The Mad1–Mad2 balancing act – a damaged spindle checkpoint in chromosome instability and cancer

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Summary

Cancer cells are commonly aneuploid. The spindle checkpoint ensures accurate chromosome segregation by controlling cell cycle progression in response to aberrant microtubule–kinetochore attachment. Damage to the checkpoint, which is a partial loss or gain of checkpoint function, leads to aneuploidy during tumorigenesis. One form of damage is a change in levels of the checkpoint proteins mitotic arrest deficient 1 and 2 (Mad1 and Mad2), or in the Mad1:Mad2 ratio. Changes in Mad1 and Mad2 levels occur in human cancers, where their expression is regulated by the tumor suppressors p53 and retinoblastoma 1 (RB1). By employing a standard assay, namely the addition of a mitotic poison at mitotic entry, it has been shown that checkpoint function is normal in many cancer cells. However, in several experimental systems, it has been observed that this standard assay does not always reveal checkpoint aberrations induced by changes in Mad1 or Mad2, where excess Mad1 relative to Mad2 can lead to premature anaphase entry, and excess Mad2 can lead to a delay in entering anaphase. This Commentary highlights how changes in the levels of Mad1 and Mad2 result in a damaged spindle checkpoint, and explores how these changes cause chromosome instability that can lead to aneuploidy during tumorigenesis.

Key words: Mitosis, Spindle checkpoint, Cell cycle, Aneuploidy, Cancer, Mad1, Mad2

Introduction

Eukaryotic cells possess a defined complement of chromosomes. Aneuploidy, which is an abnormal change in chromosome number, results from the loss or gain of chromosomes during cell division. Frequently, solid tumors are aneuploid (for example, see Storchova and Kuffer, 2008). This has led to the hypothesis that the development of aneuploidy is a factor that contributes to tumorigenesis (Ganem et al., 2007; Suijkerbuijk and Kops, 2008; Pavelka et al., 2010; Torres et al., 2010; Tang et al., 2011). Furthermore, aneuploidy, and the mechanisms that generate aneuploidy, such as chromosome instability, could serve as targets for anti-cancer therapies.

One pathway that contributes to preventing chromosome instability is the spindle checkpoint (Yu, 2006; Musacchio and Salmon, 2007; Khodjakov and Rieder, 2009; Murray, 2011). The spindle checkpoint is a surveillance mechanism that monitors chromosomes to ensure that they are attached properly to spindle microtubules. In classic experiments, a delay in cell cycle progression was observed until all chromosomes were attached to the spindle, and release from this delay required the chromosomes to be placed under tension (Nicklas and Koch, 1969; Li and Nicklas, 1995; Nicklas, 1997). The genetic basis of the spindle checkpoint was first discovered in two independent screens in which sets of mutant alleles in spindle checkpoint genes were isolated in the budding yeast *Saccharomyces cerevisiae* (Hoyt et al., 1991; Li and Murray, 1991). Early hypotheses about the state of the spindle checkpoint in cancer cells were varied and included proposals for a complete loss of checkpoint function (Elledge, 1996) as well as for a 'damaged' checkpoint (Murray 1992; Li and Benezra, 1996), where 'damaged' refers to a partial loss or gain of checkpoint function. Initial observations favored the

idea that spindle checkpoint function is lost in cancer cells (Cahill et al., 1998; Jin et al., 1998; Cahill et al., 1999). However, later work found that the checkpoint is essential, even in cancer cells (Kops et al., 2004; Michel et al., 2004). Current hypotheses, thus, favor the idea that a damaged spindle checkpoint contributes to chromosome instability. However, the extent of the contribution, if any, that a damaged checkpoint makes to cancer formation is still under debate (Dalton and Yang, 2009; Gascoigne and Taylor, 2008; Thompson and Compton, 2008; Bakhoum et al., 2009; Khodjakov and Rieder, 2009; Rossio et al., 2010b; Schvartzman et al., 2010; Murray, 2011).

On the molecular level, an increase or decrease in proteins that have a function in the checkpoint can lead to spindle checkpoint damage. Here, we will focus on two core spindle checkpoint proteins: Mad1 and Mad2 (also known as MAD1L1 and MAD2L1 in humans). First, we introduce the spindle checkpoint and highlight the roles for Mad1 and Mad2. We then review how changes in Mad1 or Mad2 levels, as well as in the Mad1:Mad2 ratio, alter spindle checkpoint functions. Finally, we discuss changes in Mad1 and Mad2 levels in cancer cells, and conclude by proposing models for how aberrant checkpoint function caused by these changes could lead to chromosome instability.

The spindle checkpoint – a mechanism to ensure accurate chromosome segregation

In metaphase, chromosomes that are correctly attached – through their kinetochores – to microtubules that originate from opposite spindle poles are 'bi-oriented' and are placed under tension by spindle forces (Fig. 1A) (Nicklas, 1997; Maresca and Salmon, 2010). These metaphase chromosomes undergo repeated cycles of 'breathing', which is defined as oscillations in the distance

