor suppressor genes might evolve through a compensatory mechanism that is selected for by CIN, instead of comprising a primary cause of carcinogenesis. These data indicate a role of CIN that is far more important and complex than the induction of copy number alterations in oncogenes alone.

In this review, I will discuss some of the current models describing the early steps in sporadic carcinogenesis, with a special focus on the formation of chromosomal instability (CIN). CIN comprises alterations in whole chromosomes (W-CIN), described in detail in a large number of publications and therefore reviewed only briefly here, and structural alterations (S-CIN) still subject to speculation and thus evaluated in more detail. Furthermore, the most important downstream effects of CIN will be discussed; W-CIN and S-CIN invariably result in copy number alterations, and therefore in an imbalanced expression pattern for a large number of genes.

This expression imbalance produces a gene dosage effect in cells affected by CIN, in the form of an unfolded protein response (Herr and Debatin, 2001). A prolonged unfolded protein response might be an important selection mechanism, promoting resistance to apoptosis and cell transformation in CIN tumors. The relation between gene dosage and cell transformation will be the subject of the final paragraphs of this review.

CIN comprises aneuploidy and breakage

The chromosomal instability observed in approximately 85% of all solid tumors can be divided in two major components: gains and losses of entire chromosomes termed aneuploidy or whole chromosome instability (W-CIN), and segmental defects (S-CIN) that comprises formation of breaks and fusion of chromosome sections (Geigl et al., 2008).

These two phenomena regularly occur together in a single tumor sample or cell line, and quantitation has shown that the levels of W-CIN and S-CIN are proportional (Duesberg et al., 1998; Fabarius et al., 2003). In tumors that show MIN, neither W-CIN nor S-CIN is normally found (Abdel-Rahman et al., 2001). Notwithstanding the correlation between the two phenomena, W-CIN is understood in far more detail than S-CIN; most carcinomas show variations in chromosome number that arise from the losses and gains of entire chromosomes during mitosis.

The cell division is one of the most dramatic events in the cell cycle, and other processes such as RNA transcription are largely suppressed during mitosis (Egli et al., 2008) so the cell can dedicate its resources to chromosome segregation. Since the cell temporarily suspends some vital processes, the total duration of mitosis is limited. To ensure rapid but correct chromosome segregation in mitosis, metazoans have evolved an elaborate regulatory network controlling chromosome capture by the mitotic spindle.
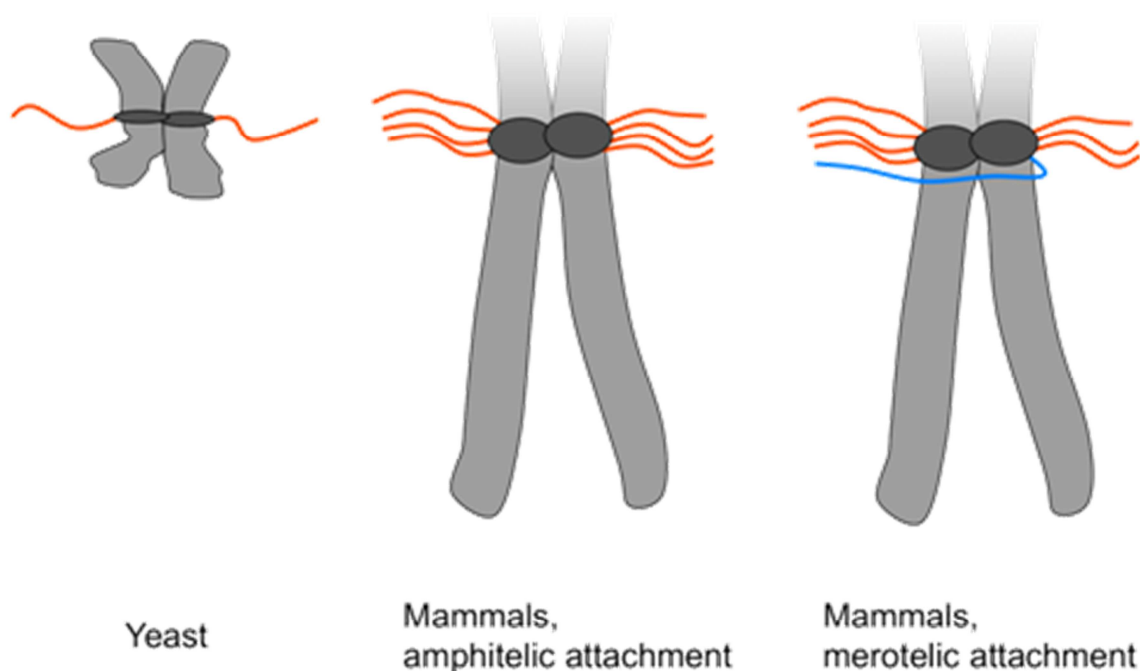
Yeast   Mammals,   Mammals,
                         amphitelic attachment   merotelic attachment

Figure 1. Comparison of yeast and mammalian chromosome segregation. The small chromosomes in lower organisms (left) require just one microtubule or fiber (red) for efficient separation in anaphase, but the large chromosomes in higher organisms (middle) are segregated only by the combined force of multiple K-fibers. The amplification of the number of fibers needed for chromosome segregation leads to occasional merotelic attachments (blue, right) detected poorly by the SAC, but potentially leading to W-CIN and S-CIN.

