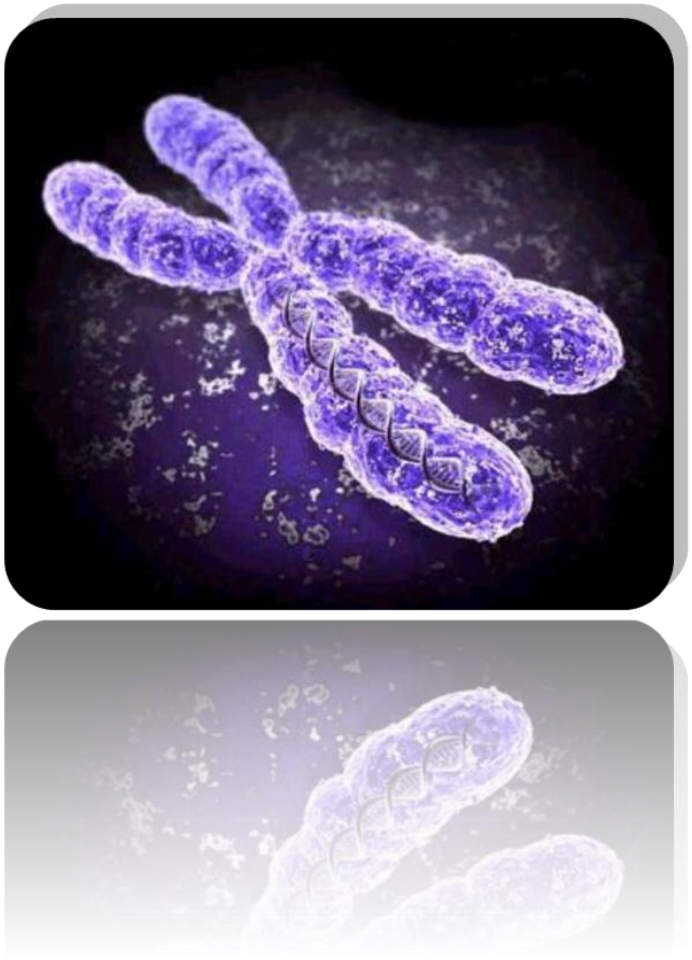
Genetic instability

its causes and its contribution to cancer



Janneke Peeters

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Genetic instability

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Abstract

Alterations within the genome, referred to as genetic instability, can occur at the whole chromosome level, i.e. whole chromosomal instability (W-CIN), as well as at the structural DNA level, i.e. genomic instability and structural chromosomal instability (S-CIN). Both types of genetic instability are frequently observed in tumor cells and are causally related to tumor formation. Until recently, it was thought that the mechanisms underlying these two types of genetic instability are distinct and occur independent of each other. However, several recent publications suggest that instability at the whole chromosome level can be a driving force of structural instability. This relationship between W-CIN and structural instability sheds new light on the mechanisms by which W-CIN can contribute to tumorigenesis.

1. Introduction

Genetic instability is defined as a cluster of events capable of causing alterations within the genome, either temporary or permanent (Perera and Bapat, 2007). There is compelling evidence that genetic instability is associated with tumor formation. One of the first theories about genetic instability and cancer came from Theodor Boveri in 1914. He observed that malignant tumor cells contained abnormalities in chromosome constitution and suggested that this is the cause of increased cell proliferation (reviewed by Manchester, 1995). Given the high amount of mutations observed in human cancers, an additional mutator phenotype for cancer cells has been proposed. This means that due to mutations in genes encoding proteins that are responsible for maintaining stability of the genome, there is an increased tendency to acquire even more mutations (Loeb, 1991). Finally, there are reports that indicate that rare truncations and inappropriate recombinations underlie the generation of cancer (Duesberg, 1987). Altogether, these findings have led to the conclusion that genetic instability associated with tumorigenesis can occur at two different levels, namely on the whole chromosomal level and on the structural level of DNA.

In this thesis, structural instability and whole chromosomal instability (W-CIN) and the mechanisms underlying these instabilities will first be explained. Secondly, attention will be given to the contribution of both types of instability to tumor formation. It has long been thought that structural instability and W-CIN are distinct processes and occur independent of each other. However, some recent publications suggest that this might not be the case. Literature data will be discussed which suggest that W-CIN can be a driving force of structural instability. These latest findings shed new light on the role of W-CIN in cancer.

2. Structural instability

Structural instability can be subdivided in changes on the nucleotide level, i.e. genomic instability, or at the chromosomal level, i.e. structural chromosomal instability (S-CIN). A detailed description of these two processes, the underlying mechanisms, and their contribution to cancer will be discussed below.

2.1 Genomic instability

Genomic instability involves single nucleotide changes, e.g. deletions, insertions, and substitutions. These changes can either be caused by defects in DNA mismatch repair (MMR) mechanisms or by exogenous mutagenic agents (Alberts *et al*., 2002; 249-250, 270 -271). During the normal process of DNA replication, despite proof-reading activity, some errors are made by DNA polymerase, for example the insertion of a wrong nucleotide. To prevent that impaired base pairing, due to insertion of a wrong nucleotide, results in erroneous protein formation, MMR enzymes travel down the DNA to check base pairing, remove the wrong base, and replace it with the correct base. Defects in MMR enzymes result in a type of genomic instability that is referred to as microsatellite instability (MIN). Every individual has some repetitive sequences in its DNA, named microsatellites. In general, microsatellites are repeating sequences composed of 1-6 base pairs. In case of a mutation in one or more MMR enzyme(s), microsatellites become highly unstable and shorten or elongate, hence the name microsatellite instability (Alberts *et al*., 2002; 249-250, 1353-1354). DNA damage due to exogenous agents, such as UV-light, is repaired by the nucleotide excision repair (NER) mechanism (Alberts *et al*., 2002; 270-271). A defective NER mechanism leads to an increase in alterations within the DNA; the resulting genomic instability is named NER-associated instability (NIN) (Lengauer *et al.*, 1998).

2.2 Structural chromosomal instability (S-CIN)

S-CIN involves rearrangements of chromosomes, i.e. (un)balanced translocations, deletions, amplifications, and inversions. A balanced translocation involves equal exchange of chromosome components, without loss of genetic information. In case of an unbalanced translocation, there is an unequal exchange and thus eventually loss of genetic information. Exchange of chromosome parts can alter gene activity, can lead to the generation of fusion proteins or truncated proteins, etc. (Lengauer *et al.*, 1998).