A Spindle checkpoint 'satisfied'   **B Spindle checkpoint 'unsatisfied'**
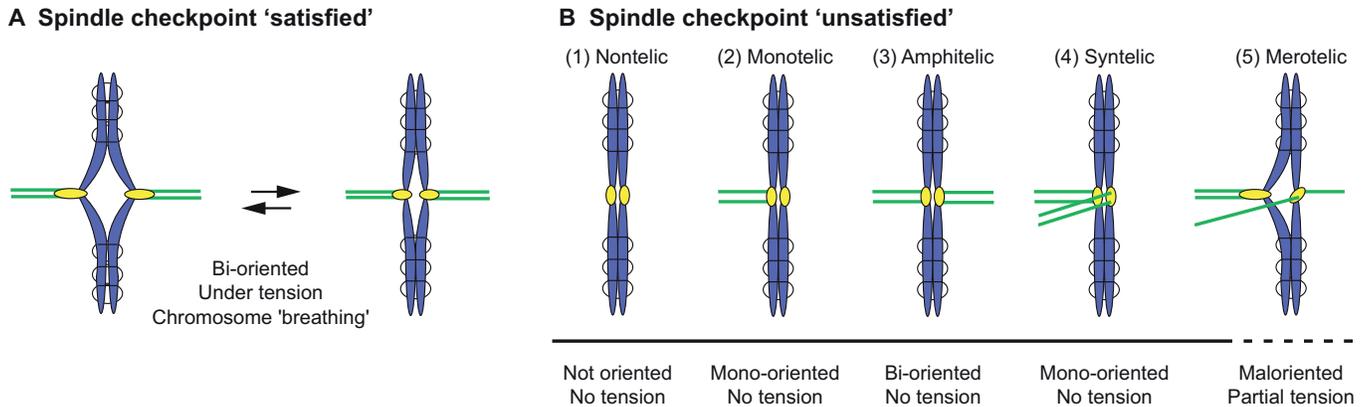

Fig. 1. The spindle checkpoint responds to chromosome attachment and tension. Potential metaphase states for paired sister kinetochores (yellow ovals) on chromosomes (blue) that are held together by cohesin rings (black rings). (**A**) An example of a chromosome that is attached to microtubules (green lines), bi-oriented, and under tension (stretched yellow ovals). The bi-oriented paired sister kinetochores undergo rounds of 'breathing', oscillating between fully stretched and semi-relaxed states (arrows). (**B**) Chromosome arrangements that could lead to the spindle checkpoint being 'unsatisfied': (1) an unattached chromosome, (2) a chromosome where one kinetochore has achieved attachment to microtubules, but the sister kinetochore remains unattached, and (3 and 4) both kinetochores are attached to microtubules, but the kinetochores are not under tension. During syntelic attachment (4), both kinetochores have attached to microtubules from the same spindle pole. During merotelic attachment (5), at least one kinetochore (distorted yellow oval) displays a mixed orientation of microtubule attachment. Merotelic attachments typically do not lead to an unsatisfied checkpoint. However, kinetochores that display syntelic attachment mixed with a small number of merotelic attachments can lead to an unsatisfied checkpoint during error correction (broken line).

between paired sister kinetochores (Inoué and Salmon, 1995; Wan et al., 2009; Wan et al., 2012). When all chromosomes are bi-oriented and under tension, the cell cycle progresses into anaphase.

The spindle checkpoint ensures accurate chromosome segregation by executing two functions (Khodjakov and Rieder, 2009). First, the checkpoint 'monitors' whether chromosomes are correctly attached to microtubules. 'Monitoring' starts at mitotic entry and lasts until entry into anaphase, and has been referred to as a state in which the mitotic checkpoint is active. Experimentally, this function is often observed as seeing that Mad2 has localized to kinetochores. Second, the checkpoint generates a signal from kinetochores that inhibits cell cycle progression. When the signal prevents cell cycle progression, the checkpoint is said to be 'unsatisfied', and when the checkpoint signal is too weak to inhibit cell cycle progression, the checkpoint is said to be 'satisfied'. The checkpoint is 'unsatisfied' when cells enter mitosis, and becomes 'satisfied' at the transition from prometaphase to metaphase. Notably, Mad2 frequently localizes to metaphase kinetochores, which suggests that spindle checkpoint 'monitoring' remains active until the end of metaphase (Waters et al., 1998; Khodjakov and Rieder, 2009). However, this low level of Mad2 localization in metaphase is apparently not sufficient to generate a checkpoint signal that is strong enough to inhibit the initiation of the steps that promote cell cycle progression (Clute and Pines, 1999).

A single unattached kinetochore is sufficient for the spindle checkpoint to be 'unsatisfied' and for cell cycle progression to be halted (Fig. 1B) (Rieder et al., 1994; Li and Nicklas, 1995; Rieder et al., 1995; Nicklas, 1997). The presence of kinetochores that are attached to microtubules, but not under tension, also causes the checkpoint to be 'unsatisfied'. These attachments occur naturally during prometaphase, but they can also be induced by the addition and subsequent removal of microtubule-depolymerizing agents from cells (Janicke and LaFountain, 1984; Salmon et al., 2005). Both amphitelic attachment, whereby the system lacks tension, and syntelic attachment, which refers to sister kinetochores that are

mono-oriented, lead to an 'unsatisfied' checkpoint (Fig. 1B) (Maresca and Salmon, 2010). In response to a lack of kinetochore tension, an error correction mechanism that depends on the activity of the kinase Aurora B promotes the detachment of microtubules from the kinetochore, and thus gives chromosomes another chance to achieve the correct attachment (Lampson and Cheeseman, 2011).

The error correction mechanism is also activated in response to merotelic attachment, which refers to a state whereby both kinetochores are attached, but at least one kinetochore is attached to microtubules from both spindle poles (Fig. 1B) (Salmon et al., 2005; Cimini et al., 2006). Merotelic attachment leads to one distorted kinetochore that is closer to the spindle equator and not 'breathing', while its paired sister-kinetochore oscillates normally (Fig. 1B) (Cimini et al., 2004). The inability of cells to completely prevent the occurrence of lagging chromosomes in anaphase has led to the hypothesis that the spindle checkpoint cannot detect merotelic attachments (Fig. 1B, broken line) (Cimini et al., 2001; Cimini et al., 2002; Salmon et al., 2005). Chromosomes that are mostly syntelic, but display a small amount of merotelism, could lead to the checkpoint becoming 'unsatisfied' during error correction. However, these kinds of attachments are rare (Janicke and LaFountain, 1984; Salmon et al., 2005).

