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# Are aneuploidy and chromosome breakage caused by a CINgle mechanism?

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**Genetic instability is a hallmark of cancer. Most tumors show complex patterns of translocations, amplifications and deletions, which have occupied scientists for decades. A specific problem arises in carcinomas with a genetic defect termed chromosomal instability; these solid tumors undergo gains and losses of entire chromosomes, as well as segmental defects caused by chromosome breaks. To date, the apparent inconsistency between intact and broken chromosomes has precluded identification of an underlying mechanism. The recent identification of centromeric breaks alongside aneuploidy in cells with spindle defects indicates that a single mechanism could account for all genetic alterations characteristic of chromosomal instability. Since a poorly controlled spindle can cause merotelic attachments, kinetochore distortion, and subsequent chromosome breakage, spindle defects can generate the sticky ends necessary to start a breakage-fusion-bridge cycle. The characteristic breakpoint of spindle-generated damage, adjacent to the centromere, also explains the losses and gains of whole chromosome arms, which are especially prominent in low-grade tumors. The recent data indicate that spindle defects are an early event in tumor formation, and an important initiator of carcinogenesis.**

**Key words:** cancer, chromosomal instability, mitosis, spindle defects, breakage-fusion-bridge

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## Introduction

The vast majority of carcinomas suffer from continuous losses and gains of entire chromosomes during mitosis. At the same time, tumors undergo ongoing structural changes in their genomes, including nonreciprocal translocations, deletions, inversions and other types of transpositions of chromosomal material. Detailed analysis of tumors by genetic techniques has led to the subdivision of carcinomas in two large classes, each with a specific type of instability. The first class of carcinomas shows losses and gains of whole chromosomes and chromosome fragments, termed chromosomal instability (CIN). The second group of tumors undergoes elongation and shortening of DNA tracts, called microsatellite instability (MIN), but suffers no gross changes in chromosome number. Whereas CIN appears to be the most widespread phenotype, found in approximately 85% off sporadic carcinomas, roughly 15% off all carcinomas have a MIN phenotype, which appears to bypass the requirement for CIN.[1-3]

CIN tumors show a remarkable diversity of genetic defects, which can even vary between cells in a single tumor. Whereas the mutations that lead to MIN accrue in a limited number of genes mostly involved in replication and repair on the base pair level,[1] the mutations that give rise to CIN cover a broad range of functions. In yeast, a search for mutations that cause CIN identified more than 100 candidate genes,[4] many of which have multiple orthologs in humans. The apparent complexity of CIN has puzzled scientists for a long time; what initially appeared to be missegregation of growth-promoting and -inhibiting properties corresponding to whole chromosomes[5] has turned out to involve complex genetic alterations unique to individual tumors and patients. A common way around the complexity of the problem is to assume that aneuploidy alone or segmental losses

alone equal CIN, but this reductionist view fails to describe most tumors correctly. An important conceptual leap has therefore been to include all genetic alterations associated with CIN in a single definition, according to which CIN refers to the combined instability of entire chromosomes and large chromosome fragments.[6] Since a single definition should correspond to a single mechanism, this has an important consequence for CIN; whereas aneuploidy explicitly refers to intact chromosomes, chromosome fragments have their origin in a break. The recent discovery of centromeric DNA damage in cells with spindle defects has provided evidence for a single mechanism explaining both aneuploidy and breakage.[7] Here we will discuss some recent data pointing to the mitotic spindle as the main, and possibly only, culprit in CIN.

## The Two Faces of CIN

Although most CIN tumors show a complex pattern of genomic alterations, this could be just a consequence of multiple rounds of breakage and repair.[3] CIN can thus be seen as the combination of entire chromosome aneuploidy and breakage-induced instability, acting during multiple cell divisions. The first indication that a common cause could bring about both features of CIN came from the quantitation of genetic instability in cancer cells,[8,9] which showed that structural instability is proportional to the aneuploidization rate. Many tumor samples, too, show a connection between aneuploidy levels and the complexity of genetic alterations; both are proportional to tumor grade.[10,11] Since the recent single definition of CIN includes both of these characteristics,[6] could a single mechanism account for both?