In yeast, a search for mutations that cause CIN has identified more than a hunderd candidate genes (Spencer et al., 1990), many of which have multiple orthologs in humans. The complexity of the network that controls chromosome segregation however reflects the magnitude of the problem faced, and spontaneous chromosome segregation errors occur at a measurable rate even in the tightly controlled mitotic spindle, leading to W-CIN (King, 2008; Compton, 2011). The problem lies in the need to control simultaneous segregation of all chromosomes - the transition form metaphase to anaphase - at the cellular level but capture of each chromosome at the level of the individual kinetochore. Tension is used to test attachment of each kinetochore individually, and a single unbound kinetochore is enough to delay anaphase (Rieder et al., 1994). Whereas segregation of the relatively small chromosomes of yeast can be accomplished with a single microtubule, the large mammalian chromosomes require large bundles of microtubules, termed K-fibers, for rapid segregation. When multiple fibers are used to segregate a single chromatid, tension no longer is a faithful measure for kinetochore attachment (Figure 1). Total lack of kinetochore capture still results in absence of tension, but capture of a single kinetochore by microtubules protruding form different spindle poles - termed merotelic attachment - might provide enough tension to signal anaphase progression but could lead to incorrect chromosome segregation (Cimini and Degrassi, 2005). The need for multiple microtubules

for the segregation of each sister chromatid thus has lead to a biological problem that as of yet still has not been resolved. Over the last decade, merotelic kinetochore attachment has been accepted as a major cause of W-CIN in human cancer.

In contrast to W-CIN, for which the role of the mitotic spindle has been consolidated in the last years, possible mechanisms of S-CIN still are part of a dynamic field of discovery. Over the last years, several hypotheses have considered a link between CIN and cellular processes ranging from DNA replication (Crasta et al., 2012) to telomere erosion (Stewart and Bertuch, 2010) and cytokinesis (Janssen et al., 2011). Thus, W-CIN is the result from a reasonably homogeneous group of experiments involving chromosome segregation, but S-CIN might be caused by a range of experimental conditions. Notwithstanding the variety of theories, the simplest explanation entails a common origin for W-CIN and S-CIN (Martínez-A and van Wely, 2010).

Experimentally induced S-CIN and the role of mitosis

Whereas aneuploidy has been tied to defects in mitotic chromosome segregation early on, the origins of S-CIN have remained unknown for a long time. Even today, it remains a matter of debate if W-CIN and S-CIN are caused by a single defect or have different origins (Martínez-A and van Wely, 2010). The different models agree that chromosome breakage is a crucial step in the generation of S-CIN, as translocations are

the result of a chromosome fragment fused to a genomic location other than the one it originated from. The origins of DSB in CIN cells however are explained in a number of different ways. Below, I will discuss some models that explain the formation of chromosome breaks under conditions that induce W-CIN.

Since W-CIN and S-CIN are frequently observed in single samples, a logical point to start a search for DSB is the mitotic spindle. Chromosomes are attached to spindle microtubules through the kinetochore-centromere interface, and the metaphase to anaphase transition marks the moment in which pulling forced physically segregate the sister chromatids. In merotelic attachments, kinetochores are bound to microtubules that project from both spindle poles and experience part of the tension that signals for degradation of cohesion molecules (Figure 1). Since merotelic attachments inefficiently activate the SAC, some cells progress into anaphase even though chromosomes are coupled to both spindle poles (Cimini et al., 2003), exposing the kinetochore to additional tension that leads to a physical deformation (Cimini et al., 2001). This situation is a consequence of the larger chromosomes in mammals and the need for many parallel microtubules to segregate chromosomes. Adding microtubules to segregate the sister chromatids is an evolutionary compromise to prevent excessive duration of anaphase, and the result is that the combined strength of microtubules capturing a typical mammalian kinetochore exceeds the pulling force needed to break the DNA backbone (Guerrero et al., 2010b).

Mutants with a reduced control of the SAC show a combination of W-CIN and S-CIN (Trachana et al., 2007; Guerrero et al., 2010b). In these mutants, the deformation of kinetochores in merotelic attachments is noticeable in the connected centromere chromatin, which also undergoes physical stretching. If the merotelic attachment persists and the microtubules exert enough force, breakage of centromere DNA follows (Guerrero et al., 2010b). In agreement with a model for merotelic attachments that places microtubules projected to the opposite pole at the periphery of the kinetochore (Cimini et al., 2001), many breakpoints localize to the edges of the centromere (Martínez et al., 2012). A chromosome damaged in mitosis can thus be divided in an arm bearing a functional centromere and an arm lacking centromere activity (Martínez-A and van Wely, 2010), explaining why different DSB repair pathways generate neoacrocentric chromosomes (Gagos et al., 2008) and tandem fusion to the telomeres of healthy acceptor chromosomes (Martínez and van Wely, 2011). The contribution of DSB repair in S-CIN will be discussed further below.

Whereas the initial description of mitotic DNA damage identified DSB formation in freely cycling cells, later studies have applied spindle poisons to increase the number of cells in mitosis (Janssen et al., 2011; Crasta et al., 2012). A release from the metaphase block allows for the identification of DNA damage in subsequent cell cycle phases, but ignores a possible contribution of the early stages of cell division. Surprisingly, although two studies used compounds that alter microtubule behavior, either monastrol or nocodazole (Janssen et al., 2011; Crasta et al., 2012), very different results were obtained. The first use of spindle poisons sprouted the hypothesis that cytokinesis accounts for cleavage of trapped chromosomes and thereby causes S-CIN (Janssen et al., 2011). After monastrol release, damaged chromosomes were indeed found close to the cleavage furrow, in contrast to damage signals in spindle control mutants, which localize to micronuclei and occasionally inside the main nucleus (Guerrero et al., 2010a). Colocalization of damaged chromosomes and cleavage furrow however is a logical consequence of merotelic attachments, since the position of the actin ring in cytokinesis is determined largely by spindle midzone microtubules that transverse the previous metaphase plate (Wheatley and Wang, 1996). In conclusion, damaged chromosomes probably are trapped close to cleavage furrow probably by a prior event, and are no direct consequence of actin ring constriction.