Molecular mechanism of the spindle checkpoint – the central role of Mad1 and Mad2

On a molecular level, how does the spindle checkpoint delay cell cycle progression when proper kinetochore attachment is not achieved? A primary cell cycle regulator at the metaphase–anaphase transition is the anaphase-promoting complex or cyclosome (APC/C), which, together with the essential co-activator cell division cycle 20 (Cdc20), promotes ubiquitylation of substrates and thereby marks them for proteolysis by the 26S proteasome (Li et al., 1997; Hwang et al., 1998; Lin et al., 1998; Wassmann and Benezra, 1998; Fang et al., 1998). Current hypotheses propose that the metaphase–anaphase transition is

determined by the 'flow' of Cdc20, which can be defined as a series of sequential protein–protein interactions, through one of two distinct pathways (Fig. 2) (Varetti et al., 2011). From mitotic entry until anaphase, the pool of Cdc20 is renewed by protein synthesis. By 'flowing' through one pathway, Cdc20 can activate the APC/C, which allows the APC/C to bind to its substrates, such as securin and cyclin B, and thereby leads to anaphase. Alternatively, when bound by Mad2, Cdc20 'flows' through the checkpoint that leads to cell cycle arrest by initially inactivating the APC/C and subsequent degradation of Cdc20 by the 26S proteasome. The checkpoint controls the metaphase–anaphase transition by controlling which of these pathways Cdc20 follows. The first step in checkpoint signaling, namely the binding of Cdc20 by Mad2, is controlled by Mad1 and Mad2.

Mad1 and Mad2 physically interact and function together in a hetero-tetrameric complex to initiate the checkpoint signal (Hardwick and Murray, 1995; Chen et al., 1998; Chen et al., 1999). When a kinetochore is not properly attached, the Mad1–Mad2 complex binds to the kinetochore, where Mad1 becomes hyper-phosphorylated and activated by the kinase monopolar spindle 1 (Mps1) (Winey and Huneycutt, 2002; Hewitt et al., 2010). The Mad1–Mad2 complex at the kinetochore serves as a 'template' that catalyzes the formation of the Mad2–Cdc20 complex (Fig. 2) (Sironi et al., 2001; Chung and Chen, 2002; Sironi et al., 2002; De Antoni et al., 2005; Nezi et al., 2006; Mapelli et al., 2007; Yang et al., 2008a; Kulukian et al., 2009; Lad et al., 2009; Fava et al., 2011). Formation of the Mad2–Cdc20 complex involves the conversion of Mad2 from an 'open' into a 'closed' Cdc20-bound conformation (Fig. 2) (Luo et al., 2000; Luo et al., 2004). Mad2 binding to Mad1 also occurs through the 'closed' Mad2 conformation (Luo et al., 2002). The conversion of Mad2 from its 'open' into its 'closed' form involves an extensive conformational change and has been shown to be the rate-limiting step in spindle checkpoint signaling (De Antoni et al., 2005; Vink et al., 2006; Hewitt et al., 2010; Maldonado and Kapoor, 2011; Lau and Murray, 2012). The structures of the 'open' and 'closed' states have been solved, but

the intermediate transition state(s) remains elusive. The 'closed' Mad2 protein forms a loop around Cdc20 that is referred to as a 'safety-belt' (Sironi et al., 2002). At the kinetochore, or after being released from the kinetochore, the Mad2–Cdc20 heterodimer binds to a pseudo-substrate that comprises the checkpoint proteins Mad3 and Bub3 (budding uninhibited by benzimidazoles 3) (Fig. 2). This Mad2–Cdc20–Mad3–Bub3 complex is called the mitotic checkpoint complex (MCC). The MCC inhibits the APC/C and thereby leads to cell cycle arrest (Fig. 2) (Yu, 2006; Musacchio and Salmon, 2007; Chao et al., 2012).

Mad2 is recycled to its 'open' conformation by p31[comet] (also known as MAD2L1BP) (Fig. 2), which promotes the disassembly of the MCC, re-activates the APC/C, and might also allow the Mad3–Bub3 complex to be recycled (Habu et al., 2002; Xia et al., 2004; Yang et al., 2007; Miniowitz–Shemtov et al., 2010; Hagan et al., 2011; Jia et al., 2011; Ma and Poon, 2011; Teichner et al., 2011; Varetti et al., 2011; Westhorpe et al., 2011). It has been observed that Cdc20 is ubiquitylated in a p31[comet]-dependent manner (Díaz-Martínez and Yu, 2007; Reddy et al., 2007; Stegmeier et al., 2007; Nilsson et al., 2008; Ge et al., 2009; Visconti et al., 2010; Varetti et al., 2011). One hypothesis is that Cdc20 ubiquitylation does not necessarily lead to Cdc20 protein degradation, but rather regulates the disassembly of the MCC in a process that is promoted by ubiquitylation mediated by UBE2C (ubiquitin-conjugating enzyme 2C, also known as UBCH10) and is opposed by de-ubiquitylation mediated by ubiquitin C-terminal hydrolase 44 (USP44) (Reddy et al., 2007; Stegmeier et al., 2007). An alternative hypothesis is that p31[comet]-dependent dismantling of the MCC, and subsequent removal from the APC/C, is coupled with Cdc20 polyubiquitylation and the direct degradation of Cdc20 by the 26S proteasome (Fig. 2), which also involves CUE domain containing 2 (CUEDC2) (Pan and Chen, 2004; Chen, 2007; Díaz-Martínez and Yu, 2007; King et al., 2007; Zeng et al., 2010; Gao et al., 2011; Ma and Poon, 2011; Varetti et al., 2011). This second hypothesis suggests that continuous Cdc20 protein synthesis is required for checkpoint
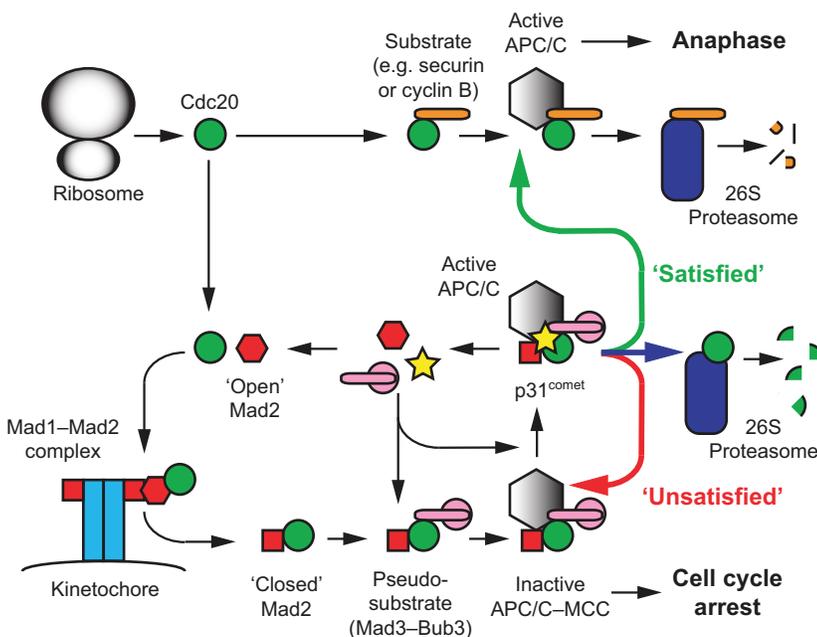

