

# The spindle checkpoint, aneuploidy, and cancer

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**Cancer cells contain abnormal number of chromosomes (aneuploidy), which is a prevalent form of genetic instability in human cancers. Defects in a cell cycle surveillance mechanism called the spindle checkpoint contribute to chromosome instability and aneuploidy. In response to straying chromosomes in mitosis, the spindle checkpoint inhibits the ubiquitin ligase activity of the anaphase-promoting complex or cyclosome (APC/C), thus preventing precocious chromosome segregation and ensuring the accurate partition of the genetic material. We review recent progress toward the understanding of the molecular mechanism of the spindle checkpoint and its role in guarding genome integrity at the chromosome level.**

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## Introduction

Chromosomal instability (CIN) leading to an aberrant chromosome number (aneuploidy) is a hallmark of cancers (Jallepalli and Lengauer, 2001). The mechanism and molecular determinants of CIN are not clearly understood (Jallepalli and Lengauer, 2001). However, there is a growing body of evidence to suggest that defects in the spindle checkpoint, a surveillance mechanism crucial for the proper segregation of chromosomes during every cell division, might promote aneuploidy and tumorigenesis (Wassmann and Benezra, 2001). Mutations in certain genes involved in the spindle checkpoint have been identified in a variety of human cancers (Cahill *et al.*, 1998). Although these mutations have not been found very frequently and a causal connection between these mutations and CIN has not been established unequivocally, the understanding of the spindle checkpoint might provide valuable insights into CIN and facilitate the design of novel therapeutic approaches to treat cancer.

### *Chromosome segregation and the spindle checkpoint*

The alignment of chromosomes on the cell equator during mitosis is achieved by a stochastic process in

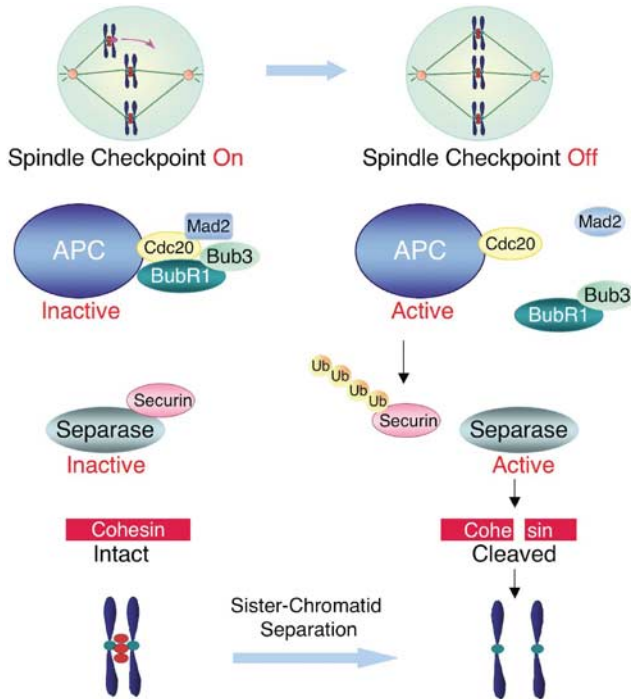
which kinetochores of the chromosomes are captured by randomly elongating and contracting microtubules emanating from the two spindle poles (Cleveland *et al.*, 2003). Once the two kinetochores of a sister-chromatid pair attach to microtubules from the opposite poles, they congress to the center of the cells (Cleveland *et al.*, 2003). Due to the inherent randomness of this process, it is crucial that chromosome segregation ensues only when all the chromosomes have congressed to the metaphase plate to ensure the even partition of the genetic material into the two daughter nuclei (Nasmyth, 2002; Cleveland *et al.*, 2003). This is accomplished by a surveillance mechanism known as the spindle checkpoint, which senses the lack of attachment and/or tension at kinetochores and in response inhibits chromosome segregation (Millband *et al.*, 2002; Musacchio and Hardwick, 2002; Yu, 2002). Through detailed analysis of the timing of various mitotic events in PtK cells by live cell imaging, Rieder *et al.* (1995) showed that a single unattached kinetochore can delay the segregation of the already aligned chromosomes. The time lag between nuclear envelope breakdown and anaphase onset varies from cell to cell and is highly dependent upon how long the last chromosome takes to congress to the metaphase plate (Rieder *et al.*, 1995). Anaphase occurs approximately 20 min after the alignment of the last chromosome in PtK cells. More interestingly, laser ablation of the last unattached kinetochore allows mitosis to proceed in the absence of microtubule attachment, suggesting that an inhibitory checkpoint signal is produced by this kinetochore to block chromosome segregation (Rieder *et al.*, 1995).

### *Molecular components of the spindle checkpoint*

Prior to anaphase, sister-chromatids are kept together by a proteinaceous bridge formed by a multiprotein complex termed cohesin (Nasmyth *et al.*, 2000; Nasmyth, 2002) (Figure 1). Anaphase is triggered by the proteolysis of one of the cohesin subunits, Scc1, by a protease called separase (Nasmyth *et al.*, 2000; Nasmyth, 2002). Separase is normally kept inactive by an associated inhibitor termed securin (Zou *et al.*, 1999; Nasmyth *et al.*, 2000; Nasmyth, 2002). After the proper attachment of all sister-chromatids to the mitotic spindle, the spindle checkpoint is satisfied. Securin is then ubiquitinated by the anaphase-promoting complex or cyclosome (APC/C), a multi-subunit E3 ubiquitin

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ligase (Yu *et al.*, 1998; Harper *et al.*, 2002; Peters, 2002; Yu, 2002). The destruction of ubiquitinated securin by the proteasome then leads to separase activation, Scc1 cleavage, loss of chromosome cohesion, and anaphase onset (Nasmyth *et al.*, 2000; Nasmyth, 2002).



**Figure 1** Molecular mechanism of chromosome segregation. At the metaphase–anaphase transition, APC/C<sup>Cdc20</sup> ubiquitinates securin. Degradation of securin activates separase. Separase then cleaves the Scc1 subunit of cohesin, allowing chromosome segregation. In response to sister-chromatid not properly attached to the mitotic spindle, the spindle checkpoint promotes the assembly of checkpoint protein complexes that inhibit the activity of APC/C, leading to the stabilization of securin, preservation of sister-chromatid cohesion, and a delay in the onset of anaphase

Ubiquitination of securin by APC/C requires the binding of the WD40-repeat-containing Cdc20 protein, which recruits substrates to APC/C (Fang *et al.*, 1998b; Hilioti *et al.*, 2001; Pflieger *et al.*, 2001; Harper *et al.*, 2002; Peters, 2002). It turns out that the spindle checkpoint interferes with the productive interaction between APC/C and Cdc20 (Yu, 2002). Thus, the complex between APC/C and Cdc20 (APC/C<sup>Cdc20</sup>) is the most downstream target of the spindle checkpoint (Fang *et al.*, 1998a; Yu, 2002). Unattached kinetochores emit a diffusible signal that inhibits the cytoplasmic pool of APC/C<sup>Cdc20</sup>, thus preventing the separation of already aligned chromosomes (Shah and Cleveland, 2000; Yu, 2002; Cleveland *et al.*, 2003). Although the exact nature of this diffusible signal and the mechanism of its generation are not clear, great progress has recently been made toward the understanding of the identities and interactions of some of the proteins involved in this process.