The second argument to implicate cytokinesis in chromosome breakage was the time of DSB formation. Strikingly, DNA damage was first observed within minutes of release from the mitotic block, based on the appearance of the DSB marker 53BP1 (Janssen et al., 2011), even though cytokinesis is not started until half an hour later. This time difference excludes cytokinesis from the possible causes of breaks directly after drug release. Kinetic studies have shown that 53BP1 accumulation is a relatively slow process; 53BP1 takes 5-15 minutes to appear on induced DSB (Schultz et al., 2000).

This timeframe might be even longer in cell division, because 53BP1 is excluded from DSB during most of mitosis (Giunta and Jackson, 2011). The combined data indicate that DSB likely appear within minutes of drug release, and that resolution of merotelic attachments in the monopolar spindle are the most probable cause of DNA damage.

A final model has suggested errors in DNA replication as a source of S-CIN (Crasta et al., 2012). For over a decade, DNA replication errors have been associated with concrete chromosomal loci termed fragile sites, in which DSB can be induced experimentally by treatment with DNA polymerase inhibitors (Arlt et al., 2003).

Fragile sites are thought to represent loci that require more time to replicate than the remainder of chromatin, because a particular basepair composition or epigenetic state restricts passage of DNA polymerase.

Whereas fragile sites do not seem to represent a problem in normally growing cells, the accelerated growth rate of cancer cells would specifically exacerbate the replication errors in fragile site loci and result in localized DSB (Crasta et al., 2012).
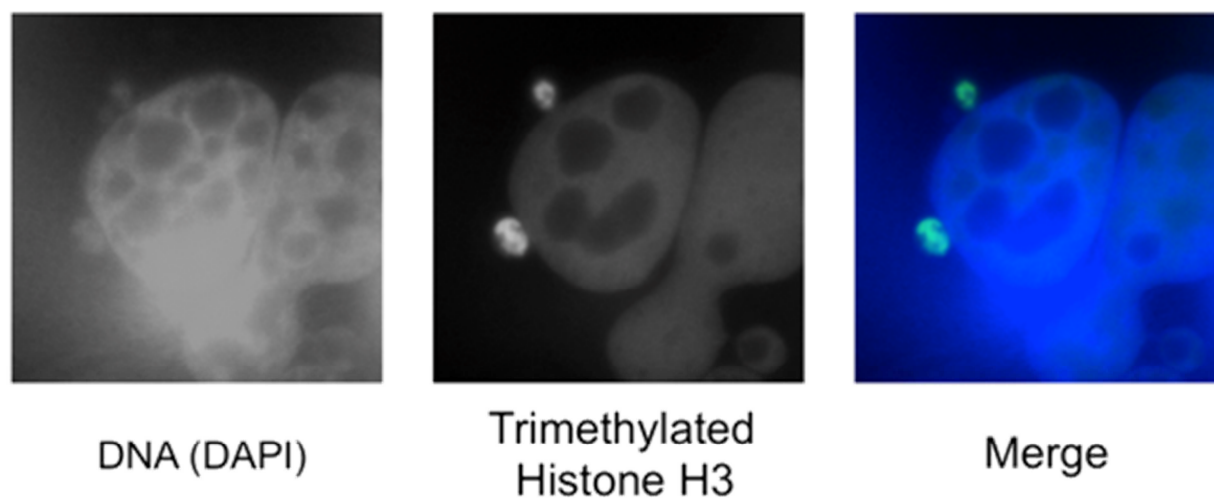
Figure 2. Epigenetic alterations in micronuclei suggest fundamental changes in chromatin physiology. CIN cells were labeled with antibodies against trimethylated Histone H3 (green), counterstained with the DNA marker DAPI (blue), and studied by fluorescence microscopy. Whereas the main nuclei showed low homogeneous labeling of chromatin, excluding the nucleoli, micronuclei revealed excessive levels of Histone trimethylation.

Although fragile sites were initially attributed to stalled replication forks, later results have shown that the rate of origin firing, but not that of replication fork progression, is lowered in fragile sites (Letessier et al., 2011). Since experimentally induced breaks at fragile sites depend on compounds that induce stalling of replication forks, these last results have opened a serious breach between laboratory and clinic. In the study that suggested interplay between S-CIN and DNA replication, DSB were detected without induction of stalled replication forks (Crasta et al., 2012). Instead, micronuclei formed after release from a block with microtubule depolymerizing agents accumulated DNA damage in the following cell cycle. However, whereas breaks in micronuclei were not obvious directly after cell division, they became evident in later cell cycle phases. Reduced levels of the MCM2 helicase subunit and delayed BrdU incorporation indicate that micronuclei formed in this way acquire DNA damage because DNA replication cannot keep up with normal cell cycle progression. Although the delayed replication and associated DSB in micronuclei are an interesting observation, they have not been attributed to a well-defined molecular mechanism; the epigenetic composition of chromatin in micronuclei seems fundamentally different from that in the main nucleus (Figure 2), but the question what induces these differences remains to be answered.