Fig. 2. Mad1 and Mad2 regulate the metaphase–anaphase transition by controlling the flow of Cdc20 into the checkpoint pathway. Cdc20 (green circle) is synthesized continuously during prometaphase and metaphase. If the spindle checkpoint is 'satisfied' (thick green arrow), after binding a substrate (orange rod), Cdc20 can activate the APC/C and promote entry into anaphase. If a kinetochore is not attached properly, the spindle checkpoint is 'unsatisfied' (thick red arrow). Under these conditions, first Mad2 (red square) and then the Mad3–Bub3 complex (pink rod and circle) bind to Cdc20 to form the MCC. The MCC binds to the APC/C and inactivates it, thereby causing cell cycle arrest. The APC/C is re-activated by p31[comet] (yellow star), which promotes the disassembly of the MCC and the ubiquitylation of Cdc20. The flow of Cdc20 through the checkpoint pathway is unidirectional and ultimately results in proteasomal degradation (thick blue arrow).

function during prometaphase and metaphase, and support for this idea has recently been found experimentally (Varetti et al., 2011). When the inactive APC/C is re-activated and released from the MCC by p31^{comet}, if the active APC/C continuously encounters Cdc20 that is bound to a substrate, the checkpoint is said to be 'satisfied'. This will ultimately promote entry into anaphase (Fig. 2). By contrast, if the active APC/C continuously encounters another MCC complex, the spindle checkpoint is said to be 'unsatisfied' and the APC/C will become inactive again, which results in the cell cycle remaining arrested (Fig. 2).

Alterations in the levels of Mad1 or Mad2 cause aberrant spindle checkpoint function

Mad1 or Mad2 levels have been manipulated in several experimental models. In budding yeast, the overexpression of Mad1 or Mad2 leads to an increase in chromosome loss (Warren et al., 2002), and overexpression of Mad2 at levels 20-fold higher than the endogenous levels results in mitotic arrest (Rossio et al., 2010a). Decreasing the level of Mad2 by half also increases the rate of chromosome loss and impairs the response from the spindle checkpoint to the loss of kinetochore tension in metaphase but not the response to the presence of a spindle poison at mitotic entry (Lee and Spencer, 2004; Barnhart et al., 2011). In aneuploid budding yeast, a ratio of 1:2 between chromosome X (which carries the *MAD2* gene) and chromosome VII (which carries the *MAD1* gene) strongly correlates with genomic instability (Zhu et al., 2012). In the fission yeast *Schizosaccharomyces pombe*, overexpression of Mad2 causes mitotic arrest (He et al., 1997; Kim et al., 1998), and a decrease in the protein levels of Mad1 or Mad2 has been found to disrupt spindle checkpoint function (S. Heinrich and S. Hauf, personal communication). In *Caenorhabditis elegans*, an increase in the level of Mad2 increases the length of the mitotic delay that is induced by a lack of tension (Essex et al., 2009). In *Xenopus laevis* egg extracts, an excess of Mad1 removes checkpoint function (Chung and Chen, 2002), and an increase in Mad2 levels causes mitotic arrest (Chen et al., 1998; Fang et al., 1998; Howell et al., 2000). In pre-implantation mouse embryos, a decrease in the level of Mad2 leads to chromosome instability, and overexpression of Mad2 induces a mitotic delay and chromosome instability (Wei et al., 2011). In adult mice, lowering either Mad1 or Mad2, or overexpressing Mad2, disrupts checkpoint function, and increases aneuploidy and tumorigenesis (Michel et al., 2001; Iwanaga et al., 2007; Sotillo et al., 2007). In tissue culture cells, an excess of a microinjected Mad1 protein fragment containing the Mad2-binding site leads to loss of checkpoint function (Howell et al., 2000; Canman et al., 2002; Cimini et al., 2003), and overexpression of Mad2 causes mitotic delay, chromosome instability and leads to the stabilization of kinetochore–microtubule attachments (Howell et al., 2000; Sironi et al., 2001; De Antoni et al., 2005; Kabeche and Compton, 2012).