The initiator of S-CIN is often the presence of chromosome breakages. Broken chromosomes can occur via different mechanisms. Common causes are defects in DNA double-stranded break (DSB) repair mechanisms, a shortening of telomeres, or the presence of fragile sites (Bailey and Murnane, 2006; Lukusa and Fryns, 2008; van Gent *et al.*, 2001). The latter refers to heritable, specific

chromosome regions that are likely to break when DNA replication is partially inhibited. Chromosome breakage results in the formation of a chromosome fragment with a centromere, i.e. centric, and a fragment without a centromere, i.e. acentric. Acentric fragments cannot bind to the spindle pole and are either lost during cell division or fuse to intact chromosomes. This kind of chromosome fusion results in a translocation, which is stably transmitted to daughter cells (Mitelman *et al.*, 2007).

In contrast to acentric chromosome fragments, centric chromosome fragments can lead to the formation of unstable translocations. The underlying mechanism is the breakage-fusion-bridge (BFB) cycle (McClintock, 1941). Consider a centric chromosome containing gene A, with a broken end (Figure 1, panel 1). In the absence of a correct DNA repair mechanism, this break is not repaired and DNA replication occurs, leading to a chromosome pair with one damaged sister chromatid that is inherited by one of the daughter cells (Fig. 1, panel 2 and 3). If this daughter cell goes through S-phase, the broken chromosome is replicated, after which fusion can occur between the broken ends of two sister chromatids during the subsequent prophase (Fig. 1, panel 4). Due to the presence of a centromere in each chromatid, a dicentric chromosome is formed. Microtubules attached to these centromeres pull them apart to opposing spindle poles, a process called anaphase, resulting in a bridge configuration. At a certain moment, the applied force of the spindle poles exceeds the strength of the chromosome and a break is introduced (Fig. 1, panel 5 and 6). Such breaks can occur at any point between the two centromeres, thus leading to a diverse range of centric chromosome fragments (Fig. 1, panel 7). In panel 7 an example is shown of the formation of a centric chromosome fragment containing a duplicated gene. Chromosome fragments are randomly distributed between the daughter cells and thus there is loss of genetic information. During subsequent cell division of these daughter cells and their offspring, centric chromosome fragments can fuse to other fragments, adapt a bridge conformation and can be ruptured. This recurring process of chromosome breakage, fusion and bridging is thus referred to as the BFB cycle. Eventually this results in gene deletion, gene amplification or gene translocation when fusion occurs between non-sister chromatids (Alberts *et al*., 2002; 1345). In addition to linear chromosomes, circular chromosomes that have exchanged genetic information with their sister chromatids can also fuse and form bridges. These bridges can break at any given point and thus result in chromosome rings genetically different from each other and their mother cell (Gisselsson *et al.*, 2000).

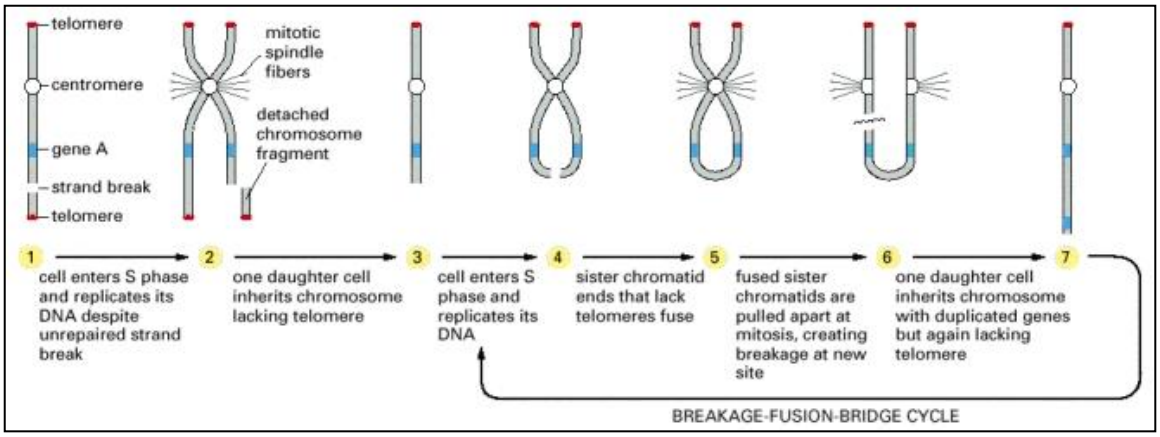

Figure 1 | Breakage-fusion-bridge (BFB) cycle. A chromosome containing a DSB near its end enters S-phase without being repaired, due to defective DNA repair mechanisms. S-phase occurs erroneously, but one daughter cell will inherit the chromosome with the broken end. After replication in the daughter cell, the broken ends of two sister chromatids can be fused together during prophase, resulting in a dicentric chromosome. Separation of the two sister centromeres results in a bridge conformation. Due to the applied force on the centromeres, eventually a break is introduced within the chromosome. Repetition of the BFB cycle in further cell divisions will lead to structural chromosomal rearrangements (Alberts *et al*., 2002; 1345).

2.3. Relationship between structural instability and cancer

One can imagine that mutations in MMR and NER enzymes result in increased mutations within the DNA. If these mutations occur in tumor suppressor genes or proto-oncogenes, this can eventually result in cancer. This has been demonstrated using mice deficient for certain MMR or NER enzymes. For example, mice deficient for the MMR genes *Pms2, Mlh1* or *Msh2* display an increase in the formation of spontaneous tumors and develop tumors at an earlier age than wild-type mice (reviewed by Prolla *et al.*, 1996). This is in agreement with the observation that patients with hereditary non-polyposis colorectal cancer (HNPCC) contain inactivating mutations in the MMR genes *MSH2* and *MLH1* (Peltomaki and de la Chapelle, 1997). Deletion of the NER gene *Xeroderma pigmentosum complementation group A* (*Xpa*) in mice leads to an increased susceptibility to carcinogen-induced tumors (de Vries *et al.*, 1995). Indeed, mutations in NER genes are commonly found in patients with Xeroderma pigmentosum that have a high number of skin tumors (Cleaver, 1986). MMR and NER mutations are recessive, indicating that in order to promote tumorigenesis both alleles must be defective. In addition, for NIN, an exogenous mutagen is necessary (Lengauer *et al.*, 1998).