The well-characterized components of the spindle checkpoint include Mad1, Mad2, Mad3 (BubR1), Bub1, Bub3, and Mps1 (Figure 2) (Yu, 2002; Cleveland *et al.*, 2003). In this list, we have not included other kinetochore proteins that might be responsible for the assembly of a functional kinetochore and therefore for the recruitment of the above-mentioned checkpoint proteins (Cleveland *et al.*, 2003) (also see below). The Mad (mitotic arrest deficient) and Bub (budding uninhibited by benzimidazole) proteins were identified by yeast mutagenesis screens for mutants unable to survive a temporary exposure to microtubule toxins nocodazole or benzimidazole (Hoyt *et al.*, 1991; Li and Murray, 1991). Mps1 was identified initially as a centrosomal protein required for the assembly of bipolar spindles, but it was later shown to play a role in the spindle checkpoint as well (Winey and Huneycutt, 2002). Homologs for these proteins were subsequently identified in higher organisms including mammals (Yu,

Checkpoint Proteins	Domain Structures	Functions
Mad1	1 718 Coiled Coil	Binds to Mad2 and recruits it to kinetochores; Essential for generation of Mad2-Cdc20 complex; Forms a complex with Bub1-Bub3 in mitosis.
Mad2	1 205 HORMA	Binds to Cdc20 and inhibits APC/C <sup>Cdc20</sup> ; Binds to Mad1.
Bub1	1 1085 TPR GLEBS Kinase	Binds to Bub3 constitutively; Forms a complex with Mad1 and Bub3 in mitosis; Phosphorylates Mad1 in vitro.
BubR1 (Mad3)	1 1050 TPR GLEBS Kinase	Binds to Bub3 constitutively; Activated by CENP-E Inhibits APC/C <sup>Cdc20</sup> in a Mad2-independent manner Forms a complex with Cdc20, Bub3, and Mad2
Bub3	1 328 WD40	Binds to BubR1 and Bub1.
Cdc20	1 499 WD40	Recruits substrates to APC/C; Molecular target of the spindle checkpoint; Forms a complex with Mad2, BubR1, and Bub3.

**Figure 2** Domain structures and functions of a subset of the human spindle checkpoint proteins. The numbers indicate the number of amino acids present in each of the proteins. TPR, tetratricopeptide repeat; HORMA is a domain found in Hop1, Rev7, and Mad2; and GLEBS is a motif that binds to Bub3 and Rael

2002). Functional disruption of these proteins in mammalian cells through dominant-negative mutants, antibody injection, or RNA interference (RNAi) results in the abrogation of the spindle checkpoint, leading to chromosome mis-segregation, aneuploidy and a failure to arrest in mitosis in the presence of microtubule poisons, such as nocodazole and taxol (Taylor and McKeon, 1997; Gorbsky *et al.*, 1998; Abrieu *et al.*, 2001; Luo *et al.*, 2002; Stucke *et al.*, 2002; Yu, 2002). These findings confirm that the mammalian checkpoint proteins identified through sequence homology are indeed functional homologs of their yeast counterparts (Yu, 2002).

#### *Direct inhibition of APC/C by the mitotic checkpoint complex*

How does the spindle checkpoint block the activity of APC/C<sup>Cdc20</sup>? A decade of genetic and biochemical studies have indicated that the most downstream event in checkpoint signaling is the inhibition of Cdc20, which is believed to be the substrate recognition subunit of APC/C. Inhibition of Cdc20 is accomplished by the mitotic checkpoint complex (MCC) that is composed of Mad2, BubR1, Bub3, and Cdc20 (Sudakin *et al.*, 2001; Tang *et al.*, 2001; Fang, 2002; Yu, 2002). We will briefly review the evidence supporting this conclusion.

#### *Function of Mad2*

The function of Mad2 was elucidated first. Overexpression of Mad2 causes a mitotic arrest in yeast and mammalian cells, presumably through inhibition of APC/C<sup>Cdc20</sup> (Hwang *et al.*, 1998; Kim *et al.*, 1998; Fang *et al.*, 1999). Initial insights into the function of Mad2 came from findings in both budding and fission yeast that Mad2 binds directly to Cdc20 (Hwang *et al.*, 1998; Kim *et al.*, 1998). Mutations in Cdc20 that render it unable to bind Mad2 allow cells to undergo mitosis even in the presence of Mad2 overexpression (Kim *et al.*, 1998). Mad2, Cdc20, and APC/C were then shown to form a ternary complex in mammalian cells (Fang *et al.*, 1998a; Wassmann and Benezra, 1998). Further insights into Mad2 function came from *in vitro* biochemical assays demonstrating that bacterially purified recombinant Mad2 inhibits the ubiquitin ligase activity of APC/C immunopurified from *Xenopus* egg extracts (Li *et al.*, 1997; Fang *et al.*, 1998a). The Mad2-binding motif of Cdc20 was then narrowed down to a stretch of 12 amino acids in the N-terminal region of Cdc20 (Luo *et al.*, 2002). As expected, introduction of a peptide containing this motif into cells leads to the inactivation of the spindle checkpoint (Zhang and Lees, 2001; Luo *et al.*, 2002). Structure of Mad2 in complex with a peptide closely resembling this motif has revealed that Mad2 undergoes major conformational changes on binding to this peptide (Luo *et al.*, 2000, 2002). There might be a major kinetic barrier between these two Mad2 conformations and spontaneous transition between these two Mad2 states might be extremely slow *in vivo* (Luo

*et al.*, 2000, 2002). Furthermore, it must be pointed out here that the binding affinity between Mad2 and the full-length Cdc20 is lower than that of the aforementioned Mad2-binding peptide of Cdc20 or the N-terminal fragment of Cdc20 lacking the WD40 repeats (Tang *et al.*, 2001; Zhang and Lees, 2001). Therefore, it is very likely that additional mechanisms, probably involving the unattached kinetochores and other checkpoint proteins, exist to facilitate the binding between Mad2 and Cdc20 *in vivo*. In this vein, Mad2 localizes to unattached kinetochores in prometaphase (Chen *et al.*, 1996; Li and Benezra, 1996). A fluorescence recovery after photobleaching (FRAP) experiment reveals that Mad2 association with the unattached kinetochores is highly dynamic with a half-life of approximately 20 s (Howell *et al.*, 2000). This is consistent with the notion that unattached kinetochores might serve as a catalytic device to transform inactive Mad2 molecules into an active form that can bind to Cdc20 and inhibit the activity of APC/C (Shah and Cleveland, 2000; Gorbsky, 2001; Yu, 2002).