Several studies have addressed whether the effects observed as a result of changes in Mad1 depend on the presence of Mad2, or whether the effects that occur as a result of changes in Mad2 depend on the presence of Mad1. A mitotic delay induced by excess Mad2 in *Xenopus* is independent of Mad1, and the addition of excess Mad1 does not remove the delay (Chen et al., 1998; Fang et al., 1998; Chung and Chen, 2002). In budding yeast, *C. elegans* and mammalian tissue culture cells, the cell cycle delay that is induced by an excess of Mad2 depends on the presence of Mad1 (Sironi et al., 2001; Essex et al., 2009; Rossio et al., 2010a). However, in budding yeast, the requirement for

Mad1 can be bypassed by tethering Mad2 to Cdc20 (Lau and Murray, 2012). In tissue culture cells, the stabilization of kinetochore–microtubule attachments induced by excess Mad2 does not require Mad1, although the effect appears to be reduced when Mad1 is absent (Kabeche and Compton, 2012).

The role of the Mad1:Mad2 ratio in spindle checkpoint function

The 'wild-type' Mad1:Mad2 ratio is still unknown in most model systems. From the molecular model of the spindle checkpoint (Fig. 2), it would make most sense if there was an excess of Mad2 relative to Mad1, which is the case in a vertebrate cell line, where Mad2 has been shown to be present in a 10-fold excess relative to Mad1 (Shah et al., 2004). However, dual manipulation of Mad1 and Mad2 has led to surprising observations: Mad1 is both an activator and an inhibitor of checkpoint function, and the checkpoint defect that occurs as a result of low Mad2 levels relative to Mad1 can be corrected by a restoring the normal Mad1:Mad2 ratio. In an *in vitro* biochemical system, the addition of an excess of a Mad1 fragment containing the Mad2-binding site disrupts the interaction between Mad2 and Cdc20, an effect that can be reversed by adding excess Mad2 (Sironi et al., 2002). In budding yeast, a decrease in Mad2 leads to an increase in chromosome loss and the inability to respond to the loss of chromosome tension. However, both phenotypes can be reversed by a compensatory decrease in Mad1 (Barnhart et al., 2011). In *Xenopus* extracts, excess Mad1 disrupts checkpoint function, and this disruption can be reversed by the addition of excess Mad2 (Chung and Chen, 2002). Thus, at least for some aspects of checkpoint function, the Mad1:Mad2 ratio is more important than the absolute amounts of the proteins.

By contrast, experiments in mice have shown that a decrease in Mad2 cannot be compensated for by a corresponding decrease in Mad1, and double heterozygous knockout mutants display an increase in aneuploidy (Iwanaga et al., 2007). This observation could be the result of, in part, the specific knockout allele of Mad1 that was employed in these experiments, which results in the removal of exon 10 and leads to a partial decrease in the remaining Mad1 protein that is expressed from the wild-type locus in heterozygous mice (Iwanaga et al., 2007). Alternatively, it is possible that different organisms simply display different sensitivities to partial disruption of checkpoint function. In budding yeast and *Drosophila melanogaster*, Mad2 is not essential, whereas it is required in mice and mammalian cell lines, which appear to be more sensitive to changes in checkpoint function and are more prone to chromosome loss (Dobles et al., 2000; Salmon et al., 2005; Haller et al., 2006). Chromosome loss rates in homozygous mouse mutants might simply be too high to maintain viability (Michel et al., 2001; Iwanaga et al., 2007); this might reflect differences in the spindle assembly rates between different organisms (Khodjakov and Rieder, 2009; Rieder, 2011). Indeed, the work performed using mice has provided the most compelling evidence demonstrating that damaging the spindle checkpoint, by changing the levels of Mad1 or Mad2, can induce the development of aneuploidy and tumorigenesis.

Changes in Mad1 and Mad2 promote tumorigenesis

Many experiments over the past decade have provided evidence for changes in the levels of spindle checkpoint components in cancer cells, although there can be a lot of variation between cells

and/or samples and it can be difficult to establish proper normalization controls. Indeed, it has been suggested that the results that provide evidence for a decrease in checkpoint mRNA transcripts or protein levels leading to a decrease in checkpoint function should be discounted (Schvartzman et al., 2010). Although the results from such studies should be considered with care, the volume of evidence [including eight examples for Mad1 (Han et al., 1999; Coe et al., 2006; Osaki et al., 2007; Wang et al., 2008; Schvartzman et al., 2010), and 27 examples for Mad2 (Suijkerbuijk and Kops, 2008; Wang et al., 2008; Burum-Auensen et al., 2010; Schvartzman et al., 2010; Wang et al., 2010; Díaz-Rodríguez et al., 2011; Furlong et al., 2012; Kato et al., 2011; Wang et al., 2012)] for changes in the levels of Mad1 or Mad2 in cancer cells is intriguing. However, a single question from these observations emerges: do changes in Mad1 or Mad2 promote tumorigenesis, or are such changes simply the result of cancer development?

The most compelling answer to this question comes from work in mice. Decreasing Mad1, or increasing or decreasing Mad2 not only leads to aberrant checkpoint function, but also promotes aneuploidy and induces tumorigenesis (Michel et al., 2001; Sotillo et al., 2007; Sotillo et al., 2010; Schvartzman et al., 2011). Recent work has also placed the aberrant overexpression of Mad2 directly downstream of the loss of function of two tumor suppressor pathways that depend on the tumor suppressor proteins p53 and RB1, and the observed defects in chromosome instability and tumorigenesis could be compensated for by lowering Mad2 expression (Chun and Jin, 2003; Hernando et al., 2004; Chi et al., 2009; Schvartzman et al., 2011). In combination with the earlier work, these observations demonstrate, for example, that an increase in Mad2 is both necessary (Schvartzman et al., 2011) and sufficient (Sotillo et al., 2007) for the development of aneuploidy and tumorigenesis.