S-CIN is frequently observed in solid tumors and blood cancers. Single, balanced translocations are associated with blood cancer, while solid tumors often contain numerous unbalanced translocations, amplifications, deletions, and inversions that differ between the cells of a tumor (Mitelman database, 2011). Gene amplifications and deletions can contribute to cancer by altering the gene dosage of genes that have either a tumor-promoting or tumor-suppressing effect (Trent, 1990). In addition, alteration of the gene copy number of one or multiple genes can result in a protein imbalance, which can favor tumor formation. This will be discussed in more detail further on

in this thesis. Translocations can promote tumor formation through two different mechanisms: either by deregulation of gene expression at one of the two breakpoints or by the generation of a hybrid, chimeric gene via the fusion of two gene segments originally located on different chromosomes (Mitelman *et al.*, 2007). B-cell and T-cell malignancies are often characterized by the first mechanism, more specifically by translocations that place a random gene adjacent to immunoglobulin enhancers or regulatory elements of T-cell receptor genes, respectively. For instance, Burkitt's lymphoma cells contain a certain translocation that juxtaposes the *MYC* gene with an immunoglobulin gene, resulting in a constitutively active *MYC* gene (Dalla-Favera *et al.*, 1982; Neel *et al.*, 1982; Taub *et al.*, 1982). A direct link between this genetic alteration and tumor formation has been shown in mice (Adams *et al.*, 1985). Chimeric genes often comprise gene segments of transcription factors and tyrosine kinases, resulting in an altered catalytic activity of these proteins. A well-known chimeric gene results from the fusion of the *BCR* and *ABL1* genes, leading to a constitutively active kinase. The BCR-ABL fusion protein is a marker of chronic myelogenous leukemia (CML) and several acute forms of leukemia (Kurzrock *et al.*, 1988). A causal relationship between the BCR-ABL gene product and leukemia has been shown using mice transgenic for the BCR-ABL fusion protein (Daley *et al.*, 1990; Elefanty *et al.*, 1990; Heisterkamp *et al.*, 1990).

3. Whole chromosome instability (W-CIN)

W-CIN is defined as a persistent rate of gain or loss of whole chromosomes. A consequence of W-CIN is aneuploidy, i.e. a state in which the chromosome number differs from the normal number of chromosomes (Thompson *et al.*, 2010). It is important to note that W-CIN and aneuploidy are not the same; W-CIN is a 'rate' while aneuploidy describes a 'state'. This difference can be illustrated with the Down syndrome. Due to a defect in meiosis, one of the gametes contains an extra copy of chromosome 21, which results in the formation of a zygote containing three copies of chromosome 21, i.e. trisomy 21. Subsequently, each cell of a patient with Down syndrome will be trisomic for chromosome 21, but there is no rate of chromosome gain or loss within these cells. Therefore, cells of patients with Down syndrome do not display W-CIN, but each cell is aneuploid (Geigl *et al.*, 2008). W-CIN has an adverse effect on cell growth; therefore certain compensatory mechanisms that allow efficient proliferation of aneuploid cells are, together with W-CIN, responsible for the generation of highly aneuploid cells (Thompson and Compton, 2008).

3.1. Mechanisms underlying W-CIN

The gain or loss of whole chromosomes can be caused by several events. Proper attachment of centrosome-derived microtubules to the kinetochores of chromosomes is an important process for correct chromosome segregation. Kinetochore-microtubule attachment is a dynamic process,

during which individual microtubules continuously associate and dissociate from the kinetochore (reviewed by Thompson *et al.*, 2010). Early in mitosis in mammalian cells, single kinetochores frequently bind to microtubules deriving from both spindle poles. Such a binding is called a merotelic attachment, i.e. merotely (Fig. 2, right panel). Due to the dynamicity of kinetochore-microtubule attachment, these merotelic attachments can be corrected before the onset of anaphase. Thus, bi-orientation is ensured and the chromosomes are properly segregated (Cimini *et al.*, 2003). However, if such merotelic attachment is not corrected, the chromosome lags behind the properly segregating chromosomes during anaphase. These so-called lagging chromosomes may end up being missegregated. Cancer cells with a W-CIN phenotype have more stable kinetochore-microtubule attachments compared to normal diploid cells (Bakhoum *et al.*, 2009). This leads to a decreased ability to correct merotelic attachments and therefore lagging chromosomes are frequently observed in these cells (Thompson and Compton, 2008). Interestingly, a recent study, which tracked the fate of lagging chromosomes, showed that these chromosomes rarely missegregate and predominantly end up in the correct daughter cell as micronuclei. Instead, W-CIN is rather caused by chromosomes with multimerotelic attachments, i.e. attachments of which most of the microtubules are oriented towards the wrong pole. These chromosomes do not lag in anaphase but segregate to the same spindle pole as their sister chromatid and thereby induce aneuploidy (Thompson and Compton, 2011).

Recent studies have shown that merotely is also caused by supernumary centrosomes, a phenotype previously proposed to cause W-CIN by promoting multipolar divisions (Ganem *et al.*, 2009; Silkworth *et al.*, 2009; Nigg, 2002). The number of centrosomes determines the number of spindle poles that are being formed during mitosis. Therefore, it has long been thought that multiple centrosomes lead to multipolar cell division instead of bipolar cell division and thus result in the formation of more than two aneuploid daughter cells or mitotic cell death (Fig. 2, lower panel) (Nigg, 2002). However, a recent study has demonstrated that cells with supernumerary centrosomes rarely undergo multipolar cell divisions (Ganem *et al.*, 2009). The idea is that cells with multiple centrosomes coalesce their centrosomes into bipolar spindles poles late in metaphase, but that the multipolar prometaphase favors the formation of merotelic attachments. This increases the risk at lagging chromosomes and thus contributes to W-CIN (Fig. 2, lower panel) (Ganem *et al.*, 2009; Silkworth *et al.*, 2009).