The best understood aspect of CIN is aneuploidy; most carcinomas show variations in chromosome number that arise from continuous losses and gains of entire chromosomes during mitosis.[12] Aneuploidy is one of the first oncogenic events in mouse models of adenomatous polyposis coli,[13] and mathematical methods predict that aneuploidy is required for sporadic carcinogenesis.[14] Aneuploidy was long believed to contribute to tumor development by changing the copy number of tumor suppressor genes and oncogenes, or was even considered a harmless side effect of tumorigenic transformation, but recent advances place aneuploidy right at the start of carcinogenesis. Although aneuploidy is sufficient to trigger tumor formation in several animal models, the rate at which aneuploidy is generated proves a critical factor; whereas heterozygosity for *Cenp-E* (centromere protein E) causes the spontaneous appearance of tumors, its homozygous deletion result in high levels of CIN that compromise cell survival.[15] A similar observation has been made for mitotic checkpoint proteins such as Bub1, Mad1, Mad2 and Cdc20.[16-19] Even though mutations in mitotic checkpoint genes are not tolerated well, and are found in only a small number of tumors,[1,20] spindle control is compromised in virtually all CIN tumors.[21,22] These data have led to the suggestion that changes in the expression levels of checkpoint proteins, and not their mutation, account for aneuploidy.[23] Additionally, proteins that help to control the mitotic spindle without forming part of the core mitotic checkpoint promote spindle defects; a search for mutations that cause CIN in yeast identified more than 100 candidate genes,[4] and in mammals, mutations in genes like *Apc* cause a large proportion of non-hereditary tumors. The involvement of non-checkpoint genes, able to induce CIN with no deleterious effect on cell survival, could be the reason that CIN is so frequent.

In contrast to the massive genetic instability in mutants of checkpoint genes, aneuploidy itself appears to be well tolerated and is not controlled by a specific checkpoint. Several well-described trisomy syndromes are compatible with life, and drug-induced tetraploidy allows near-normal cell cycle progression.[24] This discrepancy between CIN and aneuploidy is explained by the fact that CIN encompasses more than aneuploidy alone; the second feature of CIN, chromosome fragment formation, could have a critical role in tumorigenesis although it is understood only in very broad terms. The formation of chromosome fragments means that breaks are generated in the genetic material. Experimental generation of such a broken, or "reactive" chromosome end induces a phenomenon termed the breakage-fusion-bridge (BFB) cycle.[25] The BFB cycle leads to extensive genome remodeling and thus reproduces part of the CIN phenotype,[3] and BFB is now generally accepted as a mechanism that explains chromosome breakage in CIN. Once started, the BFB cycle keeps going: A chromosome fragment can fuse with another, previously intact chromosome. The formation of a dicentric chromosome in some of these fusions can lead to mitotic segregation of the two centromeres to different poles and renewed breakage. Whereas the BFB cycle is probably the pathway that propagates structural chromosome changes, how chromosomes break in the first place remains a matter of debate.

The complexity of CIN has sometimes led to the idea that it is caused by a combination of two alterations, a spindle defect responsible for aneuploidy and a second defect leading to chromosome breakage. Whereas the relation between spindle defects and aneuploidy is clear, the proposed second defect remains undefined. At least three theories—telomere attrition, defects in double-strand break (DSB) repair, and fragile sites—have been proposed for break formation, but none of these gives an adequate explanation for all aspects of CIN. Telomere shortening in mice neither reproduces the CIN phenotype nor increases carcinogenesis if not aided by additional tumor-inducing treatment.[26,27] Instead, telomere shortening causes cell senescense, effectively blocking uncontrolled proliferation. Over the last few years, it has become clear that telomere shortening is probably a minor factor in the initiation of genomic instability, although telomerase reactivation probably plays an important role in tumor progression.[28] DNA repair defects[29] or fragile sites[30] have alternatively been suggested to initiate genetic instability. In contrast to a proposed repair defect, many CIN tumors show upregulation of DSB repair pathways such as non-homologous end joining.[31,32] A related theory proposed the existence of a number of fragile sites on the chromosome.[30] Due either to the lack of repair or to an opening of the chromatin during active transcription, the chromosome would be more prone to rupture at specific sites. Genetic mapping in a large number of tumor cell lines nonetheless shows a near-random distribution

of breakpoints, and only a few fragile sites show prevalence over this randomness.[33] DSB repair accordingly lacks preference for any part of the chromosome—for example, induced DSB in centromeres and on chromosome arms are repaired with equal efficiency,[34]—and does not explain the preference for whole-arm translocations. Although current theories could thus partially explain the behavior of hereditary tumors and chromosome breakage in specific cell types, they fall short when it comes to describing CIN as a single phenomenon.

## The Spindle as a Break-Generating Device

Since the evidence gathered in recent years has shown that aneuploidy is caused by spindle defects, could the mitotic spindle also generate chromosome fragments, and are the genetic alterations in CIN tumors compatible with a model that relies on the spindle-kinetochore pathway?

A specific spindle defect, merotelic kinetochore attachment, is of interest because of its ability to exert a physical force on the chromosome. In a merotelic attachment, a single kinetochore is connected to both spindle poles. If this situation persists, an individual chromosome could lag behind on the metaphase plate until late in mitosis, and be segregated into the wrong daughter cell. Merotelic attachments appear to be an important cause of aneuploidy in CIN, are common in metaphase and early anaphase, and are not recognized efficiently by the spindle checkpoint.[35,36] A tension-based mechanism for the correction of merotelic attachments has therefore been proposed.[37] In a single merotelic attachment, approximately ten microtubules are projected to one pole and five microtubules to the opposite pole.[35] The force generated by these 15 microtubules, 750 pN, easily overcomes the tensile strength of DNA, which is around 250 pN.[38] Nonetheless, these estimates are based on theoretical calculations. Direct labeling of kinetochores after recovery from a nocodazole block[35] or in untreated cells with mild spindle defects[7] has indeed revealed centromere distortion. Considering that as few as five microtubules can match the strength of the chromosome, the cell is forced to stage a delicate balancing act between spindle severance and centromere rupture to resolve a merotelic attachment. This indicates that the mitotic spindle can cause chromosome rupture if not properly controlled.

Based on mouse models of mitotic checkpoint genes, generation of a high rate of aneuploidy apparently offers a good chance to find DNA damage. Although inactivation of checkpoint genes such as *BubR1*, *Bub3* or *Mad2* results in early embryonic death,[39] this lethality is partially rescued by the additional inactivation of *p53*.[40] Labeling of *Mad2*$^{-/-}$*p53*$^{-/-}$ embryonic fibroblasts for DSB showed DNA damage in well-defined subnuclear regions, which can be interpreted as spindle-induced DNA damage with subsequent activation of p53-mediated apoptosis.[40] Nuclear regions of DNA damage have also been found after induction of mitotic slippage by treatment with a minimally effective concentration of spindle poisons,[41,42] suppression of Cenp-E expression by interfering RNA,[41] or mutations in non-checkpoint genes such as *death inducer obliterator* (*Dido*) and *adenomatous polyposis coli* (*Apc*).[7] Mutations in genes coding for these accessory spindle proteins produce a mild phenotype compared to mutations in checkpoint genes, show little reduction in cell viability, and allow for a more detailed analysis of the associated breaks.

An apparently simple observation, but an important step for the interpretation of data from tumor samples, derives from work on primary *Dido* mutant cells. Disruption of the *Dido* gene causes merotelic attachments, centromere distortion, and lagging anaphase chromosomes.[7] Like *Mad2*$^{-/-}$*p53*$^{-/-}$ fibroblasts and nocodazole-blocked cells, *Dido* mutant cells accumulate DSB in mitosis.[7] Remarkably, the breaks generated by spindle defects localize on or adjacent to centromeres, although overall DSB repair defects can be excluded in the *Dido* mutant and centromeric DSB are repaired just as efficiently as elsewhere on the chromosome.[34] In accordance with the current view of merotelic attachments, breaks seem to affect only one of the two sister chromatids.[7] This localization was subsequently confirmed in other CIN cell lines such as *Apc* mutants. The discovery of centromeric DSB in mitosis provided the first direct indication that a poorly regulated spindle is able cause DNA damage, yielding important clues for the interpretation of data from tumor samples.

## Evidence from Tumors

Although a direct observation of centromere-localized breaks has been made in cell lines only, genetic analysis of tumor samples shows that chromosome rupture around centromeres is an active and common process in CIN. The most prominent result from a centromeric break, formed in mitosis, is the segregation of the two arms into different daughter cells. In this way, the gain of one arm and loss of the other are simultaneous. Spectral karyotyping showed that exactly this instability of chromosome arms occurs together with aneuploidy in *Dido* mutant cells.[7] Genetic mapping of breakpoints in a wide range of cell lines shows a comparable arm preference,[33] and clinical samples show frequent gains and losses of whole arms.[10,11,43-45] Whereas telomeric end-to-end fusion cannot fully explain this type of instability, a pericentromeric break that leaves the telomere intact agrees with the available data. The centromeric break does not yet explain how an arm is grafted onto another chromosome—interstitial telomeres indicate that broken arms tend to fuse to chromosome ends,[46]—but at least provides the required starting material. Although it is unlikely that spindle-induced rupture is selective for a specific centromere, not all chromosome arms undergo changes at the same rate; in many cases, one chromosome arm is lost while the other is preserved or amplified.[47] Arms that contain oncogenes such as *c-Myc* (8q), *ErbB2* (17q) or *Pik3CA* (3q) are amplified much more often than average.[33] Even though mitotic centromere rupture and gain of chromosome arms are random events, oncogenes located on amplified arms thus seem to offer a selective growth advantage.

In several types of carcinomas, low-grade tumors show mostly whole arm changes, whereas high-grade tumors have a more complex pattern of instability.[10,11] When an oncogene is present on a chromosome arm, its region could be amplified