Models for how changes in Mad1 and Mad2 levels lead to a damaged checkpoint

Excess Mad1 competes with Cdc20 for binding to Mad2. This competition could have two consequences: the generation of a pool of free Cdc20 that can promote the ubiquitylation of substrates, and a lower level of APC/C inhibition. In combination, these effects cause premature entry into anaphase (Fig. 3A). By contrast, an excess of Mad2 could lead to the continuous inactivation of the APC/C by depleting the amount of Cdc20 that is available to bind substrates, and by increasing the amount of APC/C in the inactive MCC-bound form (Fig. 3B). In most experimental systems, this effect requires Mad1, but this is not the case in *Xenopus* extracts or when Mad2 is artificially tethered to Cdc20 (Fig. 3B, dashed arrow) (Chen et al., 1998; Fang et al., 1998; Howell et al., 2000; Lau and Murray, 2012). The imbalance in the 'flow' of Cdc20 into the checkpoint pathway, which leads to its destruction by the 26S proteasome, can cause an extended mitotic arrest (Fig. 3B).

How do changes in levels of Mad1 and Mad2 lead to chromosome instability?

Hypotheses for the development of aneuploidy, including the development of tetraploids, invoke an increase in syntelic and/or merotelic chromosomes in metaphase, which leads to non-disjunctions or lagging chromosomes in anaphase (Ganem et al., 2007; Storchova and Kuffer, 2008; Suijkerbuijk and Kops, 2008; Pavelka et al., 2010; Schvartzman et al., 2010; Torres et al., 2010; Tang et al., 2011). Lagging chromosomes occur frequently in tissue culture cells (Cimini et al., 2001; Salmon et al., 2005), and are the

most common mitotic defect in human tumor cells, where the kinetochores are often distorted, which suggests merotelic attachments (Thompson and Compton, 2008). Reducing the stability of kinetochore–microtubule attachments in cancer cells also suppresses the incidence of lagging chromosomes, which reduces chromosome mis-segregation rates (Bakhoum et al., 2009).

We propose that these observed increases in chromosome instability result from damage to the spindle checkpoint. It has been argued that checkpoint defects do not contribute to the aneuploidy observed in cancer cells, because cancer cells display a functional checkpoint response when spindle poisons are present at mitotic entry (Khodjakov and Rieder, 2009). However, cells harboring either a defect that disrupts rapid switching from a 'satisfied' state back to an 'unsatisfied' state in metaphase (i.e. excess Mad1), or a defect that prevents switching in metaphase from an 'unsatisfied' to 'satisfied' state (i.e. excess Mad2), have both been observed to delay cell cycle progression and prevent chromosome loss when spindle poisons are present at mitotic entry (Chen et al., 1998; De Antoni et al., 2005; Barnhart et al., 2011).

Cells enter mitosis with all chromosomes unattached and with other G2-M checkpoints active (Rieder, 2011). Thus, rapid spindle checkpoint switching from a 'satisfied' to an 'unsatisfied' state is crucial for cell cycle arrest only in metaphase, after all chromosomes have been bi-oriented and APC/C-dependent destruction of cyclin B has been initiated (Clute and Pines, 1999). If a metaphase kinetochore loses the correct attachment(s), rapid checkpoint switching to the 'unsatisfied' state is necessary to prevent premature entry into anaphase. Spindle checkpoint switching in metaphase occurs within a minute of the loss of tension, even while microtubules are mostly still attached to kinetochores (McEwen et al., 1997; Clute and Pines, 1999; Wan et al., 2009). Excess Mad1 can result in cells with a damaged checkpoint; they can arrest the cell cycle if they enter mitosis in the presence of a spindle poison, but they cannot arrest the cell cycle in response to the loss of tension that is induced in metaphase, and display an increase in chromosome loss (Barnhart et al., 2011). These defects can be reversed by a compensatory change that restores the Mad1:Mad2 ratio, even when the total amounts of the Mad1 and Mad2 proteins are only half of the normal levels (Barnhart et al., 2011).

Mitotic delays that are induced by a variety of mechanisms also lead to chromosome mis-segregation (Dalton and Yang, 2009; Rossio et al., 2010b; Schvartzman et al., 2010), and mitotic delays have been observed in cancer cells (Sisken et al., 1982; Sisken et al., 1985; Yang et al., 2008b). We propose that a checkpoint that cannot be 'satisfied' because of the presence of excess Mad2, even when chromosomes are correctly bi-oriented, results in merotelic attachments through three routes: (1) as a result of the loss of microtubule attachment during chromosome 'breathing', (2) as a result of an expansion in the kinetochore microtubule-binding surface area, and (3) as a result of a disruption in the Aurora B-dependent error correction mechanism (Fig. 4).