Micronucleus formation by spindle poison requires that a lagging chromosome remains at the metaphase plate long enough to be excluded from the main nucleus, indicating that merotelic spindle attachments are maintained during a considerable part of mitosis. The forces exerted by a prolonged merotelic attachment can cause physical stretching of chromatin (Cimini et al., 2001), to which the micronucleus might respond by epigenetic alterations, delayed replication, and DSB

accumulation. Whereas the same effects are detectable right after mitosis in SAC control mutants (Guerrero et al., 2010b), the treatment with microtubule-destabilizing agents (Crasta et al., 2012) might relax the tension enough to reduce centromere stretching and delay a DNA damage response in the micronuclei.

The proposed mechanisms for experimentally induced mitotic DSB appear to have different origins, but all are related to the mitotic spindle; whereas some models rely on genetic mutants that suffer from spindle defects (Guerrero et al., 2010b), other studies form monopolar spindles (Janssen et al., 2011) or require spindle assembly from a depolymerized state (Crasta et al., 2012). The different outcomes, consequently, might also be spindle related; replication stress was found in micronuclei that arise through spindle defects (Guccini et al., 2012), cytokinesis related breakage was induced by suppressing kinases that control the SAC (Yamagishi et al., 2012), and merotelic kinetochore attachments are a consequence of defects in the control of mitotic microtubules (Cimini and Degrassi, 2005; Knowlton et al., 2006). A plausible explanation for the variety of observations therefore is a common mitotic origin that results in a number of possible outcomes according to the parameters that are assayed. To determine which of the proposed mechanisms of DSB formation is relevant for carcinogenesis in humans, a survey of S-CIN in tumor samples is fundamental.

The contribution of DSB repair to CIN

Formation and repair of DSB are considered an important aspect of CIN irrespective of the model system used, because the translocations that characterize S-CIN cannot occur without breakage and fusion (Martínez and van Wely, 2011). Breakage

generates genomic fragments that might contain essential genes, the transmission of which is not guaranteed when separated from the rest of the chromosomes. Since DSB compromise cellular physiology, the natural tendency of cells is to repair any encountered breaks as soon as possible. The imperative to repair DSB is strong enough to put a brake on the normal cell cycle; not only do DSB slow interphase progression to prevent replication of damaged chromosomes, they also slow the metaphase to anaphase transition where the chance of segregating chromosome fragment into different daughter cells is a real danger (Dotiwala et al., 2010). To repair DNA damage, mammalian cells bear a combination of pathways that perform overlapping and complementary roles (Bernstein et al., 2002). Whereas pathways specialized in the excision of damage at the basepair level important factors in MIN tumors (Roøyrvik et al., 2007), homologous recombination (HRR) and particularly non-homologous end-joining (NHEJ) repair the DSB produced in S-CIN (Guerrero et al., 2010b; Martínez and van Wely, 2011). Although a potential relation between HRR, NHEJ and S-CIN has been the subject of study for decades, no causal relation has been uncovered. Mutants in either HRR or NHEJ need more time to repair DSB when challenged (for example by irradiation), but the natural rate of DSB formation seems to be too low to cause significant accumulation of damage (Muller, 1950). In agreement with a low rate of spontaneous breaks, the frequency of sporadic - not induced- tumors is only moderately elevated in most repair mutants (Stiff et al., 2004). Thus, HRR and NHEJ have an important role in the processing of breaks but do not contribute significantly to their de novo formation. The situation is very different when the rate of DSB formation is raised; even relatively low doses of radiation or cytotoxic compounds can overwhelm existing repair mechanisms. Commonly, such treatments induce apoptosis in the target cells, but the small proportion of surviving cells might replicate in the presence of chromosomal translocations (Traut, 1963).

Repair mechanisms likely contribute to the processing of chromosome fragments, because S-CIN is a direct consequence of breakage. Most CIN tumors have normal or even increased levels of DSB repair (Pucci et al., 2001; Hosoi et al., 2004; Cheng et al., 2010), probably to cope with the consequences of mitotic chromosome breaks. The importance of DSB repair is further emphasized by the lethal effects of repair deficiency in a CIN background (Guerrero et al., 2010b); whereas mild repair defects or mild CIN are tolerated individually, their combination causes synthetic lethality. Abrogation of NHEJ has particularly severe consequences for CIN cells (Guerrero et al., 2010b). This is understandable when one takes the mitotic origin of CIN-related DSB into

account; Not only is NHEJ the dominant repair pathway in mammalians, HRR is inactive right after mitosis due to the lack of substrate (Rothkamm et al., 2003; Valerie and Povirk, 2003), and chromosome fragments in micronuclei are excluded from HRR due to the physical separation from a homologous counterpart. Notwithstanding the relatively mild effects of repair mutants if an additional source of DSB is absent, NHEJ has important tumor-suppressive properties because it can rapidly fuse chromosomal fragments and thus prevent loss or translocation of genome segments.

Whereas HRR uses homologous substrates by obligation, NHEJ does not use sequence similarity. Although some subclasses of NHEJ use microhomology to compare the extremes of DNA fragments (Iliakis et al., 2004), this is not a prerequisite, and many fragments are joined without previous control. DSB repair by NHEJ occasionally involves the deletion of addition of a few bases at the site of fusion (Lukacsovich et al., 1994). These deletions and additions might eventually lead to frameshift mutations, but the potential to generate intragenic mutations of NHEJ appears insignificant compared to the base-pair alterations in MIN. However, the absence of control for homology also has important consequences for S-CIN, because this capacity to fuse unrelated DNA fragments is - together with the appearance of DSB - a critical step in the generation of chromosomal translocations.