The mitotic checkpoint (MC) is a surveillance system that ensures proper chromosome segregation during mitosis. The MC does this by delaying movement of chromosomes to opposite poles of the cell until all chromosomes are attached via their kinetochore to microtubules from opposite spindle poles. One of the main components of the MC is the mitotic checkpoint complex (MCC), which in humans is composed of BUBR1, BUB3, and MAD3, and inhibits the anaphase

promoting complex/cyclosome (APC/C) by modulating CDC20, the activator of APC/C. In addition, there are a lot of other proteins associated with the MC and downstream signaling (reviewed by Suijkerbuijk and Kops, 2008). A weak MC can be caused by several events, for instance by altered levels of MC components, disturbed post-translational modification of MC proteins or loss-of-function mutations in one of the checkpoint genes (reviewed by Weaver and Cleveland, 2006; Kops *et al*., 2005; Suijkerbuijk and Kops, 2008). However, a clear correlation between altered expression levels of MC components and aneuploidy has not been observed yet and loss-of-function mutations are not frequently detected in human cancers. A weak MC leads to premature sister chromatid separation, generating one daughter cell with an extra chromosome and one daughter cell missing a chromosome (Fig. 2, left panel). An overactive MC, due to a gain-of-function mutation, is thought to lead to prolonged mitosis, which increases the risk of merotely and eventually results in the generation of lagging chromosomes (Sotillo *et al.*, 2007; Diaz-Rodriquez *et al*., 2008). In addition to components of the MC, defects in several non-mitotic checkpoint proteins involved in cell-cycle regulation are associated with W-CIN. Most of these proteins are tumor suppressor proteins, for example adenomatous polyposis coli protein (APC), retinoblastoma (RB), p53, RE1-silencing transcription factor (REST), and Von Hippel-Lindau protein (pVHL) (reviewed by Thoma *et al.*, 2011). For instance, p53 and RB, proteins that both inhibit the G1- to S-phase transition of the cell cycle, are involved in centrosome duplication during mitosis (Fukasawa *et al.*, 1996; Meraldi *et al.*, 1999). REST, RB, and pVHL are important for maintaining appropriate levels of the MC protein MAD2, the first two being inhibitors of MAD2 and the latter an activator (Guardavaccaro *et al.*, 2008; Thoma *et al.*, 2009; Hernando *et al*., 2004).

An extensive study identifying genes associated with W-CIN in colorectal cancers revealed that genes that regulate sister chromatid cohesion are frequently mutated in these cancers (Barber *et al.*, 2008). Sister chromatid cohesion is established by cohesin, a multimeric protein complex that keeps the sister chromatids together and gets cleaved by separase after proper alignment and attachment of chromosomes to microtubule from opposing spindle poles, i.e. the metaphase (Alberts *et al*, 2002; 1001). It is thought that cohesin molecules form a ring-like structure that embraces the sister chromatids (reviewed by Barbero, 2011). It has been reported that separase overexpression can induce aneuploidy, indicating that premature loss of sister chromatid cohesion gives rise to W-CIN (Fig. 2, middle panel) (Zhang *et al*., 2008). Furthermore, both overexpression and knock-out of securin, the protein that inhibits separase activity before the onset of anaphase, has also been shown to result in chromosome missegregation (Yu *et al*., 2003; Jallepalli *et al.*, 2001). These results suggest that W-CIN can result from both premature sister chromatid separation and failure of chromatids to segregate. In addition to colorectal cancers, a recent publication shows that a diverse range of tumors contains an inactivating mutation or deletion in *STAG2*, encoding one of

the subunits of the cohesion complex, and that this results in aneuploidy (Solomon *et al.*, 2011). Repair of this mutation reverted the defects observed in sister chromatid cohesion showing that aberrant cohesion can be a cause of aneuploidy.