#### *Function of BubR1/Mad3*

Recently, BubR1 has emerged as another key player in APC/C inhibition as it directly inhibits APC/C<sup>Cdc20</sup> in a Mad2-independent manner (Tang *et al.*, 2001). BubR1 is the mammalian homolog of yeast Mad3 (Taylor *et al.*, 1998). Both BubR1 and Mad3 contain the so-called GLEBS motif that mediates their binding to Bub3 (Wang *et al.*, 2001). Binding between BubR1/Mad3 and Bub3 is constitutive, and is required for the localization of BubR1 to kinetochores during mitosis (Taylor *et al.*, 1998). BubR1 can directly bind to Cdc20 *in vitro* and *in vivo* (Wu *et al.*, 2000; Tang *et al.*, 2001). Recombinant BubR1 protein purified from insect cells inhibits the activity of APC/C in ubiquitination assays more effectively than does recombinant Mad2 (Tang *et al.*, 2001). Bub3 binding to BubR1 does not contribute significantly to the ability of BubR1 to inhibit APC/C (Tang *et al.*, 2001). A significant difference between BubR1 and its yeast homolog Mad3 is that BubR1 possesses a kinase domain, which is absent in Mad3. Surprisingly, the kinase domain of BubR1 is not required for its inhibition of APC/C (Tang *et al.*, 2001).

Recently, the kinase activity of BubR1 has been shown to be essential for checkpoint functions other than APC/C inhibition (Mao *et al.*, 2003). BubR1 binds to the mitotic motor protein CENP-E, which is required for the maintenance of stable kinetochore-microtubule interactions and for proper checkpoint signaling (Abrieu *et al.*, 2000). Using purified BubR1 and CENP-E proteins *in vitro*, Mao *et al.* (2003) have shown that the kinase activity of BubR1 toward itself or a nonspecific substrate, histone H1, can be stimulated by its binding to CENP-E. Immunodepletion of CENP-E from or addition of a CENP-E inactivating antibody to nocodazole-treated *Xenopus* egg extracts supplemented with sperm nuclei abolishes the kinase activity of endogenous BubR1 (Mao *et al.*, 2003). However, there has been some controversy about the actual significance

of the BubR1 kinase activity in *Xenopus* egg extracts. It had been reported earlier that BubR1 immunodepletion abolishes the spindle checkpoint, which can be restored by the addition of either wild-type or kinase-dead recombinant BubR1 proteins to these lysates (Chen, 2002). In contrast, Mao *et al.* (2003) found that the kinase-dead BubR1 protein cannot effectively restore checkpoint function in BubR1-immunodepleted extract. In a set of elegant experiments, Mao *et al.* (2003) were able to resolve the apparent discrepancy between the two results. They showed that addition of the wild-type BubR1 protein to 10% of its endogenous level does not restore the spindle checkpoint, but further addition of the kinase-dead mutant of BubR1 can restore the checkpoint (Mao *et al.*, 2003). Therefore, it is quite possible that, in the earlier experiments, the immunodepletion of BubR1 might not have been complete, and a small amount of persisting BubR1 could have aided the exogenously added kinase-dead BubR1 protein to restore the checkpoint (Chen, 2002). Thus, BubR1 appears to have two roles in the spindle checkpoint: a stoichiometric role involving direct physical association with Cdc20 and a yet unidentified catalytic role requiring only a small portion of the endogenous pool of BubR1. As the kinase activity of BubR1 is required for the recruitment of CENP-E and Mad2 to the kinetochore in *Xenopus* egg extracts, it is likely that the catalytic function of BubR1 is important for an upstream event of spindle checkpoint signaling (Mao *et al.*, 2003). A definitive answer to this question requires the identification of key substrates of the CENP-E activated BubR1.

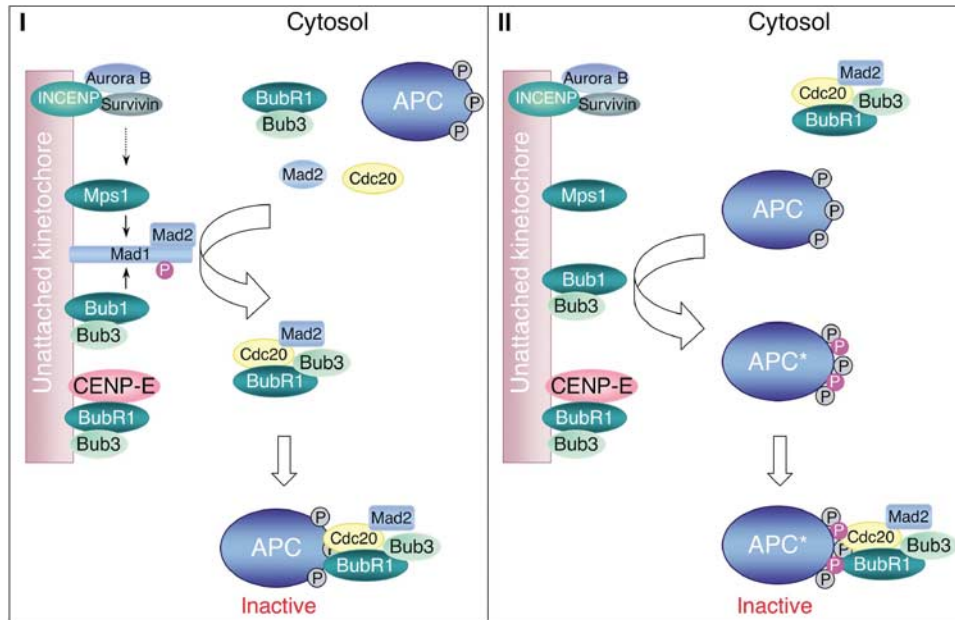
### The MCC

Although Mad2 and BubR1 can independently inhibit APC/C activity *in vitro*, there must be synergism between these two proteins *in vivo* because both of them are indispensable for a functional checkpoint. Consistent with this notion, Fang *et al.* have shown that Mad2 and BubR1 can synergistically inhibit APC/C *in vitro* (Fang, 2002). In addition, a complex of BubR1–Bub3–Mad2–Cdc20 exists in mammalian cells and in *Xenopus* egg extracts, and has been referred to as MCC (Sudakin *et al.*, 2001; Chen, 2002). A Mad3–Bub3–Mad2–Cdc20 complex has also been demonstrated to exist in both budding and fission yeast (Hardwick *et al.*, 2000; Millband and Hardwick, 2002). MCC partially purified from HeLa cells inhibits the activity of APC/C in ubiquitination assays much more effectively than does recombinant Mad2 (Sudakin *et al.*, 2001). It is also possible that Cdc20 in the MCC complex has a higher affinity for APC/C than free Cdc20 or Mad2 bound Cdc20. This might alleviate the requirement of the checkpoint to inhibit the entire cytosolic pool of Cdc20, and can explain the observation that there exists a pool of free Cdc20 not bound to either Mad2 or BubR1 in nocodazole arrested cells.

There is a controversy as to whether the MCC is generated specifically in mitosis or throughout the cell

cycle. Several groups have reported that MCC or its subcomplexes (Mad2–Cdc20 and BubR1–Bub3–Cdc20) only form in checkpoint-active mammalian cells or *Xenopus* egg extracts (Fang *et al.*, 1998a; Tang *et al.*, 2001; Chen, 2002; Fang, 2002). The amount of MCC is negligible in *Xenopus* egg extracts lacking an active checkpoint or thymidine-arrested HeLa cells, due to the lack of Mad2–Cdc20 and BubR1–Cdc20 interactions (Fang *et al.*, 1998a; Tang *et al.*, 2001; Chen, 2002; Fang, 2002). From these studies, it has been postulated that an active spindle checkpoint stimulates the formation of MCC, which then binds to APC/C in a stoichiometric fashion to inhibit APC/C (Yu, 2002) (Figure 3). As all four components of MCC have been found to localize to unattached kinetochores in a dynamic fashion (Howell *et al.*, 2000; Kallio *et al.*, 2002a), it is likely that the unattached kinetochores might be involved in the generation of MCC. In a different model, it has been argued that MCC exists throughout the cell cycle, but fails to bind to APC/C in the absence of checkpoint signaling (Hoyt, 2001; Sudakin *et al.*, 2001). Upon checkpoint activation, APC/C itself is modified, which then leads to efficient MCC binding and prolonged inhibition of APC/C (Hoyt, 2001; Sudakin *et al.*, 2001) (Figure 3). Consistent with the second model, Sudakin *et al.* have detected MCC in the interphase mammalian cells (Hoyt, 2001; Sudakin *et al.*, 2001). Furthermore, the MCC complex can be immunoprecipitated from yeast cells arrested in S phase with hydroxyurea (Fraschini *et al.*, 2001). In addition, this complex can be generated in Ndc10 mutants lacking a functional kinetochore, suggesting that the kinetochore might not be absolutely necessary for the formation of MCC (Fraschini *et al.*, 2001). Additional studies are obviously needed to resolve these differences. However, we would like to suggest a simple explanation for these discrepancies. It is conceivable that a basal level of MCC can be generated without the assistance of kinetochores at all cell cycle stages, but the level of MCC increases remarkably in mitotic cells that possess an active checkpoint. It is also possible that, in addition to stimulating the formation of MCC, an active spindle checkpoint also modifies the APC/C that further bolsters its interaction with MCC.

Does Mad2 have any APC-inhibitory activity *in vivo* in the absence of Mad3? Some key insights into this issue come from unpublished data from Spencer and colleagues (MS Lee and FA Spencer, personal communication). They performed a yeast screen to identify genes that are synthetic lethal with Mad1, Mad2, or Mad3. An identical set of genes were found to be synthetic lethal with either Mad1 or Mad2. However, several deletion mutants were identified that can survive in the absence of Mad3, but not in the absence of Mad2. This indicates that Mad2 can perform functions that do not absolutely require Mad3/BubR1. Of course, these functions could be checkpoint related or unrelated. As we do not have conclusive evidence for a noncheckpoint role for Mad2, we tend to believe that Mad2 by itself might have a residual ability to inhibit APC *in vivo*.



**Figure 3** Two distinct, yet nonexclusive, models for the generation of the APC-inhibitory spindle checkpoint signal. In model I, the unattached kinetochore serves a template catalysing the formation of the BubR1–Bub3–Mad2–Cdc20 (MCC) complex, which then diffuses away from the kinetochore to inhibit the APC. In model II, modification of the APC by the unattached kinetochore promotes its association with the MCC, leading to the inhibition of APC

## Generating the MCC

### Role of Mad1

Elegant studies in several experimental organisms have established a complicated set of protein–protein interactions among various checkpoint components. The kinetochore localization of Mad2 and its binding to Cdc20 require the function of Mad1, but not Bub1, Bub3, or BubR1(Mad3) (Hardwick *et al.*, 2000). Mad1 is constitutively bound to Mad2 through its C-terminus domain whereas its N-terminal coiled–coiled domain is required for its kinetochore localization (Chen *et al.*, 1998; Luo *et al.*, 2002). A Mad1 mutant containing the Mad2-binding site but lacking the N-terminal coiled–coiled domain disrupts the checkpoint in a dominant-negative fashion (Chung and Chen, 2002; Luo *et al.*, 2002). Surprisingly, however, Mad2 overexpression can cause metaphase arrest even in the absence of Mad1, suggesting that, at higher concentrations, Mad2 can function without the assistance of Mad1 (Luo *et al.*, 2002; Millband and Hardwick, 2002). Mad2 binds to Mad1 through a motif that is highly similar to the Mad2-binding motif of Cdc20 (Luo *et al.*, 2002). Both solution and crystal structures of Mad2 in association with this motif have been solved (Luo *et al.*, 2002; Sironi *et al.*, 2002). Mad1 binding to Mad2 induces the same conformational changes in Mad2 as does the Cdc20 peptide (Luo *et al.*, 2002; Sironi *et al.*, 2002). Thus, Cdc20 and Mad1 binding to Mad2 are mutually exclusive. *In vitro*, Mad1 can prevent Mad2 from inhibiting APC/C<sup>Cdc20</sup> and Mad1 overexpression in HeLa cells abolishes the spindle checkpoint (Luo *et al.*, 2002). Paradoxically, Mad1 participates in both the

activation and inhibition of Mad2 (Luo *et al.*, 2002). The ratio of Mad1:Mad2 is thus crucial for proper checkpoint signaling.

The exact mechanism by which Mad1 facilitates the Mad2–Cdc20 interaction is not clear. However, we have previously postulated that Mad1 facilitates the binding of Mad2 to Cdc20 by catalysing the formation of a Mad2 conformer that is more compatible for Cdc20 binding (Luo *et al.*, 2002; Yu, 2002). For this mechanism to be correct, the interaction between Mad1 and Mad2 has to be dynamic, that is, Mad2 has to turn over quickly on Mad1. As the Mad1–Mad2 interaction is constitutive throughout the cell cycle, the checkpoint may enhance the rate of Mad2 turnover on Mad1 by accelerating its release from Mad1, instead of the steady-state levels of the Mad1–Mad2 complex. We do not understand yet whether and how this is accomplished when the checkpoint is activated during mitosis. However, it is tempting to speculate that, upon recruitment to kinetochores, Mad2 might dissociate from Mad1 with the assistance of other kinetochore proteins, and the Mad2 molecules thus released might exist in a conformational state capable of Cdc20 binding. It is not known whether the formation of the Mad2–Cdc20 complex occurs at the kinetochores or the active Mad2 species generated at kinetochores diffuse into the cytoplasm to bind to Cdc20.

### Role of upstream checkpoint kinases

Consistent with the possibility that the Mad1–Mad2 interaction is regulated by the checkpoint, Mad1 is a substrate of the upstream checkpoint kinases such as

Mps1 and Bub1 (Hardwick *et al.*, 1996; Seeley *et al.*, 1999). Mps1 is a protein kinase required for the spindle checkpoint. Immunodepletion of Mps1 from checkpoint-active *Xenopus* egg extracts or its disruption in mammalian cells with kinase-dead mutants or RNAi causes disruption of the spindle checkpoint (Abrieu *et al.*, 2001; Stucke *et al.*, 2002). In Mps1 RNAi cells, Mad1 and Mad2 are no longer present at the unattached kinetochores (Stucke *et al.*, 2002). Mps1 overexpression causes a mitotic arrest in yeast that is dependent on the presence of other checkpoint genes including Bub1, Bub3, Mad1, Mad2 and Mad3 (Hardwick *et al.*, 1996). In yeast, activation of the checkpoint either by nocodazole treatment or by overexpression of Mps1 leads to hyperphosphorylation of Mad1 and the formation of a Mad1–Bub1–Bub3 complex (Hardwick *et al.*, 1996; Brady and Hardwick, 2000). Moreover, hyperphosphorylation of Mad1 depends on Mps1 *in vivo* and Mad1 can be directly phosphorylated by Mps1 *in vitro* (Hardwick *et al.*, 1996). These data suggest that phosphorylation of Mad1 by Mps1 may play a role in regulating the dynamics of the Mad1–Mad2 interaction and consequently the assembly of the MCC.

Bub1 is another checkpoint kinase that might be involved in regulating the Mad1–Mad2 interaction. Similar to BubR1, Bub1 contains a GLEBS motif and is constitutively bound to Bub3 (Wang *et al.*, 2001). Bub1 localizes to the kinetochores in prometaphase and its kinetochore localization persists at a lower level even in anaphase cells (Jablonski *et al.*, 1998). In budding yeast, the N-terminus domain of Bub1 is sufficient for its kinetochore localization and checkpoint function (Warren *et al.*, 2002). The kinase domain of Bub1 has been found to be dispensable for its checkpoint function (Warren *et al.*, 2002). Instead, it is required for proper chromosome segregation (Warren *et al.*, 2002). However, the kinase activity of Bub1 has recently been shown to be essential for the spindle checkpoint in fission yeast (Yamaguchi *et al.*, 2003). Obviously, this discrepancy needs to be resolved with additional studies. Nevertheless, Bub1–Bub3 forms a complex with Mad1 in mitosis (Brady and Hardwick, 2000). Coupled with the fact that human Bub1 phosphorylates human Mad1 *in vitro* (Seeley *et al.*, 1999), it is conceivable that phosphorylation of Mad1 by Bub1 may be required for the dissociation of Mad2 from Mad1 and the subsequent formation of the Mad2–Cdc20-containing complex.

#### Role of Cdc20 phosphorylation

In addition to the conformational activation of Mad2, modification of Cdc20 may also be required for the efficient formation of MCC. Recently, MAPK phosphorylation of Cdc20 in the Mad2-binding domain has been shown to be required for Mad2 and BubR1 binding to Cdc20 in *Xenopus* egg extract (Chung and Chen, 2003). Addition of Cdc20 mutant proteins lacking these phosphorylation sites to *Xenopus* egg extracts immunodepleted of endogenous Cdc20 does not restore spindle checkpoint, presumably due to a defect in the formation

of the MCC (Chung and Chen, 2003). Further studies are needed to check whether this phenomenon is conserved in mammalian somatic cell cycles.

### The sensing mechanism of the spindle checkpoint

#### Attachment versus tension

There has been an ongoing debate on the exact nature of the mitotic defects that are sensed by the spindle checkpoint. There are two main models: (1) the most upstream sensor of the spindle checkpoint monitors the occupancy status of microtubule-binding sites on the kinetochore; or (2) it senses the tension across the sister-chromatid pair generated by the attachment of kinetochore microtubules emanating from the opposite poles of the mitotic spindle. Multiple attempts have been made in different organisms to uncouple microtubule attachment at the kinetochores from the subsequent development of tension across the kinetochores. Treatment of mammalian cells with microtubule toxins, such as taxol, vinblastine or noscapine at low concentrations or, in the case of PtK cells, exposure to hypothermia, alters microtubule dynamics in such a way that kinetochore microtubule attachment remains largely unaffected, but the tension across the paired kinetochores is absent (Waters *et al.*, 1998; Hoffman *et al.*, 2001; Skoufias *et al.*, 2001; Shannon *et al.*, 2002; Zhou *et al.*, 2002). These cells show a prolonged mitotic arrest under these conditions, suggesting that microtubule attachment per se might not be sufficient to silence the checkpoint. Moreover, in cells with monastrol, an inhibitor of the mitotic motor Eg5, some of the syntelically attached chromosomes still show Mad2 staining, indicative of an active spindle checkpoint (Kapoor *et al.*, 2000). This is consistent with the notion that microtubule attachment alone is not sufficient for satisfying the checkpoint. Further support for this notion comes from studies on budding yeast cells lacking Cdc6, a protein essential for the initiation of DNA replication (Stern and Murray, 2001). The  $\Delta$ CDC6 yeast cells do not arrest in S phase because the replication intermediates that are sensed by the replication checkpoints are missing in these cells (Stern and Murray, 2001). However, they arrest in mitosis in a spindle checkpoint-dependent manner. The kinetochores in these cells are attached to microtubules as indicated by their proximity to spindle poles and an equal segregation frequency to the mother and daughter cells (Stern and Murray, 2001). Obviously, these kinetochores do not experience any tension because they are unpaired. This suggests that microtubule attachment at kinetochores alone may not be sufficient to satisfy the spindle checkpoint, which also senses the lack of tension across the kinetochores (Stern and Murray, 2001).

On the other hand, other experiments suggest that attachment by itself is sufficient to silence the spindle checkpoint. For example, laser ablation of the last unattached kinetochore allows cells to undergo mitosis (Rieder *et al.*, 1995). In this situation, the undamaged

kinetochore that pairs with the laser ablated one is attached to microtubules, and should not experience any tension. Yet the cells escape mitosis, suggesting that lack of tension at this kinetochore is apparently unable to maintain the checkpoint signal (Rieder *et al.*, 1995). Studies in maize cells paint a more complicated picture (Yu *et al.*, 1999). In this organism, the spindle might monitor microtubule attachment in mitosis, but tension in meiosis (Yu *et al.*, 1999). Furthermore, the interpretation of studies that implicate the lack of tension as a mechanism of checkpoint activation is complicated by the interdependence between tension and microtubule attachment at the kinetochores. It is well accepted that tension promotes the stabilization of kinetochore microtubule attachment (Nicklas *et al.*, 2001). A formal argument can be made that, although kinetochore attachments appear to be grossly normal under all these conditions, the attachment is not stable and some of the microtubule-binding sites may become unoccupied at a given time. It is the transient lack of attachment that then triggers the checkpoint. It is almost technically impossible to rule out this possibility and thus definitively answer the question of whether the checkpoint senses attachment versus tension.

#### *Distinctive checkpoint responses to different defects*

Nevertheless, studies toward resolving this issue have provided significant insights into the functions of several checkpoint proteins in response to different spindle defects. For example, both Mad2 and BubR1 are required for the maintenance of mitotic arrest in response to taxol, vinblastine or hypothermic treatment (Shannon *et al.*, 2002). Microinjection of antibodies against either of these proteins causes premature escape from mitosis under these conditions (Shannon *et al.*, 2002). Mad2 is absent from the tension-free attached kinetochores in all the above-mentioned situations, indicating that kinetochore localization of Mad2 responds primarily to attachment, and not tension (Waters *et al.*, 1998). In insect spermatocytes, if attachment of one kinetochore of a sister-chromatid pair is repeatedly disrupted by microneedle manipulation, Mad2 persists on the unattached kinetochore, but not on the attached one, despite the fact that both kinetochores in this case lack tension (Nicklas *et al.*, 2001). It is intriguing that, although Mad2 is undetectable at the kinetochores, the spindle checkpoint is still active and the cytoplasmic pool of Mad2 appears to be essential for its maintenance. Thus, it is gradually being recognized that Mad2 localization to kinetochores is not always essential for an active checkpoint. Similar observation has been made in mammalian cells depleted for the kinetochore protein Hec1 using RNAi (see below) (Martin-Lluesma *et al.*, 2002). In these Hec1 RNAi cells, Mad2 is absent from the kinetochores, but the cells are still arrested in mitosis and this arrest depends upon the presence of Mad2 (Martin-Lluesma *et al.*, 2002). How can these Mad2 depleted kinetochores maintain an active spindle checkpoint? Again, it is possible that a residual amount of Mad2 undetectable

by the currently available techniques is present on the kinetochores and is responsible for the observed checkpoint activation. However, a somewhat convoluted but more provocative explanation might be that there are two pathways for the formation of Mad2-containing APC/C inhibitors. An intact unattached kinetochore with high concentrations of Mad2 is required for an initial nucleation of the Mad2-containing inhibitors. A subsequent process that involves the Mad2-lacking kinetochores then maintains the existing pool of these Mad2-containing inhibitors, which might be sufficient for the maintenance of the already activated checkpoint. For example, the Mad2-lacking kinetochores might maintain the pre-existing inhibitory complex by inhibiting the putative activities that might disrupt the MCC. Another possibility is that BubR1 might be performing some functions on its own in these cells for which kinetochore bound Mad2 is not required. Consistent with BubR1 playing a compensatory role, in taxol-arrested cells, the BubR1 levels on the kinetochores are higher than normal cells while the kinetochore concentrations of Mad2 are lower (Hoffman *et al.*, 2001).

#### *Molecular sensors of the spindle checkpoint*

Regardless of the nature of the defects that the spindle checkpoint responds to, recent studies on the chromosome passenger proteins, including Aurora B, INCENP, and Survivin, have begun to shed light on the molecular mechanisms by which the spindle checkpoint senses these defects (Stern, 2002; Tanaka, 2002). Aurora B, INCENP, and Survivin physically interact and form a complex, which localizes to the kinetochores in prometaphase, to the cell equator during metaphase, and to the midbody during cytokinesis (Bolton *et al.*, 2002; Murata-Hori *et al.*, 2002; Tanaka, 2002). Functional disruption of these proteins in several model organisms have revealed their involvement in multiple mitotic events, including chromosome congression, chromosome segregation, the spindle checkpoint, and cytokinesis (Kallio *et al.*, 2002b; Murata-Hori *et al.*, 2002; Murata-Hori and Wang, 2002; Hauf *et al.*, 2003; Lens *et al.*, 2003). These proteins are also required to establish bipolar attachment of chromosomes, probably by destabilizing kinetochore microtubule attachments that lack tension (Biggins and Murray, 2001; Tanaka *et al.*, 2002). As mentioned above, Cdc6 budding yeast mutants enter mitosis without a prior round of replication and arrest in mitosis in a Mad2-dependent manner (Stern and Murray, 2001). Cdc6 mutants can attach the kinetochores to microtubules originating from either of the spindle poles at equal frequency whereas double mutants lacking both Cdc6 and Ipl1 (Aurora B in *Saccharomyces cerevisiae*) segregate all their chromosomes to the mother cell (Tanaka *et al.*, 2002). This indicates that Ipl1 promotes biorientation by destabilizing monotelic or syntelic kinetochore microtubule attachments that lack tension (Tanaka *et al.*, 2002). It was later shown that Ipl1-mediated phosphorylation of Dam1, a component of the DASH complex, is

required for the establishment of biorientation (Kang *et al.*, 2001; Li *et al.*, 2002). More interestingly, the Ipl1 mutants are not only defective in establishing chromosome biorientation, but also in spindle checkpoint signaling (Biggins and Murray, 2001). Despite problems in establishing biorientation, the Cdc6 and Ipl1 double mutants can escape mitosis, indicative of a defective spindle checkpoint. Surprisingly, these cells can arrest in the presence of nocodazole, which depolymerizes microtubules and leaves all kinetochores unattached. This suggests that Ipl1 might be selectively required for checkpoint activation responding to the absence of tension (Biggins and Murray, 2001).

Inhibition of Aurora B by various means in different systems has subsequently confirmed the findings made in budding yeast. Recently, two small molecule inhibitors of Aurora B, hesperadin and ZM447439, have been developed (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003). Treatment with these inhibitors or Aurora B RNAi allows the cells to escape mitosis in the presence of Taxol, but not in the presence of nocodazole (Kallio *et al.*, 2002b; Ditchfield *et al.*, 2003; Hauf *et al.*, 2003). Similar findings have been observed in cells depleted for Survivin using RNAi (Lens *et al.*, 2003). There are two possible explanations for this. In the first scenario, the spindle checkpoint might have two arms: one responds to attachment that is independent of Aurora B, and the other monitors tension for which Aurora B is essential. The tension sensing machinery might be dysfunctional in the absence of Aurora B. In nocodazole-treated cells, the lack of attachment might be sensed by the Aurora B-independent pathway, leading to mitotic arrest. In the Taxol-treated cells, the kinetochores remain attached to microtubules and the 'attachment-sensing arm' of the checkpoint is satisfied. Aurora B thus becomes indispensable for maintaining the mitotic arrest in these cells. In a second scenario, the spindle checkpoint can only detect lack of microtubule occupancy at the kinetochores. Aurora B is required for destabilizing kinetochore microtubule attachments in the absence of tension to generate transiently unattached kinetochores. In Taxol-treated cells, Aurora B continuously keeps on severing kinetochore-microtubule connections, and the resulting transiently unattached kinetochores activate the checkpoint. In the absence of Aurora B, all the kinetochores are attached to microtubules despite a lack of tension and thus the checkpoint is silenced. Although it is unclear how the Aurora B-INCENP-Survivin complex senses spindle defects and promotes biorientation, it has become increasingly clear that this complex is a crucial part of defect sensing mechanism of the spindle checkpoint.

How the Aurora B complex communicates with the other downstream checkpoint components is a subject of active investigation. Recent studies have provided some tantalizing clues. For example, RNAi-mediated depletion of Aurora B or Survivin in mammalian cells diminishes the kinetochore localization of BubR1 (Lens *et al.*, 2003). Moreover, BubR1 is normally hyperphosphorylated in checkpoint-active cells. This hyperphosphorylation of BubR1 is absent in Aurora B-depleted

cells (Ditchfield *et al.*, 2003). Thus, it is possible that Aurora B directly phosphorylates BubR1. Mps1 might be another downstream target of Aurora B, as Ipl1 is required for the mitotic arrest exerted by overexpression of Mps1 in yeast (Biggins and Murray, 2001). Coupled with the fact that certain Mps1 mutations are synthetic lethal with certain Dam1 mutations (Jones *et al.*, 1999), it is very likely that Mps1 and Ipl1 are involved in some common functions.

## Recruitment of checkpoint proteins to the kinetochores

### Recruitment of Bub1 in yeast

What is the mechanism by which the checkpoint proteins are recruited to the kinetochores? Recent studies have begun to shed light on this baffling question in the spindle checkpoint field. Recently, the yeast Skp1 protein has been identified as a Bub1-binding protein in a yeast two-hybrid screen (Kitagawa *et al.*, 2003). The physical interaction between the two proteins is direct, as demonstrated by *in vitro* binding assays using recombinant proteins (Kitagawa *et al.*, 2003). Furthermore, Skp1 was shown to be required for the recruitment of Bub1 to kinetochores in yeast (Kitagawa *et al.*, 2003). Skp1 is an essential component of the CBF3 complex that is absolutely required for the assembly of a functional kinetochore. CBF3 mutants do not arrest in mitosis despite their inability to segregate their chromosomes, because they are essentially kinetochore-null and thus cannot recruit any of the checkpoint proteins (Kitagawa *et al.*, 2003). Therefore, the Skp1 mutations can perturb the checkpoint either due to a generalized defect in the assembly of the functional kinetochore or specifically due to a lack of interaction with Bub1. To distinguish between these two possibilities, Kitagawa *et al.* (2003) identified a Skp1 mutant that is still proficient in kinetochore assembly but does not interact with Bub1. This mutant does not show any significant chromosome mis-segregation phenotype, which is a hallmark of kinetochore dysfunction, but are checkpoint deficient under specific circumstances. Furthermore, this mutant can efficiently arrest in mitosis under circumstances where kinetochore microtubule attachment is disrupted, for example, in the presence of nocodazole. However, these cells cannot arrest in situations where kinetochore microtubule attachment is normal and there is only a lack of tension at the kinetochores. The authors further tested the effect of this mutation in the presence of three other mutations: Ctf8, Scc1 and Cdc6 (Kitagawa *et al.*, 2003). In Ctf8 and Scc1 mutants, the kinetochores can still attach to microtubules. But the tension at kinetochores is diminished because the linkage between sister-chromatids is compromised. Similarly, in Cdc6 mutants, as mentioned before, the kinetochores are unpaired and consequently lack tension although these kinetochores are capable of microtubule attachment. The Skp1 mutant cells fail to arrest in mitosis in all three situations. The inability of Skp1 mutants to arrest in these situations further

bolsters the idea that the set of proteins required to sense the absence of attachment might be different from that involved in sensing tension. Unfortunately, mammalian Skp1 has not been shown to be involved in kinetochore function (Freed *et al.*, 1999; Gstaiger *et al.*, 1999). Whether Skp1 plays a role in recruiting Bub1 to kinetochores in mammalian cells remains to be determined.

### Recruitment of Mad1

Progress has also been made toward the mechanism by which Mad1 is recruited to kinetochores in prometaphase. Martin-Lluesma *et al.* (2002) have recently shown that human Hec1 interacts with human Mad1 in a yeast two-hybrid screen. Mad1 no longer localizes to kinetochores in mitotic cells that are depleted for Hec1 RNAi (Martin-Lluesma *et al.*, 2002). However, no direct interactions between Hec1 and Mad1 have been detected either *in vivo* or *in vitro*, suggesting that the interaction between Hec1 and Mad1 observed in the yeast two-hybrid assay might be bridged through other components (Martin-Lluesma *et al.*, 2002). Hec1 is the mammalian homolog of the budding yeast Ndc80, which forms a complex with Nuf2, Spc25, and Spc24 (Wigge and Kilmartin, 2001). The Ndc80 complex in yeast is a key kinetochore component and is required for proper chromosome segregation (Wigge and Kilmartin, 2001). Mutants singly disrupted for Ndc80 and Nuf2 arrest in mitosis, indicating that the checkpoint is active in either case. However, simultaneous disruption of both genes leads to an inactivation of the checkpoint, allowing the double mutant cells to escape mitosis without proper chromosome segregation. Similarly, Spc24 and Spc25 mutants are checkpoint defective, suggesting a role for the Ndc80 complex in the establishment of the spindle checkpoint in yeast. A similar Ndc80 complex has been characterized and shown to perform functions ranging from kinetochore assembly, chromosome congression, and the spindle checkpoint in *Xenopus* (McClelland *et al.*, 2003). The Ndc80 complex must also exist in mammalian cells, as cells depleted for Nuf2 using RNAi show similar phenotypes as the cells depleted for Hec1 (DeLuca *et al.*, 2002; Hori *et al.*, 2003). It is possible that the mammalian homologs of Spc24 and Spc25 are responsible for bridging the interactions between Hec1 and Mad1. Consistent with this notion, yeast Spc25 and Mad1 proteins have been shown to interact in a yeast two-hybrid screen (Newman *et al.*, 2000). Unfortunately, the mammalian homologs of Spc25 and Spc24 cannot be identified through sequence similarity alone. It will be interesting to identify these missing components of the vertebrate Ndc80 complex and to test whether the intact Ndc80 complex is responsible for recruiting Mad1 to the kinetochores in mitosis.

Inactivation of other kinetochore components also impairs the kinetochore localization of checkpoint proteins. For example, RNAi-mediated depletion of CENP-I, a mammalian kinetochore protein homolo-

gous to the *Saccharomyces pombe* Mis6 protein, causes a transient mitotic delay that is dependent upon Mad2 (Liu *et al.*, 2003). The kinetochore localization of Mad1 and Mad2 is also compromised in cells subjected to CENP-I RNAi (Liu *et al.*, 2003). In a very recent report, the Ran GTPase has been shown to be involved in the recruitment of the kinetochore proteins in *Xenopus* extracts (Arnaoutov and Dasso, 2003). Addition of high levels of RCC1, an exchange factor for Ran, or depletion of Ran GAP1 perturbs the spindle checkpoint *Xenopus* extract supplemented with sperm nuclei and nocodazole. In this case, the checkpoint defect might be a consequence of diminished levels of Mad2, Bub1, and CENP-E proteins at the kinetochores. Thus, Ran might regulate the loading of checkpoint proteins onto the kinetochores. However, instead of specific functions in recruiting certain checkpoint proteins, CENP-I and Ran might be generally required for the intactness of mature mitotic kinetochores. Disruption of their functions might cause pleiotropic defects at the kinetochores that in turn leads to the loss of kinetochore localization of various checkpoint proteins.

### Silencing the spindle checkpoint

#### *Dynein-mediated depletion of checkpoint proteins from kinetochores*

We also have some clues about the mechanisms leading to the silencing of the checkpoint after all the chromosomes are properly aligned. Studies in *Drosophila* neuroblasts and mammalian PtK cells have implicated the minus end directed motor, dynein, in the process of checkpoint inactivation (Howell *et al.*, 2001; Wojcik *et al.*, 2001). In particular, dynein-mediated transport of the checkpoint proteins from the kinetochores to the spindle poles might be required for shutting off the checkpoint. First, Wojcik *et al.* (2001) showed that dynamitin, a subunit of the dynactin complex, accumulates at the unaligned kinetochores in prometaphase in *Drosophila* cells and migrates to the poles once the chromosomes align on the metaphase plate. The dynactin complex is physically associated with dynein and is thought to recruit and bind cargo proteins to dynein. Thus, the localization pattern of dynactin might also reflect the localization pattern of functional dynein, which is certainly consistent with it playing a role in transporting certain checkpoint proteins from the kinetochores to the spindle poles. They further showed that a cargo of the dynein/dynactin complex is the kinetochore protein, Rough Deal (Rod), which has been implicated in the spindle checkpoint and exhibits similar cellular localization pattern as observed for dynactin (Wojcik *et al.*, 2001). Rod was initially identified in *Drosophila* and its functional homologs were later found in mammalian cells (Basto *et al.*, 2000; Chan *et al.*, 2000). In *Drosophila*, the redistribution of Rod to the spindle poles following chromosome alignment is blocked in a specific hypomorphic dynein mutant

(Wojcik *et al.*, 2001). In contrast to the wild-type cells, high levels of Rod can be observed on the kinetochores even in metaphase in these mutants. These cells exhibit a metaphase block that is dependent upon an active spindle checkpoint, suggesting that dynein-mediated depletion of Rod at the kinetochores is required for switching off the checkpoint (Wojcik *et al.*, 2001).

In a complementary study, Howell *et al.* (2001) showed that several checkpoint proteins, including Mad2 and BubR1, are also transported to the spindle poles in a dynein-dependent manner. Inhibition of dynein in metaphase cells by microinjection of an antidynein antibody or recombinant p50 dynamitin protein leads to metaphase arrest. This arrest is dependent upon the spindle checkpoint because microinjection of anti-Mad2 antibodies allows these cells to escape from this mitotic arrest. Dynein is required for various aspects of mitosis, including chromosome congression and proper spindle formation and positioning. However, chromosome congression occurs normally in cells microinjected with p50 dynamitin in prometaphase. Electron microscopy further revealed that the number of chromosomes attached to each kinetochore in dynamitin-injected cells is similar to that of the wild-type cells. Thus, the metaphase arrest and persistence of Mad2 on the kinetochores in these cells with a compromised dynein function is not due to defects in kinetochore microtubule attachment. Instead, it is more likely that these cells are unable to shut off the spindle checkpoint due to a failure to deplete Mad2 and other checkpoint proteins from the kinetochores.

#### *Cmt2 binding and phosphorylation of Mad2*

The dynein-mediated redistribution of kinetochore proteins provides clues as to how the generation of the wait anaphase signal is abolished in metaphase, but does not explain how the already existing APC/C inhibitory complexes in the cytosol are dismantled. The MCC might dissociate spontaneously or there might exist active mechanisms to break it up. A newly identified protein, Cmt2, has been implicated in the disassembly of the Mad2-containing checkpoint complexes (Habu *et al.*, 2002). Cmt2 was identified as a Mad2-binding protein in a yeast two-hybrid assay and was subsequently shown to interact with Mad2 *in vivo* (Habu *et al.*, 2002). The timing of Cmt2 binding to Mad2 coincides with the dissociation of Mad2 and Cdc20. Overexpression of Cmt2 disrupts the checkpoint and allows cells to escape mitosis in the presence of nocodazole. Depletion of Cmt2 by antisense oligonucleotides results in a transient delay in metaphase followed by cell death. These findings suggest that Cmt2 counteracts the function of Mad2 and might be required to silence the checkpoint (Habu *et al.*, 2002). Another mechanism of turning off Mad2-dependent checkpoint signaling involves phosphorylation of Mad2. Wassmann *et al.* (2003) showed that Mad2 is phosphorylated at multiple sites in its C-terminal region *in vivo*. Phosphorylation of Mad2 increased as cells exit from a nocodazole-mediated mitotic arrest. Moreover, Mad2 mutants mimicking

the phosphorylated form of Mad2 were not only unable to bind to Cdc20, but also inhibited the spindle checkpoint signaling in a dominant-negative manner (Wassmann *et al.*, 2003). These results suggested that phosphorylation of Mad2 inhibits its function and might be an important mechanism for checkpoint inactivation.

#### *Defective spindle checkpoint and aneuploidy*

Genetic instability has long been thought to be a primary contributor to tumorigenesis. It can occur in two forms: microsatellite instability (MIN) characterized by a high mutational load and CIN resulting in aberrant chromosome numbers or aneuploidy. Defects in DNA damage repair pathways have been implicated in MIN whereas the mechanisms behind CIN remain poorly understood. The well-established role of the spindle checkpoint in proper chromosome segregation prompted the speculation that its dysfunction might be responsible for CIN. Cahill *et al.* (1998) demonstrated that a large number of CIN colorectal cancer cell lines are defective in the spindle checkpoint. In some of these cell lines, one of the Bub1 alleles was mutated. These mutated Bub1 alleles inhibit the function of the intact copy of the Bub1 gene in a dominant-negative fashion, as their introduction into otherwise checkpoint proficient MIN cell lines resulted in a loss of spindle checkpoint function. Mutations in checkpoint genes have subsequently been shown in many CIN cancer cell lines in numerous studies (Ohshima *et al.*, 2000; Ru *et al.*, 2002). However, a large number of aneuploid cell lines do not appear to harbor mutations in the known spindle checkpoint genes. It is possible that the spindle checkpoint defects in these cell lines might result from altered expression levels of the known checkpoint genes or mutations of as yet unidentified checkpoint genes.

Paradoxically, a complete inactivation of certain checkpoint genes in higher organisms results in early embryonic lethality. For example, Mad2 null mouse causes embryonic lethality and the Mad2 null embryos show high levels of chromosome mis-segregation and apoptosis (Dobles *et al.*, 2000). Deletion of Bub1 in *Drosophila* is also embryonic lethal (Basu *et al.*, 1999). It is unclear whether the spindle checkpoint is essential for early development of multicellular organisms or whether Mad2, Bub1, and other checkpoint genes have yet unidentified essential noncheckpoint functions. Nevertheless, Mad2 haploinsufficiency is compatible with viability (Michel *et al.*, 2001). The level of Mad2 in these cells is approximately 70% of that of the wild type, suggesting partial compensation of protein levels by the remaining allele (Michel *et al.*, 2001). The reduced Mad2 levels in Mad2<sup>+/-</sup> cells results in spindle checkpoint deficiency. These mice display a high incidence of lung tumors after long latencies (Michel *et al.*, 2001). Thus, relatively minor alterations in the levels of spindle checkpoint proteins can promote tumorigenesis. Instead of a complete inactivation, partial disruption of the spindle checkpoint is more likely to be observed in

cancers, because too frequent loss or gain of chromosomes might compromise cell viability.

## Conclusion

In summary, there is tantalizing evidence supporting the notion that a defective spindle checkpoint contributes to CIN in human cancers. Furthermore, antimotitic anticancer drugs, such as Taxol and vinblastine, kill cancer cells through exploiting defects in their spindle checkpoint. A better understanding of the mechanism of this checkpoint, in particular, how the checkpoint proteins

collaborate to inhibit the ubiquitin ligase activity of APC/C, will eventually lead to new strategies to combat cancer.

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