Cells encounter an especially difficult situation when a merotelic attachment causes enough DNA damage in a centromere to separate the two arms of a single corresponding chromosome. When the two arms are segregated into different daughter cells - copy number analysis (Beroukhim et al., 2010; Bignell et al., 2010) shows that this situation occurs regularly -, the NHEJ repair machinery only encounters one half of its normal substrate after completion of mitosis. For the lack of a suitable counterpart, NHEJ may then fuse the solitary chromosome arm to a telomere, because this is the only structure that comes preloaded with part of the NHEJ protein complex and sufficiently resembles a break (Martínez and van Wely, 2011). In this case, NHEJ might promote carcinogenesis, since the resulting product is a chromosomal translocation - although the breakpoint do not map inside a chromosome arm - and possibly alters the copy number of an oncogene in the receiving cell. Thus, NHEJ can be a tumor-suppressive as well as a tumor-promoting repair mechanism.

Many of the surprising properties of NHEJ are never observed under experimental conditions, because irradiation or cytotoxic compounds artificially increase the number of DSB far above normal levels. These induced DSB however distribute in a random manner over the genome, instead of having a particular location related to natural cellular processes. Again, the choice

of model system for the study of DSB repair might bias results. Similarly, the one-sided view given by most experimental models might have biased conclusions regarding the role of telomeres in S-CIN (Martínez and van Wely, 2011). To validate the contribution of NHEJ and telomeres to cancer, the real life situation must be evaluated through the mapping of break- and fusion-points in clinical samples.

W-CIN and S-CIN in tumor samples and cell lines

Whatever the experimental method used to induce breakage, the manipulation of a model system likely induces a bias with respect to the type and localization of DNA damage. Thus, although experimental models illustrate the possibility of a particular pathway to induce DNA damage, they provide no information regarding the contribution of that pathway to CIN in real life.

If an experimentally identified pathway participates in a clinical setting, at least some aspect of that pathway must be found in tumor samples of sporadic cancer. Whereas it is common practice in the laboratory to disrupt protein function by gene targeting (Dobles et al., 2000; Guerrero et al., 2010b) or small molecule inhibitors (Janssen et al., 2011; Crasta et al., 2012), we must assume that the very first step of carcinogenesis occurs in normal cells. To bridge the gap between laboratory and clinic, a good indicator is to see by experiment if part of a molecular mechanism occurs spontaneously. Again, the phenomenon described in more detail, W-CIN, yields a number of useful clues.

W-CIN is a common phenomenon even under physiological conditions (Compton, 2011). For example, aneuploidy is detected in approximately a third of all miscarriages (Hassold and Hunt, 2001), and nearly 80% of all human embryos contain aneuploid cells at the blastomere stage (Vanneste et al., 2009; Voet et al., 2011). W-CIN is also found regularly in some adult tissues, for example the brain (Rehen et al., 2005). The ease by which W-CIN is detected in healthy individuals shows that chromosome missegregation is a common origin of genetic defects, and probably occurs before other alterations in carcinogenesis. Nonetheless, the frequency of aneuploidy nowhere is as high as in sporadic carcinomas (approximately 85%), indicating that CIN is a real requirement for cancer development. If one accepts that chromosome missegregation is a common phenomenon preceding other steps in tumorigenesis, a single mechanism leading to the combination of W-CIN and S-CIN - merotelic kinetochore attachments - must reveal its presence in tumor samples. Although merotelic attachments occur spontaneously non-transformed cultured cells (Cimini et al., 2001; Cimini et al., 2003), no direct methods

have confirmed their presence in animals or humans. Lagging chromosomes are a key aspect of the three models for S-CIN presented above, and the clinical manifestation of lagging chromosomes - micronuclei – are an important clinical tool for cancer diagnosis (Kirsch-Volders et al., 1996; Kirsch-Volders et al., 2002). These clinical cytogenetic techniques however provide suboptimal resolution to differentiate W-CIN and S-CIN, and therefore cannot support just one of the proposed mechanisms; genetic mapping of translocation breakpoints might help to make this distinction.

Whereas merotelic kinetochore attachments have a direct connection to the centromere region of the chromosome (Guerrero et al., 2010a), no such preference has been described for actin ring chromosome capture in cytokinesis or delayed replication in micronuclei. Since translocations are a clinical manifestation of breakage, mapping of breakpoints to the centromere would thus specify merotelic attachments as the main cause. Unfortunately, centromeres largely contain repeat sequences not covered by the human genome project (Venter et al., 2001), precluding analyses with single-base resolution such as massive parallel sequencing. The combined analysis of somatic copy number alterations (SCNA) and karyotyping nonetheless provides enough information needed to faithfully map centromeric breaks. The clinical manifestation of centromere fission, formation of individual chromosome arms, indeed is omnipresent in human cancers (Beroukhim et al., 2010; Bignell et al., 2010). Moreover, whereas the frequency of random breaks follows the statistical distribution predicted by the size of translocated chromosome fragments, whole-arm translocations occur at least ten times more often (Beroukhim et al., 2010). Other possible breakpoints, for example fragile sites brought about by replication defects, contribute only marginally to SCNA in the natural environment. The clinical data thus concur with a model for S-CIN that specifically includes centromere-localized breaks, and provide compelling evidence for the prevalence of merotelic attachments in human cancer.