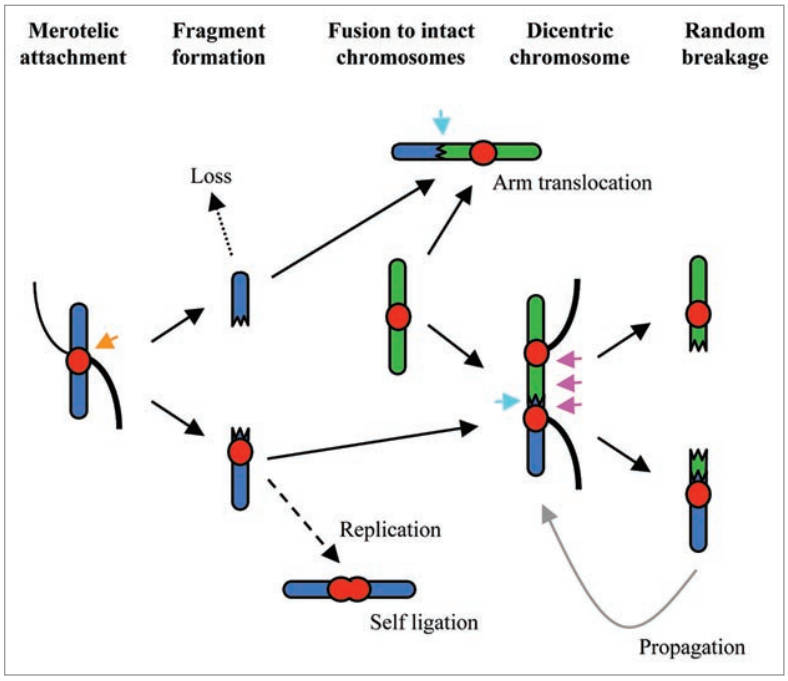


**Figure 1.** Starting the BFB cycle by merotelic attachment. An uncorrected merotelic attachment causes chromosome breakage at the centromeric region (orange arrow), resulting in two "reactive" chromosome arms. The arm without a centromere is easily lost in subsequent cell divisions, or can be rescued by a translocation. The centromere-containing arm can replicate and segregate, however, suffering only modest erosion of the broken end. The centromere-containing arm thus forms a reactive species that persists during subsequent cell divisions. The arm bearing the first break can self-ligate, forming a pseudodicentric chromosome, or fuse to a "healthy" chromosome (green), forming a true dicentric chromosome bearing an interstitial telomere (blue arrows). Capture of a dicentric chromosome by two spindle poles leads to secondary breaks, which can occur at any point between the two centromeres (purple arrows). This secondary, random breakage generates two new "reactive" arms, propagating the BFB cycle (grey arrow).

preferentially as the complexity of genetic alterations increases. The lack of a strong oncogene or tumor suppressor gene does not prevent instability of a particular arm, however, but tends to produce random secondary translocations as tumor growth progresses.[48] Mouse models of intestinal cancer, too, show gains and losses of whole chromosome arms,[13] although the corresponding syntenic regions are spread out over several chromosomes in humans. The gain or loss of an entire chromosome arm rather than of a small segment is thus a favored mechanism, and possibly represents one of the earliest events in CIN. A study that combined karyotyping and marker analysis showed that individual chromosome arms could be gained in near-diploid cells,[47] which suggests that no polyploid intermediate would be necessary to start the BFB cycle in cancer. Instead, and in agreement with the initial experiments in maize,[25] gaining a single "reactive" chromosome arm is enough to start an unstoppable chain of events (**Fig. 1**).

If centromeric breaks contribute to the early steps of tumorigenesis, they are expected to occur in premaligant or normal cells, albeit at low frequency. Analysis of merotelic attachments in cultured cells showed that centromere distortion is not uncommon in normal mitosis.[35,49] In vivo evidence of spindle-generated DNA damage has thus far been found only in embryos from *Dido* mutant mice, in the form of micronuclei containing damaged DNA.[7] In humans, we have to rely on genetic evidence for centromere rupture. Although extremely infrequent, the exact splitting of a chromosome at the centromere and formation of two autosomal rings, each corresponding to a single chromosome arm, has been observed.[50] In situ hybridization with probes flanking either side of the centromere detected centromeric breaks in a small proportion of lymphocytes from healthy individuals.[51] The occasional errors inherent to normal chromosome segregation could thus give rise to the breaks that characterize CIN, and in this way trigger the BFB cycle.

## Back to BFB

Although the role of telomerase and DSB repair are currently considered less important for the initiation of the BFB cycle, their activation in cancer and correlation to carcinogenesis cannot be denied. The observation that spindle defects can cause chromosome breakage gives rise to a very different picture, in which aneuploidy and BFB share a single origin—the spindle—that causes CIN. This common origin implies that phenotypes ascribed to aneuploidy, for example knockouts in mitotic checkpoint genes, could be caused in part by DNA damage. The uncertainty as to whether p53 is actually able to detect numerical chromosome changes could be related to this aneuploidy-associated DNA damage. Other observations are also easier to interpret when a single CIN is taken into account. A continuous de novo generation of breaks, increasing in number with the complexity of translocations, explains why DSB repair is activated in many carcinomas.[31,32] Also telomerase appears to be involved in a general repair pathway that "heals" chromosomes after induction of DSB.[52] In accordance with the previous observation, telomerase reactivation in a hereditary breakage syndrome takes place only after cells have gone through crisis.[53] This indicates that a certain degree of genomic instability is acquired before telomere regrowth, and that telomerase reactivation and enhanced DSB repair are mechanisms by which proliferating cancer cells escape apoptosis (**Fig. 2**).