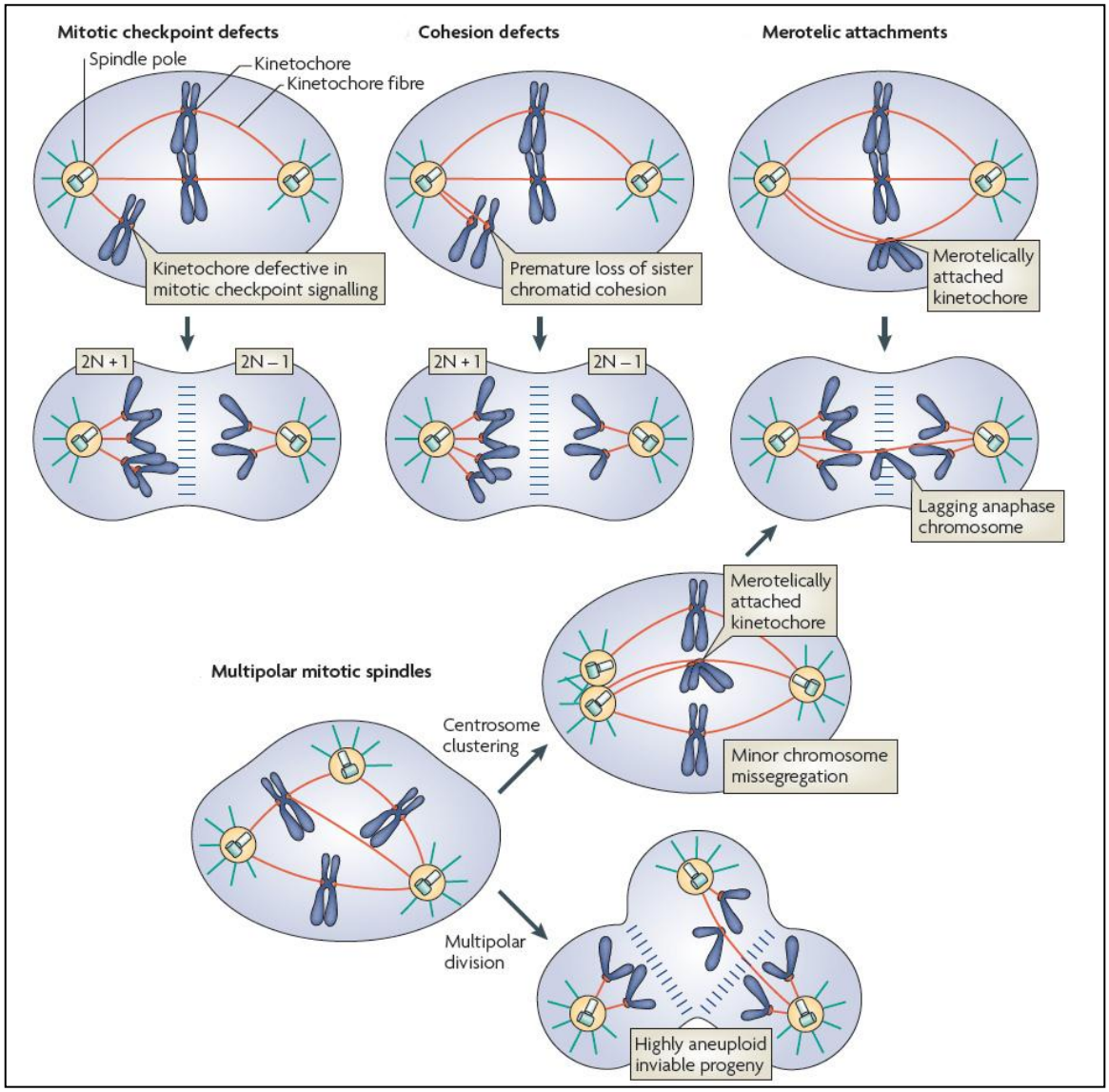

Figure 2 | Mechanisms that can induce W-CIN and lead to aneuploidy. *Left panel:* Mitotic checkpoint (MC) defects. A defective MC does not signal that there is an unattached kinetochore, and thus sister chromatid separation occurs while not all chromatids are attached to opposing spindle poles. *Middle panel:* Cohesin defects. Premature sister chromatid separation or failure of chromatids to segregate compromises proper chromosome segregation. *Left panel:* Merotelic attachments. Single kinetochores frequently bind to microtubules deriving from both spindle poles. If such attachment persists into anaphase, lagging chromosomes are generated which increase the risk at missegregation. *Lower panel*. Multiple centrosomes can induce multipolar mitosis which results in mitotic cell death, or cluster which increases the risk at merotely and results in an increase in the frequency of lagging chromosomes (Adapted from Holland and Cleveland, 2009).

3.2 Relationship between W-CIN and cancer

Aneuploidy is observed in more than 90% of the solid tumors and in 50% of leukemias and lymphomas (Mitelman database, 2011). To study the relationship between W-CIN and cancer, several mouse models have been developed in the past decade (reviewed by Foijer *et al.*, 2008).

These W-CIN mouse models are generated by disruption of the MC. Since homozygous knockouts of MC genes are embryonically lethal, W-CIN mouse models are heterozygous or hypomorphic for MC genes. Hypomorphic mouse models are characterized by a reduction of protein expression below 50%. These mouse models are of particular interest since some heterozygous mouse models do no lead to an overt phenotype because half of the protein amount is still sufficient for the MC to function properly (Foijer *et al.*, 2008).

Examining W-CIN mouse models for tumor formation has led to some puzzling results considering the contribution of W-CIN to tumorigenesis. Heterozygocity/hypomorphicity for *Bub1b*, encoding BUBR1, although causing significant aneuploidy in MEFs, did not cause spontaneous tumor formation in mice (Baker *et al.*, 2004; Dai *et al.*, 2004). In addition, mice heterozygous for *Bub3* showed the same tumor-free phenotype (Kalitsis *et al.*, 2000). However, when these mice were treated with a carcinogen, tumor formation was increased compared to similarly treated wild-type mice. This suggests that defects in the MC and the resulting W-CIN are not sufficient to induce tumorigenesis, but that they rather facilitate it. Interestingly, mice hypomorphic for *Bub1b* show a premature aging phenotype and, on average, do not exceed a lifespan of 6 months (Baker *et al.*, 2004). Although a similar phenotype is observed in mice haploinsufficient for both *Bub3* and *Rae1*, further experiments have shown that this early aging phenotype is probably related to other events than aneuploidy (Baker *et al.*, 2006).

In contrast to mouse models for *Bub3* and *Bub1b*, mice hypomorphic for *Bub1*, heterozygous for *Cenp-E*, *Mad1* or *Mad2*, or overexpressing *Mad2*, all spontaneously developed tumors (Dobles *et al.*, 2000; Iwanaga *et al.*, 2007; Jeganathan *et al.*, 2007; Michel *et al.*, 2001; Silkworth *et al.*, 2009; Weaver *et al.*, 2007). This suggests that in these cases W-CIN is sufficient to drive tumorigenesis. An assumption that can be made based on the outcomes of all the W-CIN mouse models is that some MC proteins can initiate cancer while others cannot. An explanation for this could be that the ability of MC proteins to promote cancer is related to their secondary cellular functions, outside of mitosis. For example, it has recently been reported that BUB1 is important for the DNA damage response and that reduction of BUB1 levels leads to delayed DNA repair (Yang et al., 2011). In mice that are hypomorphic for *Bub1*, this additional function is likely to contribute to tumorigenesis. For BUBR1 a role has been described in the induction of apoptosis in cells that exit mitosis without segregating their chromosomes (Shin *et al.*, 2003). In addition, both BUBR1 and BUB3 can act as repressors of gene transcription in interphase, i.e. the interval between the end and start of mitosis (Yoon *et al.*, 2004).

Examination of aneuploidy levels of MEFs and splenocytes of different W-CIN mouse models shows that there is no clear correlation between the level of aneuploidy and spontaneous tumor formation. For example, MEFs of mice hypomorphic for *Bub1* display similar levels of aneuploidy as

MEFs from mice hypomorphic for *Bub1b*, but only *Bub1* hypomorphic mice spontaneously develop tumors (Jeganathan *et al.*, 2007; Baker *et al.*, 2004). This observation seems to support the idea that tumor formation is related to secondary functions of MC proteins. However, it is important to note that due to technical limitations only the aneuploidy levels of splenocytes and MEFS can be determined and that these levels might not be representative for tumor-prone cell types since these cell types require long-term culturing instead of short-term culturing like splenocytes. This is underscored by the observation that the aneuploidy levels of splenocytes strongly differ from those of germ cells in mice that contain a mutation in one or more of the following proteins: MAD2, BUBR1, BUB3, REA1, and NUP98 (Jeganathan *et al*., 2006). This outcome suggests that different tissues exhibit different sensitivities for reduction or deletion of a certain MC protein. This is supported by the notion that spontaneous tumor formation in the majority of W-CIN mouse models preferentially occurs in the lung.