Clinical evidence for the role of NHEJ in S-CIN

SCNA and karyotyping not only underline the importance of centromere fission, but also hint at the mechanism by which NHEJ promotes carcinogenesis. Additional copies of chromosome arms limited by a centromeric break on one extreme and a telomere on the other rarely remain isolated fragments; the imperative to maintain an entire genome complement results in an almost obligatory fusion.
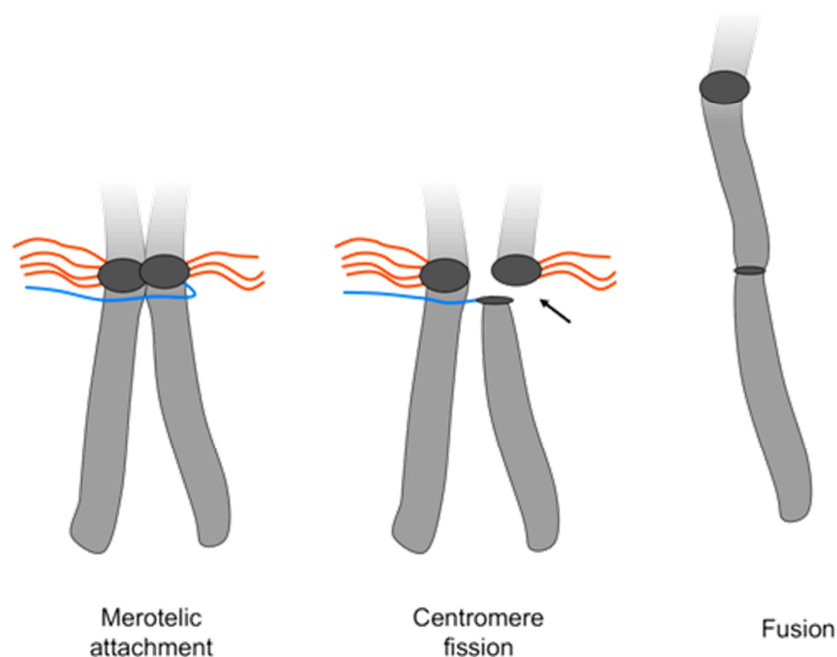
Figure 3. The combination of centromere fission and NHEJ lead to telomere capture in CIN tumors. If left uncorrected merotelic attachments (left) can exert enough force to physically stretch kinetochores and shear centromere DNA (arrow, middle) in mitosis. Upon exit from mitosis, HRR is inactive and NHEJ encounters only a "half substrate". Due to the preloading with NHEJ components, telomeres form an inefficient but viable ligation partner (right), to which isolated arms can be fused in tandem orientation.

Depending on the moment of fusion - soon after mitosis or after DNA replication - and its subcellular location - in a micronucleus or together with other chromosomes -, and isolated chromosome arm commonly fuse with itself or with telomeres of healthy chromosomes (Martínez-A and van Wely, 2010; Martínez and van Wely, 2011). Whereas centromere to centromere fusions give rise to Robertsonian translocations and isochromosomes, the centromere to telomere fusions result in tandem ligation of the broken arm on the end of a healthy chromosome (Figure 3). In contrast to earlier proposals, in which telomere erosion was the driving force behind fusion in an antiparallel orientation, tandem fusion is caused by the ligation of a "half break" to a normal telomere.

An overview of tumor karyotypes, revising nearly a hundred cases, has revealed that centromere-mediated fusions account for half of all translocations in sporadic carcinomas (Martínez and van Wely, 2011). These data confirm that healthy telomeres offer protection against fusion with other standard chromosome ends, but can be a substrate of NHEJ when no suitable partner for a break is found. Even karyotypes attributed to actin ring constriction in cytokinesis show characteristic centromere to telomere fusions (Janssen et al., 2011), corroborating the role of merotelic attachments in spindle poison-induced DSB. In some karyotypes, a small proportion of isolated, but apparently stable chromosome arms can be found, that probably represent neoacrocentric chromosomes. These might have acquired a new telomere at the breakpoint through the alternative telomere elongation pathway (Gagos et al., 2008), which would suppress further recognition by the NHEJ pathway.

A final phenomenon linked to S-CIN is the amplification of proto-oncogenes. Multiple proto-oncogenes, for example MYC and PIK3C, and some tumor-suppressor genes like INK4 show numerical changes without the acquisition of point mutations (Bignell et al., 2010), supporting the importance of S-CIN as a separate oncogenic mechanism.