Once chromosomes are bi-oriented, the paired sister kinetochores 'breathe'. In PtK1 cells, these oscillations normally last for ~23 minutes, from the time when the last chromosome is bi-oriented at the end of prometaphase until the initiation of anaphase (Rieder et al., 1994). In metaphase the average individual kinetochore–microtubule attachment half-life is 5–7 minutes (Cassimeris et al., 1990; Shelden and Wadsworth, 1996; Zhai et al., 1995; Bakhoum et al., 2009). Bi-oriented, 'breathing' metaphase chromosomes were observed to be less than 1.1 μm
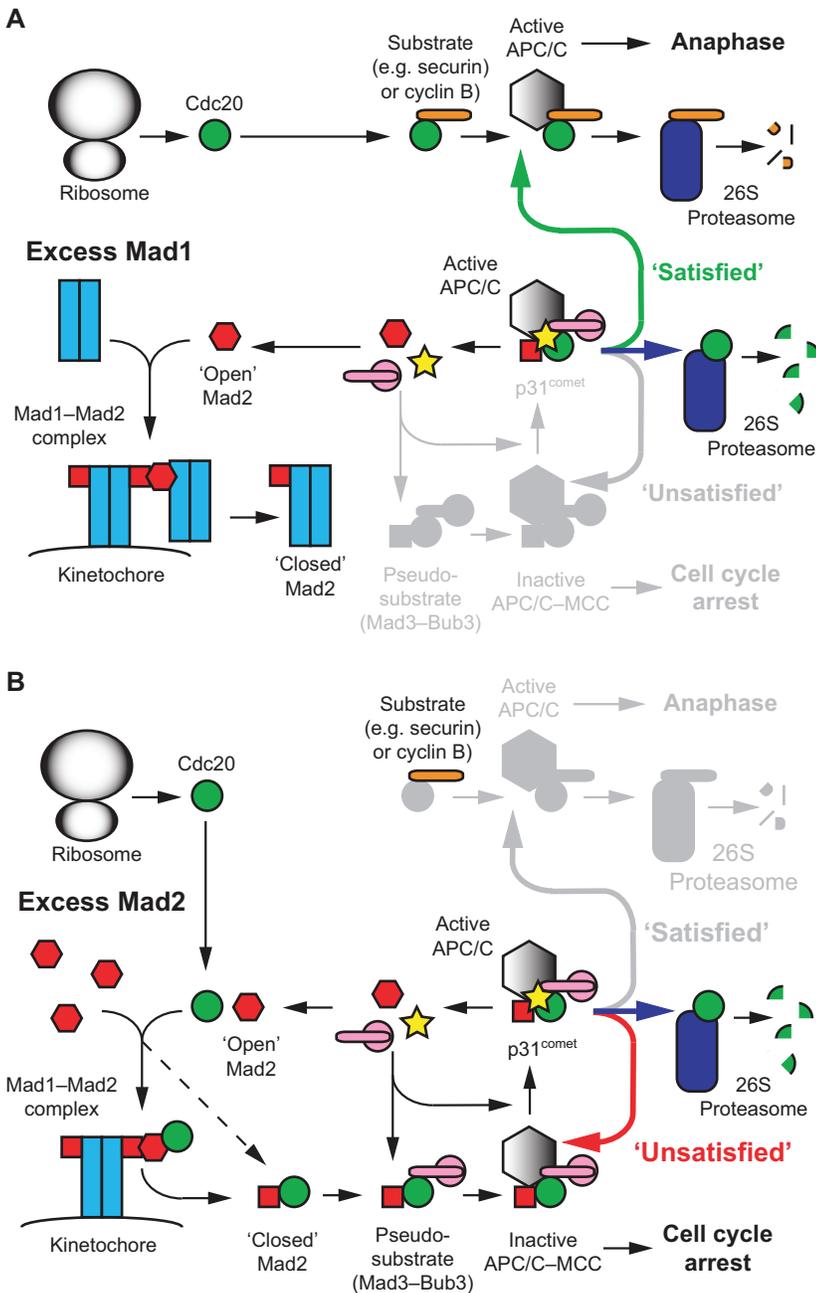
Fig. 3. Model of how excess Mad1 or excess Mad2 could alter the flow of Cdc20 into the checkpoint pathway. An excess of Mad1 or Mad2 disrupts the normal balance of Mad2 binding to Cdc20, which is the first step in checkpoint signaling. (**A**) In cells with reduced Mad2 levels or an increase in Mad1 levels (blue rectangles), much of the 'open' Mad2 (red hexagon) could be sequestered by the free Mad1 dimers. This could lead to a delay in inactivating the APC/C, and allow free Cdc20 (green circle) to promote a premature entry into anaphase. (**B**) In cells with increased Mad2 levels (red hexagons), much of the Cdc20 might be sequestered into the checkpoint pathway. This could lead to a decrease in the amount of free Cdc20, and, in combination with the continuous inactivation of the APC/C, lead to an extended mitotic arrest.

apart (i.e. in a state where they lack tension) for only 1% of the time (Wan et al., 2012). Thus, during a normal PtK1 metaphase, bi-oriented sister kinetochores on a single chromosome would, on average, only experience a lack of tension for ~14 seconds, and each kinetochore–microtubule attachment site would cycle through three or four rounds of detachment and reattachment. In an extended mitotic arrest that lasts, for example, 4 hours, each kinetochore–microtubule attachment site could cycle through 40 rounds of the detachment–reattachment cycle, and each chromosome could experience a lack of tension for several minutes, which might be sufficient to cause activation of the Aurora B error correction mechanism and thereby lead to additional microtubule detachments (Fig. 4). The subsequent attachment state of the chromosome would depend on which

spindle pole the next microtubule reattached from, which could lead to a merotelic chromosome (Fig. 4, right).

A second possible route to the development of merotelic chromosomes could result from the structural changes in kinetochores that have been observed during prolonged mitotic arrests. During mitotic arrest in the absence of microtubules, kinetochores appear to expand and curve into a 'crescent' shape. In metaphase, anaphase, and in a mitotic arrest where microtubules stay bound to the kinetochores, the microtubule-binding surface area of kinetochores also increases (McEwen et al., 1997; McEwen et al., 1998), but not to the same extent as in the absence of microtubules. This increase in surface area could allow new microtubule attachments to occur, which could lead to merotelic chromosomes (Fig. 4, bottom).
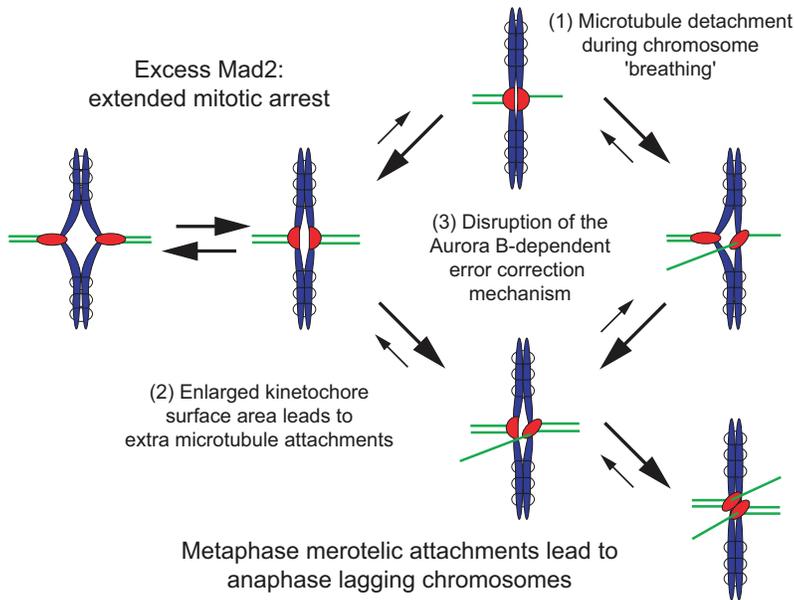
Fig. 4. An excess of Mad2 leads to merotelic chromosomes. In the presence of an excess of Mad2, cells remain arrested in metaphase (left) even when bi-orientation and tension have been achieved. This extended arrest could lead to the development of merotelic chromosome attachments through three possible mechanisms: (1) during extended periods of kinetochore 'breathing' (left), a kinetochore might relax enough to trigger the loss of tension and microtubule detachment (top), which could lead to merotelic attachment (right), (2) an expansion in the binding surface area between kinetochores and microtubules could allow additional microtubules to bind to the kinetochore (bottom middle) that would lead to merotelic attachment, and/or (3) disruption of the error correction mechanism can promote the accumulation of merotelic attachments (bi-directional arrows). If a single merotelic attachment develops, the paired sister kinetochore is also more likely to develop a merotelic attachment (bottom right).

When Mad2 is present at normal levels, a prolonged metaphase arrest, where the Aurora B error correction mechanism is functioning rapidly, does not lead to a substantial change in the number of merotelic kinetochores in metaphase (Cimini et al., 2003; Khodjakov and Rieder, 2009). This suggests that the formation of new merotelic kinetochores and their removal by the error correction mechanism are happening continuously, and the number of merotelic kinetochores remains at a steady-state level. However, once one sister kinetochore becomes merotelic, the probability that its sister kinetochore will also become merotelic increases (Fig. 4, bottom right) (Cimini et al., 2002). Indeed, an extended metaphase arrest leads to an increase in the average number of merotelic kinetochores per cell during metaphase in cells that have at least one merotelic attachment, and a corresponding increase in the average number of anaphase lagging chromosomes per cell in cells that have at least one lagging chromosome (Cimini et al., 2003).

Finally, regardless of the route through which merotelic attachment is brought about, recent observations have revealed that overexpression of Mad2 leads to the stabilization of kinetochore–microtubule attachments by disrupting the Aurora-B-dependent error correction mechanism (Kabeche and Compton, 2012). This disruption exacerbates merotelic attachments by trapping chromosomes in an aberrant merotelic state (Fig. 4). Cells eventually proceed into anaphase with lagging chromosomes because of mitotic 'slippage' or 'adaptation' (Dalton and Yang, 2009; Rossio et al., 2010b; Schvartzman et al., 2010), and display chromosome instability, which leads to the development of aneuploidy during tumorigenesis.

Conclusions and perspectives

The concept that a damaged mitotic spindle checkpoint contributes directly to the development of aneuploidy and tumorigenesis is likely to be a primary train of thought in the coming decade in cancer cell biology. One mechanism that leads to damage in the pathway is a change in the levels of Mad1 and Mad2, or an imbalance in the Mad1:Mad2 ratio. *In vivo* evidence for this interpretation has been observed in several model organisms, and has been buttressed by *in vitro* biochemistry.

Changes in the levels of Mad1 and Mad2 have been detected in many cancer cell lines and tumor biopsy samples. Steps are being taken to employ changes in Mad1 and Mad2 as cancer biomarkers and to specifically target this part of the checkpoint pathway as a new strategy in developing potential anti-cancer therapies.

In the future, the consequences of changing the levels of Mad1 and Mad2, and the ratio between them, should be explored. The levels of both Mad1 and Mad2 transcripts and/or proteins should be simultaneously measured, because a single measurement is not sufficient to determine the degree of checkpoint function. These measurements should also be coupled with measurements of other checkpoint pathway regulators, notably p31[comet] and CUEDC2 (Habu et al., 2002; Gao et al., 2011). The binding between Mad1 and Mad2 is at the heart of the checkpoint mechanism, and the recent discovery of other Mad1- and Mad2-binding partners is noteworthy (Zhang et al., 2009; Lee et al., 2010; Lussi et al., 2010; Orth et al., 2011).

At the cellular level, when investigating spindle checkpoint function in cancer cells, it is important to consider that cells might harbor a damaged spindle checkpoint that remains fully functional in response to the presence of spindle poisons at mitotic entry. It will, therefore, be essential to: (1) measure the wild-type *in situ* rate of mitotic progression from nuclear envelope breakdown to anaphase in a cell type that matches that of the cancer cells under study in tissue culture; (2) measure the rate at which the checkpoint can switch from the 'satisfied' state back to the 'unsatisfied' state when a kinetochore–microtubule attachment defect is introduced specifically in metaphase in matched normal and cancer cells; and (3) determine the consequences on chromosome segregation, with special attention paid to non-disjunction events and lagging chromosomes, after the induction of a mitotic delay induced by Mad2 overexpression.

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