The loss of a chromosome arm must be a dramatic event, so it is likely that the cell has some kind of mechanism to prevent or monitor breakage in mitosis. Several of the mitotic checkpoint proteins react to DNA damage and in response delay anaphase.[54-56] Although part of the mishap has already occurred once the break is formed, delaying anaphase at least prevents segregation of the broken halves. This mechanism not only allows additional time for repair of

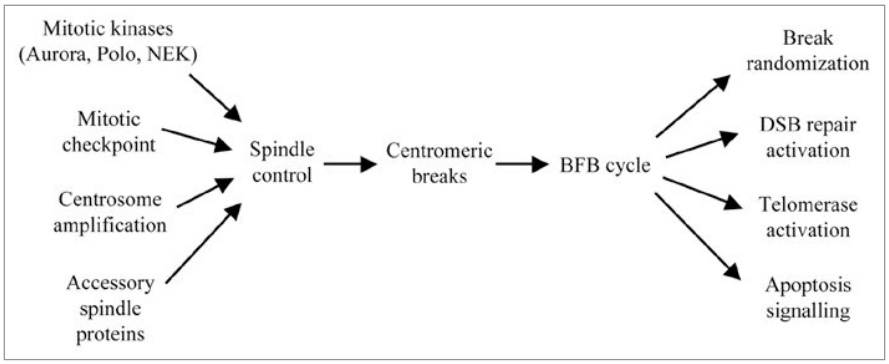


**Figure 2.** Causes and consequences of centromeric breaks. The mitotic spindle is influenced by several mechanisms, each of which can contribute to spindle defects; this probably explains the frequent occurrence of CIN compared to MIN tumors. Other alterations observed in CIN tumors, for example activation of break repair, activation of telomerase and suppression of apoptosis, are probably a cellular response to continuous break formation in the BFB cycle.

**Table 1.** Genetic defects that are explained by centromeric breaks

| Genetic alteration | Cause | Additional events |
|---|---|---|
| Aneuploidy | Spindle defects | - |
| Arm gains | Pericentromeric break, centromere-positive arm | Merotelic attachment |
| Arm losses | Pericentromeric break, centromere-nagative arm | Merotelic attachment |
| Arm translocation | Fusion of broken arm to healthy chromosome | Formation of interstitial telomere |
| Dicentric chromosome | Fusion of broken arm to healthy chromosome | Formation of interstitial telomere |
| Pseudodicentric chromosome | Self-fusion of broken centromere-positive arm | After S-phase replication |
| BFB cycle | Breakage of dicentric chromosomes | After initial fusion |

the break, but also makes the rejoining of the two ends physically possible.

## Closing Remarks

Conclusive identification of the origin of chromosomal instability, especially the moment at which a chromosome acquires its first break, is like a Big Bang theory for cancer. Just as astronomers are studying a universe billions of years old, most tumor cells we analyze have gone through so many unstable divisions that little or no relation to the first ancestor remains. Through the combination of animal models and detailed analysis of cancer cells, the potential mechanisms of carcinogenesis can nonetheless be verified by examining defects that occur repeatedly. In this way, we can conclude that centromeric breaks and a poorly controlled mitotic spindle explain most, if not all, features of CIN (**Table 1**). Animal models and cell lines derived from CIN tumors offer a reciprocal verification of the theory. In combination, the two provide a firm basis for the further study of early events in carcinogenesis.

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