In some tumors, dozens or more copies of these oncogenes are present, and other samples reveal homozygous deletion of tumor-suppressor genes. Such extremes cannot be reached by whole arm translocations alone, because this induces a severe gene dosage effect in the target cell. Instead, intra-arm breaks provide a selective mechanism that allows for the amplification or deletion of smaller chromosomal segments. Typically, proto-oncogenes such as MYC and PIK3C are located on arms that acquire a single additional copy without breakage in low-grade tumors but gain higher copy number when breakage allows for amplification of small segments (Bignell et al., 2010). Since centromere fission can generate isolated arms without or with a functional centromere, fusion of these can create a normal but extended or a dicentric chromosome, respectively (Martínez-A and van Wely, 2010). Dicentric chromosomes are highly unstable, since the two centromeres might be segregated towards different spindle poles in a subsequent mitosis, causing renewed DSB and initiating breakage-fusion-bridge cycles.
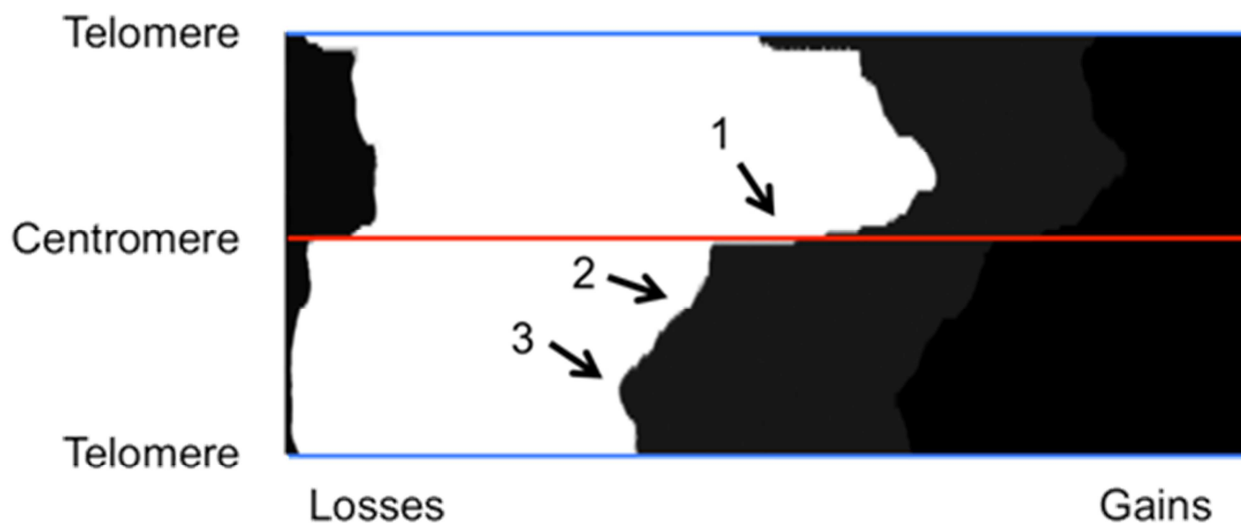
Figure 4. Signatures of centromere fission and selection in tumor samples. The frequency distribution of breakpoints from a single chromosome is shown. Whereas whole arm translocations give rise to a sharp SCNA variation that coincides with the centromere (1), subsequent random breakage and positive selection of oncogene-containing segments results in a sloping SCNA distribution (2) that peaks around the oncogene (3).

In contrast to the comparatively localized DSB generated by merotelic attachments, breaks in dicentric chromosomes can occur anywhere between the two centromeres.

Even though centromere breaks account for a large proportion of SCNA, random breakage repaired by NHEJ is evident in tumor samples and cell lines (Beroukhim et al., 2010; Bignell et al., 2010).

Karyotypes of advanced tumors generally reveal more W-CIN and S-CIN than those of low-grade cancer samples (Schröck et al., 1996; van Tilborg et al., 2002; Fabarius et al., 2003).

Moreover, the complexity of translocations increases with tumor grade, indicating ongoing cycles of breakage and fusion in S-CIN. The typical sloping pattern of the breakpoint distribution, from a low frequency close to centromeres and telomeres to a high frequency surrounding the oncogenes (Figure 4), illustrates the combination of negative selection by bulk gene dosage and positive selection by the oncogene (Kost-Alimova et al., 2007). Probably, a combination of negative and positive selection determines cellular fitness during the gradual process of transformation in carcinogenesis.

Gene dosage and cellular fitness

Since the discovery of oncogenes, several decades after the first description of aneuploidy, a debate has taken place how copy number alterations can lead to cancer. Whereas the mutagenic effect of defects in DNA mismatch repair has contributed to the rapid acceptance of MIN as a potential cause of cancer, as similar relation between CIN and cancer has met with much more resistance. In part, the acceptance of oncogenes as a carcinogenic mechanism is based on historical arguments; the study of carcinomas required the

induction and serial transfer of tumors from one animal to another of the same species until the development of cell culture. In the first half of the 20th century, transfer of virally induced tumors in birds and rodents proved to be a reliable way of tumor maintenance (van Epps, 2005). A logical consequence of this historical heritage was the discovery of viral oncogenes and their human equivalents in the early days of molecular genetics (Pulciani et al., 1982). In contrast to birds and rodents, however, the majority of human carcinomas seem to have no viral origin - some exceptions are hepatitis B and human papilloma virus - but instead arise spontaneously.

Although the normal equivalents of viral oncogenes in the human genome, termed proto-oncogenes, frequently acquire mutations, this by no means is a universal requirement for carcinogenesis (Davies et al., 2002). In MIN tumors, the mutagenic effect of defects in mismatch repair might produce a disposition for the acquisition of mutations in proto-oncogenes, but the same mutations in sporadic cancer might be acquired after the primary tumor has been established (Nowak et al., 2002).

If oncogenic activation induces physiological alterations that surpass the limit of cellular adaptation, cellular senescence is induced (Sager, 1991). Cells thus seem to have an intrinsic resistance against change as part of a general tumor-suppressive program. The resistance to change not only restricts the activation of individual gene products but also responds to a general gene dosage effect that results from the gains and losses of large chromosome fragments.

Additional chromosomes in cultures cells are gradually lost, until the cells reverted to a mostly diploid state (Thompson and Compton, 2008).

W-CIN is a reversible condition, at least in vitro, and normal ploidy apparently is the condition of greatest cellular fitness.

The burden of gene dosage is reflected in clinical conditions and in S-CIN, too, where the frequency a chromosome arm participates in translocations is inversely correlated with the number of genes on that arm (Beroukhim et al., 2010).