The two aforementioned findings suggest that genetic context is a major contributing factor in determining whether aneuploid cells transform into tumor cells (Fig. 3). This provides a likely explanation for the distinct outcomes of the W-CIN mouse models. The importance of genetic context is corroborated by the observation that deletion of the tumor suppressor gene $p16^{Ink4a}$ in mice hypomorphic for *Bub1b* ($Bub1b^{-/H}$) results in a significant increase in the incidence of tumors, specifically in the lung. When another tumor suppressive gene was deleted, $p19^{Arf-/-}$, such increase in tumor formation was not observed (Baker *et al.*, 2008). Another striking example of the importance of genetic context in modulating the effect of W-CIN on tumor formation is observed when *Bub1b* haploinsufficiency ($Bub1b^{-/+}$) is combined with a mutated *Apc* allele ($Apc^{+/Min}$). This compound haploinsufficiency results in an increase in the amount of colon tumors while there is a decrease of small intestinal tumors (Rao *et al.*, 2005). These results indicate that W-CIN can also have a tissue restricted tumor suppressive effect. Mice deficient for securin ($Pttg^{-/-}$) combined with *Rb* haploinsufficiency show a similar tissue restricted inhibition of tumor development (Chesnokova *et al.*, 2005). Even more strikingly are the observations in mice heterozygous for *Cenp-E* (Weaver *et al.*, 2007). As described earlier, these mice showed an increase in spontaneous tumor formation, especially lymphomas and lung tumors. However, compared to wild-type mice, these mice displayed a decrease in spontaneous liver formation. In addition, the *Cenp-E* $^{-/+}$ mice seemed to be protected against carcinogen-induced tumors and when combined with a $p19^{Arf-/-}$ background, tumorigenesis was delayed. In conclusion, the relationship between W-CIN and cancer is quite complex. Whether W-CIN and the resulting aneuploidy in a certain tissue lead to tumor formation seems to depend on the genetic context, which determines the sensitivity of a certain tissue to develop aneuploidy.

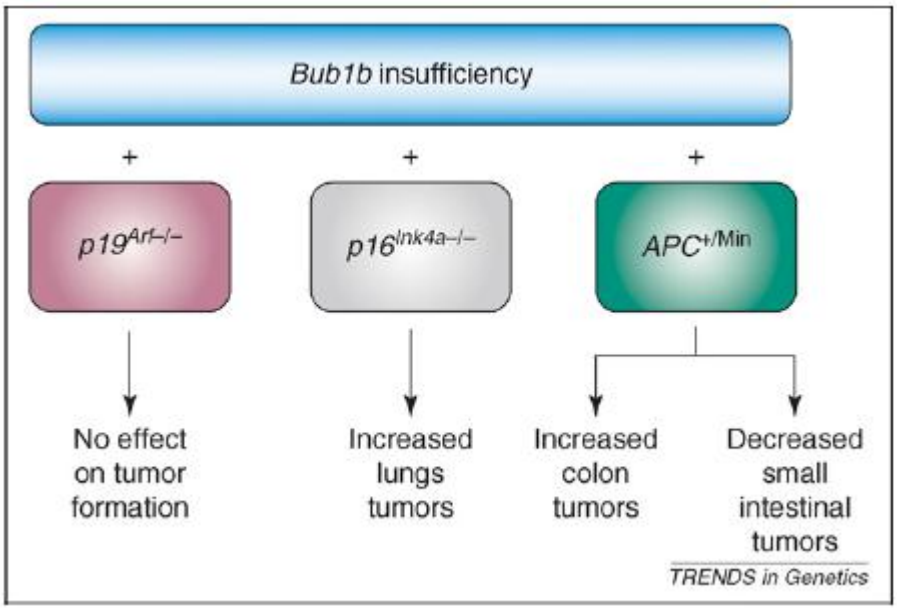

Figure 3 | The importance of genetic context in modulating the effect of W-CIN on tumor formation. *Bub1b* insufficient mice display W-CIN. Deletion of the tumor suppressor gene $p16^{Ink4a}$ in these mice accelerates tumor formation in the lung, while deletion of the tumor suppressor gene $p196^{Arf-/-}$ has no effect on tumor formation. Likewise, mutation of one of the *Apc* alleles increases colon tumor formation, while the number of small intestinal tumor formation is reduced (Adapted from Ricke et al., 2008).

4. Tumor-promoting mechanisms of W-CIN

As shown in the previous paragraph, W-CIN is associated with tumor formation. Over the past few years, several mechanisms have been proposed explaining the link between W-CIN and cancer. These mechanisms, which will be discussed in detail below, include alteration of gene copy number/protein imbalance, loss of heterozygosity of certain tumor suppressor genes, and the acquisition of aneuploidy-tolerating mutations. In addition to this, based on recent publications, a newly proposed mechanism linking W-CIN to cancer will be discussed.

4.1 Tumor-promoting mechanisms of W-CIN: initial thoughts

Due to the gain or loss of whole chromosomes, W-CIN and aneuploidy have an enormous impact on gene expression. The subsequent alteration of the expression of numerous genes, i.e. gene dosage alteration, leads to an imbalance in protein levels. The most direct consequence is alteration of the efficiency of the function of the protein(s). In addition, a protein imbalance can result in an increase in promiscuous interactions, impaired protein folding due to the sequestration of chaperones, and an increase in the abundance of other proteins due to sequestration of turnover mechanisms (reviewed by Sheltzer and Amon, 2011). If the gene dosage of a subunit of a multimeric protein complex that functions in regulating gene expression is altered, this changes the stoichiometry of the subunits of the multimeric complex. This will have an effect on assembly and function of the complex as a whole and thus affect gene expression. This latter principle is referred to

as the gene balance hypothesis (Birchler *et al.*, 2005; Birchler and Veitia, 2007). All the aforementioned consequences of protein imbalance can strongly promote tumorigenesis if they result in activation of tumor-promoting pathways or inhibition of tumor-protective pathways. However, results of experiments performed in aneuploid yeast cells and analysis of protein levels of genes encoded by chromosome 21 in Down Syndrome patients have shown that cells try to compensate for dosage alterations, both at the protein and the transcriptional level (Cheon *et al.*, 2003a; Cheon *et al.*, 2003b; Cheon *et al.*, 2003c; Cheon *et al.*, 2003d; Torres *et al.*, 2007). These findings suggest that either the temporary protein imbalance that is present before compensation occurs contributes to tumor formation or that other mechanisms are involved in explaining the link between aneuploidy and cancer (see below).

Another example of the impact of aneuploidy on gene expression associated with tumor formation comes from a study that showed that W-CIN could promote tumorigenesis by the induction of tumor suppressor loss of heterozygosity (Baker *et al.*, 2009). Mice insufficient for the MC protein BUB1 were crossed onto a $p53^{+/-}$ background and showed a dramatic increase in tumor formation compared to mice displaying only low BUB1 levels or haploinsufficient for *p53*. Examination of the tumor cells of $Bub1^{low}p53^{-/+}$ mice revealed a complete loss of the wild-type *p53* allele. Remarkably, the lymphoma cells showed gain of an extra copy of chromosome 11 (containing the knockout *p53* allele). Similar results were obtained for mice displaying a mutated *Apc* allele, $Apc^{+/Min}$, combined with low BUB1 levels, although in this case the effect was colon specific. Since both mouse models show high chromosome missegregation rates due to low BUB1 levels, it seems likely that this is the cause of the subsequent chromosome loss and gain. Nevertheless, low BUB1 levels do not promote tumorigenesis in all mouse models haploinsufficient for a tumor suppressor gene. When crossed onto an $Rb^{-/+}$ background, no increase in tumor formation was observed while chromosome missegregation rates were high and loss of heterozygosity occurred. Even more strikingly, in $Pten^{-/+}$ mice low BUB1 levels suppressed the development of prostate cancer (Baker *et al.*, 2009). Altogether, these results show that W-CIN can promote tumorigenesis via loss of heterozygosity of tumor suppressor genes, but again underscores the importance of genetic context and tissue context (see also paragraph 3.2). For example, W-CIN due to *Bub1b* insuffiency might promote loss of heterozygosity of the $p16^{Ink4a}$ allele and the wild-type *Apc* allele, the latter specifically in the colon, and thereby induce tumor formation in these mice.

In mammalian and yeast cells it has been reported that chromosome gain has a detrimental effect on cellular fitness (Torres *et al.*, 2007; Williams *et al.*, 2008). Diploid mouse embryonic fibroblasts (MEFs) trisomic for a certain chromosome and haploid yeast cells disomic for one or more chromosomes, both display a decreased proliferation capacity. This phenotype was more pronounced in MEFs and yeast cells carrying an extra copy of a large chromosome. In aneuploid yeast

strains a delay in G_1-phase seemed to underlie the proliferation defect. In addition, in both cell lines general alterations in cellular metabolism were detected, although these alterations differed between aneuploid MEFs and yeast cells (Torres *et al.*, 2007; Williams *et al.*, 2008). For disomic yeast strains it was also observed that they were extremely sensitive to conditions that interfere with transcription, translation, and protein folding (Torres *et al.*, 2007). Altogether, these results point to a decrease in cellular fitness, which seems at odds with a role for aneuploidy in cancer. However, compensatory mutations may occur that enable toleration of the adverse effects of aneuploidy and subsequently allow the advantageous effects of aneuploidy to become apparent. Characterization of the genetic changes in disomic yeast strains with an increased proliferation capacity revealed that there are two classes of aneuploidy-tolerating mutations: strain-specific mutations and genetic alterations that are shared by several strains (Torres *et al.*, 2010). An example of the latter is a *UBP6* mutation. UBP6 possesses proteasome-inhibitory activity and thus delays protein degradation (Leggett *et al.*, 2002). Due to the presence of an extra chromosome, there is excessive protein production, which poses a major burden on cellular protein quality-control mechanism. Inactivation of *UBP6* allows increased degradation of excessive proteins and since excessive protein production underlies the adverse effects of aneuploidy, this enables toleration of aneuploidy (Torres *et al.*, 2007; Torres *et al.*, 2010). In combination with growth-promoting mutations this can eventually lead to a situation in which aneuploidy promotes tumorigenesis (Torres *et al.*, 2008). Such a growth-promoting mutation could, for instance, be a mutation in the p53 pathway. Induction of chromosome missegregation in a diploid cancer cell line in culture generates aneuploid cells. These cells are delayed in the cell cycle due to elevated levels of p53 and one of its targets, p21. Deletion of *p53* releases the growth inhibitory effect and allows accumulation of aneuploid cells in culture (Thompson and Compton, 2010).

4.2 Tumor-promoting mechanisms of W-CIN: a new insight

Several recent, independent publications indicate that, besides the above-mentioned mechanisms, W-CIN can promote tumor formation via another mechanism: i.e. by the induction of structural instability.

Compelling evidence for this mechanism comes from a study performed in aneuploid yeast strains (Sheltzer et al., 2011). Thirteen yeast strains, each carrying a different additional chromosome, were analyzed for the development of genetic alterations. Remarkably, each strain displayed one or more forms of instability. For example, increased spontaneous mitotic recombination, enhanced forward mutagenesis, an increased number of DNA DSBs, and an increased rate of MIN were observed. These observations indicate that genomic repair mechanisms might be impaired. Indeed, defects in recombinational repair and DNA repair mechanisms were detected.

Together, these events lead to enhanced sensitivity to certain genotoxic agents. The cause of this genomic instability in aneuploid yeast strains has been shown to be a stoichiometric imbalance in protein levels (Sheltzer et al., 2011). In conclusion, this study shows that aneuploidy can drive a mutator phenotype. Since a mutator phenotype is a common feature of cancer cells, and aneuploidy is a consequence of W-CIN, this sheds new light on the contribution of W-CIN to tumor formation. These findings are also an argument for the aforementioned necessity of aneuploid cells to acquire mutations to compensate for the adverse effects on cellular fitness, in particular protein homeostasis, in order to compete with non-aneuploid cells.

In addition to the discovery that aneuploidy can drive structural instability at the nucleotide level, another paper shows that chromosome segregation errors, i.e. W-CIN, can be a cause of structural chromosomal aberrations (Janssen *et al.*, 2011). In chromosomally unstable cancer cells, the most frequently occurring cause of missegregation are lagging chromosomes, mainly due to merotelic attachments (Thompson and Compton, 2008; Cimini *et al.*, 2001). To study the consequences of W-CIN on chromosome integrity, this type of segregation errors were experimentally induced in nontransformed human retinal pigment epithelial (RPE-1) cells by treating them with an MPS1 inhibitor or Monastrol. Monastrol acts by inhibiting the motor protein Eg5 that is essential for the formation and maintenance of a bipolar mitotic spindle (Sawin *et al.*, 1992). Cells recovering from Monastrol treatment show an increase in the formation of erroneous kinetochore-microtubule attachments which gives rise to W-CIN (Bakhoum *et al.*, 2009; Cimini *et al.*, 2001). As an alternative model system, tumor cell lines displaying W-CIN were used. In both models, in addition to an increase in chromosome missegregation, the number of DNA DSBs was increased in lagging chromosomes. The DSBs were generated during cytokinesis, probably by forces applied by the cleavage furrow since the DBSs were predominantly positioned at DNA locations that were located within the cleavage furrow. As described earlier, chromosomal breakage can lead to the induction of the BFB cycle, which gives rise to deletions, amplifications, and translocations. Indeed, the prevalence of structural chromosomal aberrations in cells displaying W-CIN was significantly enhanced, despite the induction of a DNA damage response (Janssen *et al.*, 2011). These results indicate that chromosomal instability on the whole chromosomal level, W-CIN, can give rise to chromosomal instability at the structural level, S-CIN. Since S-CIN is frequently observed in both solid tumors as well as blood cancers, this might explain tumor formation in the previously described mouse models for W-CIN.

Results of a study performed by Guerrero and colleagues seem to be in line with the observation that W-CIN can induce structural instability (Guerrero *et al.*, 2010). In this study MEFs derived from mice bearing a mutation in the *death inducer obliterator* (*Dido*) gene were used. The main product of *Dido* is Dido3, a centrosome-associated protein (Futterer *et al.*, 2005). Dido

disruption results in early degradation of BUBR1. In addition to its checkpoint function, BUBR1 plays a role in establishing kinetochore-microtubule attachments (Elowe *et al*., 2007; Huang *et al*., 2008). Besides an impaired MC, *Dido* mutants thus suffer from spindle abnormalities. Together with an increase in the frequency of lagging chromosomes, it has therefore been suggested that *Dido* mutant MEFS display W-CIN (Trachana *et al.*, 2007). As a consequence of this instability, both *Dido* mutant MEFS and *Dido* mutant embryo's contained an increased amount of micronuclei with a concomitant increase in the amount of DNA DSBs compared to the wild-type. Since *Dido* disruption affects spindle assembly, and spindle defects have been reported to cause DNA damage in cancer cells, it is possible that the DSBs observed in the micronuclei were caused by spindle abnormalities (Dalton *et al.*, 2007). This is supported by the observation that the micronuclei contained centromeres and the majority of DSBs co-localized with these centromeres (Guerrero *et al.*, 2010). Since spindle abnormalities can underlie W-CIN and the *Dido* mutant MEFs are thought to be chromosomally instable, these findings support the idea that W-CIN be a cause of structural instability.

More supporting evidence for a possible relationship between W-CIN and S-CIN comes from a study in which several colorectal cancer cell lines were karyotyped. Cancer cell lines with a stable, near-diploid karyotype displaying MIN contained almost no rearranged chromosomes. In contrast, cell lines characterized by unstable chromosome numbers exhibited many chromosomal structural rearrangements (Abdel-Rahman *et al.*, 2001). Even more strikingly, the rate at which structural chromosomal instability is altered in cancer cells increases exponentially with the degree of aneuploidy (Fabarius *et al.*, 2003). Additional support comes from chromosomally instable mice that overexpress *Mad2*. These mice show an increased number of broken chromosomes and chromosome fragment fusions compared to wild-type cells (Sotillo *et al.*, 2007). Although these observations seem to support a link between W-CIN and S-CIN, it must be noted that it is unclear whether structural instability is a direct effect of W-CIN in all of these cases.

5. Discussion

Genetic instability is strongly associated with cancer. Genetic instability can occur both at the whole chromosome level as well as at the structural DNA level. Until recently, it was thought that these two types of genetic instability occur independent of each other, and contribute to tumor formation via distinct mechanisms. However, recent literature data show that instability at the whole chromosome level can be a driving force of structural instability. This relationship between W-CIN and structural instability gives rise to a novel perspective on the role of W-CIN in tumor formation.

As the W-CIN mouse models have shown, the relationship between W-CIN and cancer is quite complex and, due to the distinct outcomes of the models, a lot of questions still remain. A

major drawback of W-CIN mouse models is that W-CIN cannot be quantified in the whole, living organism. Hence, comparison of different models is difficult. Aneuploidy levels are often used as an indication, but this is not directly correlated to the amount of W-CIN. Therefore, it would be worthwhile to develop techniques that allow quantification of the rate of chromosome missegregation in living organisms and thus enable determination of W-CIN and aneuploidy independently. An alternative approach to create better insight in the link between W-CIN and cancer could be the development of an inducible, quantitative, tissue-specific W-CIN mouse model. The currently existing mouse models for W-CIN display W-CIN from birth on. One can imagine that during their development, these mice suffer from several problems due to W-CIN. This might interfere with the tumorigenic effects of W-CIN. Therefore, induction of W-CIN at a certain age enables normal development and allows better examination of the relationship between W-CIN and cancer. Another advantage of an inducible mouse model would be that mice could function as their own negative control. The induction of quantitative levels of W-CIN allows comparison between different mouse models. In the existing mouse models, W-CIN is induced by either downregulating or overexpressing the levels of a certain MC component. However, mice in which the protein level of MAD2 or BUBR1 is reduced to 30% do not display the same level of W-CIN. Therefore, it is hard to draw conclusions from comparison of different mouse models. As described earlier, W-CIN seems to have a tumor promoting effect in some tissues and a tumor suppressing effect in others. By inducing W-CIN in a single tissue, the role of genetic context considering W-CIN and cancer can be studied in more detail.

The publication showing that aneuploidy in yeast can drive genomic instability, also revealed another interesting feature of aneuploidy, namely that it increased chromosome loss in several disomic yeast strains (Sheltzer et al., 2011). This shows that aneuploidy can induce W-CIN. Since aneuploidy is a consequence of W-CIN, this indicates that the connection between W-CIN and aneuploidy is reciprocal. In addition, since aneuploidy can be the result of a single missegregation event, this shows that one missegregation event can underlie the induction of a long-term W-CIN phenotype. Together with the ability to induce genomic instability and given the ability of W-CIN to be a driver of structural instability, this has major cellular consequences for an aneuploid cell and eventually the organism as a whole.

In conclusion, the correlation between genetic instability and cancer has been puzzling scientists since the beginning of the 20th century. In 1914, Theodor Boveri was the first to suggest that chromosomal abnormalities detected in malignant cells could be associated with the tumor phenotype of these cells. From that point on, additional research has confirmed that there is a relationship between chromosomal instability and cancer. However, this relationship is still not completely understood since both tumor-promoting and tumor-suppressing effects have been

reported for W-CIN. Novel findings have now created new insights, by showing that W-CIN can drive structural instability. Still, there are a lot of outstanding questions. For example, is the structural instability that is frequently observed in tumor cells a direct consequence of W-CIN? Hence, further experimentation is necessary to unravel the exact role of W-CIN in cancer formation. This information will lead to a better understanding of cancer as a whole and may be used for the development of novel cancer therapies.

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7. References

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