The observation that cancer cells preferentially lose small chromosomes (Duijf et al., 2013) shows that both gains and losses are limited by gene dosage. During some part of carcinogenesis, cancer cells must alter their behavior to overcome the intrinsic resistance to gene dosage and tolerate the large-scale CIN seen in advanced tumors. Fusion to healthy chromosomes partly explains how entire chromosome arms are gained even though this compromises cellular fitness; the loss of the fused fragment that would return the cell to its optimal state is linked to the loss of the acceptor chromosome by the fusion. Thus, the return to an optimal diploid state for the donor arm can only be achieved by a haploid state of the acceptor chromosome and results in an even more severe gene dosage penalty. For this reason, clinical samples in which additional arms fused in tandem to other chromosomes are the only karyotype alteration are quite common (Martínez and van Wely, 2011).

Some studies have suggested that tumors arise through a tetraploid state followed by gradual loss of chromosomes (Gisselsson, 2011). Such a diploid state might give rise to relatively mild gene dosage effects because all genes are amplified proportionally. Once polyploid cells have lost a dozen or so chromosomes, however, gene expression will suffer from an extreme disequilibrium unlikely to be overcome.

The recurrent translocation of the same chromosome arm, without additional genetic defects, in low-grade tumors contradicts the role of a tetraploid intermediate, but instead indicates that a gradual acquisition of additional genetic material is the normal pathway in CIN. Polyploidy usually is the result of an aborted duplication, and is accompanied by amplification of the centrosomes that form the spindle poles in mitosis (Duensing and Duensing, 2010). Due to the presence of multiple spindle poles, chromosome segregation in polyploid cells nearly always leads to a mitotic catastrophe and rapid cell death (Vitale et al., 2011).

A survey of genes that induce tolerance for aneuploidy identified pathways that promote protein turnover, especially the ubiquitin proteasome system (Torres et al., 2010). The potential of similar pathways to be treated with anticancer drugs are being studied actively (Chen and Dou, 2010).

The most important effect of gene dosage discovery until now is the production of improperly folded protein intermediates, which induces a cellular stress response that activates the pro-apoptotic p53 pathway (Herr and Debatin, 2001; Zhang et al., 2006). Nearly a century after the first description of aneuploidy (Boveri, 1914), the unfolded protein response is the first potential mechanism to explain malignization of CIN tumors; prolonged low level activation of a pro-apoptotic pathway in response to gene dosage potentially leads to the selection of mutants that suppress apoptosis through additional - numerical, structural, or sequence-dependent - mutations. Similar spill-over effects of the unfolded protein response, possibly involving the ubiquitination (Torres et al., 2010) essential for controlled protein degradation in mitosis, might be an important determinant in the development of ongoing chromosomal instability. Finally, spill-over effects might explain the shift in epigenetic modifications such as promoter methylation, since the additional genome segments compete for transcription factors that could become a limiting factor in the nucleus. Thus, the general interwoven nature of signaling pathways makes mammalian cells susceptible to imbalance, where CIN can be both driving force and consequence.

Concluding remarks

Considering the contributions of centromere fission and NHEJ - phenomena that act in an indiscriminate manner, but on single chromosomes -, it is easier to grasp the near random nature of cancer development.

Off course, one has to consider the modulating effects of individual genes on the global imbalance brought about by gene dosage. Not only the shear number of genes on a chromosome arm but also their expression level - think of tumor-specific markers - and particular function in cell survival - the "general" oncogenes and suppressor genes - play a role. In the end, a neo-Darwinistic model comprising randomized CIN and modulation by specific survival factors seems to give a reasonable description of carcinogenesis.

A question that remains to be answered is how genetic alterations that apparently reduce cellular fitness can lead to transformation and carcinogenesis.

We are accustomed to relate cancer to oncogene activation, and only see the fast-growing cells that appear at the end of the carcinogenic process, but these might be the result and not the cause of transformation. Although the final stages of carcinogenesis indeed are associated with a cell population that proliferates rapidly, has accumulated a variety of genetic defects, and acquired a certain degree a malignancy, the same does not necessarily hold true for the initial stages. The near universal presence of CIN, albeit associated with gene dosage, shows that some cells continue to proliferate in the body when their fitness is compromised.

This conclusion has been made before in a different context; radiation promotes formation of leukemia even though it has the potential to induce apoptosis. The prevalence of small chromosomes alterations in low-grade tumors - comparable to a low radiation dose - corroborates the classical stepwise model of carcinogenesis, in which gradual cycles of stimuli and

adaptations lead to drastic alterations after multiple iterations (Vogelstein et al., 1988).

Considering the reduced fitness associated with W-CIN and S-CIN, the cells where the initial steps of carcinogenesis occur must somehow tolerate gene dosage. The discovery of cancer stem cells might explain this tolerance towards gene dosage in the near future, since stem cells might unite the exact properties to form cancer precursors (van Wely and Martínez, 2012). Firstly, apoptotic control in stem cells is less than strict, since tight control might eliminate the self-renewing population and endanger long-term tissue maintenance. Next, the predisposition to differentiate might prepare stem cells for a rapid change in gene expression, accompanied by tolerance for gene dosage. Finally, the sustained replication of stem cells means they are at risk for chromosome segregation; since each mitosis has a small risk of erroneous chromosome segregation, many division result in a large cumulative risk. To maintain the balance between tissue renewal and genome stability requires making the right decision in each cell cycle during the entire life of the organism; in the end, one could consider that cancer is the effect of stem cell indecisiveness. The design of experiments that faithfully model cellular decision-making will be a challenge for the understanding of carcinogenesis in the next years.

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This article should be referenced as such: