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The kinetochore and spindle checkpoint in mammals

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TABLE OF CONTENTS

1. ABSTRACT

The two daughter cells that result from each and every cell division receive an identical set of chromosomes. This is accomplished by pulling each copy of a pair of duplicated sister chromatids to opposite poles during mitosis. Inaccuracies in this process lead to aneuploidy, which is a major cause of birth defects and can facilitate the rise of malignancies. Such inaccuracies are prevented in normal cells by the mitotic checkpoint (also known as the spindle assembly checkpoint) that halts cell cycle progression in mitosis when as little as a single chromosome is not properly attached to the mitotic spindle. This review focuses on molecular aspects of mitotic checkpoint signaling in mammals, including sensing improper attachments and transducing this information to the cell-cycle machinery.

2. THE KINETOCHORE

The site of attachment of the chromosome to the microtubule-based spindle is known as the kinetochore, a name derived from an early hypothesis that this site causes the movement of the chromosomes in anaphase (1). The kinetochore is a large structure that is assembled on the centromere of each chromatid (the site of the central constriction), and consists of an estimated 100 proteins. These proteins are generally grouped into two subgroups: the inner- and outer-kinetochore proteins (Figure 1). In general, the inner-kinetochore proteins provide the basic platform for building the protein structures of the outer-kinetochore that are responsible for the three functions performed by the kinetochore: microtubule capture, microtubule dynamics and chromosome movement, and mitotic checkpoint signaling. A schematic representation of

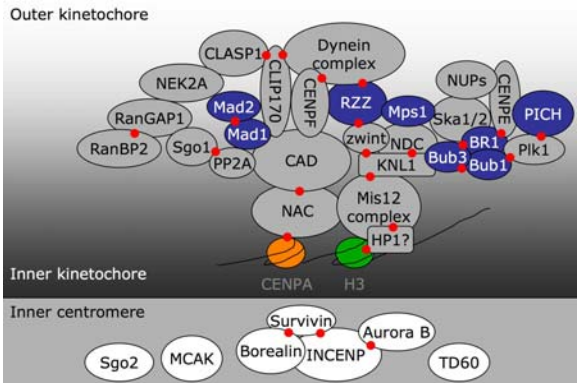

Figure 1. Schematic representation of proteins that localize to an unattached kinetochore and the inner centromere of a human cell. Direct interactions are shown by a connecting red dot. Mitotic checkpoint proteins are shown in blue (see table 1). 'CENP-A' and 'H3' denote the nucleosome containing either histone H3 variant. See text for more details.

the complexes that make up the kinetochore is shown in figure 1. The defining mark for centromere identity that drives kinetochore assembly is the centromere-specific histone H3 variant CENtromere Protein-A (CENP-A), that replaces histone H3 in most nucleosomes at the centromere (reviewed in (2)). The presence of CENP-A in nucleosomes marks the site for building a kinetochore and depletion of CENP-A prevents all but a few proteins from binding the kinetochore. In this regard, CENP-A-independent centromere-specific localization of the Mis12 complex that itself supports assembly of kinetochore-subcomplexes has been reported (3). In support of the idea that CENP-A and Mis12 independently contribute to kinetochore assembly is the observation that none of the complex members of the Mis12 complex are found in TAP purifications of CENP-A and vice versa (4-6). Note, however, that Mis12 localization to centromeres in *C. elegans* is dependent on CENP-A, suggesting that at least in worms CENP-A is the most fundamental centromere mark (4). Interestingly, two recent studies described the identification of proteins (hMis18a/b and M18BP1/hKNL2) that localize to centromeres in late anaphase and that are required for the maintenance of the CENP-A mark on centromeres (7, 8). The two studies were not in agreement, however, whether centromere-localization of this group of proteins is, in turn, dependent on CENP-A, but it was reported to have no influence on Mis12 localization in human cells (7). Besides CENP-A or Mis12, other factors also contribute to specifying the site of kinetochore assembly, including centromere-specific heterochromatin modifications (9, 10).

A group of kinetochore proteins termed the CENP-A Nucleosome Associated Complex (NAC) assembles onto CENP-A-containing nucleosomes (5, 11). This complex consists of CENP-C, -H, -M, -N, -T, and -U, which in turn recruits a CENP-A-distal complex (CAD) that comprises CENP-I, -K, -L, -O, -P, -Q, -R, and -S. Both complexes may in fact be made up of multiple stable subcomplexes (11). The NAC and CAD complexes along with CENP-A are crucial for proper chromosome segregation in mitosis. A distinct group of proteins that include AF15q14/hKNL1, hDsn1, hNnf1 and hNsl1 assembles onto Mis12 (4, 6, 12, 13). It is unknown at present whether assembly of this complex requires CENP-A. Combined, these inner-kinetochore protein complexes control the localization and/or activity of numerous proteins that are involved in the three kinetochore functions, and I will use the next sections to discuss these functions and the proteins that execute them. Please note that to avoid overlap with other reviews in this issue, I will limit discussion of data to those obtained in mammalian systems, and will only refer to other systems when necessary.

2. KINETOCHORE FUNCTIONS

2.1 Microtubule capture

The kinetochore is ready and able to bind and hold on to microtubule bundles as soon as the nuclear envelope is broken down. Microtubules that sprout from the centrosomes reach into the cytoplasm to 'search and capture' kinetochores (reviewed in (14)). The mysterious microtubule-binding capacity of the kinetochore recently received significant attention with evidence of proteins being essential for this initial capture, and whose activity may be regulated by enzymes that correct misattachments (see below). The Ndc80/Hec1-Nuf2-Spc24/25 complex (hereafter referred to as the NDC complex) forms an extended rod from the outer kinetochore into the cytoplasm (15), is required for the structural integrity of the microtubule binding sites (16) and binds microtubules directly (17). This microtubule-binding capacity is enhanced *in vitro* by the hKNL-1 protein (17). Depletion of NDC complex members by RNAi leads to weak kinetochore-spindle microtubule interactions and a subsequent prolonged mitosis (18). Importantly, acute inhibition of Ndc80 by antibody injection in prophase PtK1 cells suppressed plus-end assembly of microtubules fibers on kinetochores that had already established interactions with spindle microtubules, and caused mis-segregations (19). These studies suggest that the NDC complex is required both for initial microtubule capture and subsequent maintenance of microtubule dynamics at the kinetochore.

Intriguingly, regulation of the NDC complex is a major mechanism of how improper attachments can be corrected. The chromosomal passenger complex (CPC) that harbors the Aurora B kinase is required for correction of attachments that do not produce tension, such as those of a syntelic nature (reviewed in (20)). Aurora B phosphorylates the Ndc80/Hec1 component of the NDC complex, thereby weakening the interactions of that complex with kinetochore microtubules and allowing detachment of the microtubule from the kinetochore (17, 19).

Various other proteins contribute to microtubule capture by the kinetochore. CLIP-170 is a plus-end tracking protein, or +TIP, that localizes specifically to unattached kinetochores and that leaves the kinetochore upon attachment (21, 22). RNAi of CLIP-170 causes severe

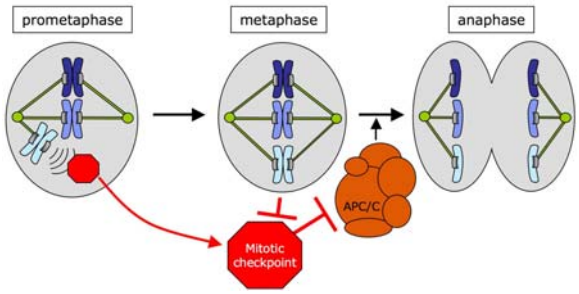

Figure 2. Principle of mitotic checkpoint signaling. An unattached kinetochore in prometaphase emits a checkpoint signal that inhibits the APC/C, whose activity is required for the metaphase-to-anaphase transition. The checkpoint is inhibited once all chromosomes have attached and congressed to the metaphase plate, causing APC/C activation and the transition to anaphase.

chromosome misalignments and mitotic arrest due to inability of the kinetochores lacking CLIP-170 to interact stably with spindle microtubules (21, 22). In addition, mammalian cells depleted of CENP-E, a kinesin 7 family member, have trouble reaching full alignment likely due to a decreased microtubule-binding capacity of kinetochores (23, 24). Kinetochores of unaligned chromosomes in CENP-E-depleted cells were unbound to microtubules, while aligned chromosomes had less microtubules bound than wild-type cells (23, 24). Finally, the outer-kinetochore protein CENP-F/mitosin was recently reported to be essential for maintaining stable kinetochore-spindle interactions (25-27). CENP-F has two microtubule binding domains (26), one of which facilitates microtubule polymerization *in vitro*. Kinetochores lacking CENP-F can initiate microtubule binding, but are unable to maintain stability (25). Some non-microtubule-binding proteins have also been reported to regulate kinetochore-microtubule interactions, and these include BubR1 (see section 6), RanBP2 (28) and Crm1 (29). For a good review on these subjects, please see (30).

2.2 Microtubule dynamics and chromosome movement

Once a kinetochore has made initial attachments to the spindle microtubules, the chromosome starts a phase of oscillations and poleward movements that eventually result in congression to the metaphase plate. The poleward movements occur without microtubule depolymerization, likely by the action of minus-end directed microtubule motor proteins such as dynein (31). Several models have been postulated for how subsequent congression to the central region of the cell is achieved. Once a chromosome is mono-oriented (attached with one kinetochore) and close to one pole, the unattached kinetochore faces the other pole and stands a higher chance of engaging microtubules from that opposing pole. Once this has occurred (the chromosome is now referred to as being 'bioriented'), a combination of polar ejection forces and kinetochore-based minus-end-directed movements on the leading kinetochore result in congression (for a thorough review see (32)). A recent study suggested a novel and exciting model for congression without the need for biorientation. In this model, the unattached kinetochore of a chromosome that is mono-oriented and close to the pole binds to kinetochore microtubules of another, already bioriented, chromosome and uses it as a guide to reach the metaphase plate (33). This would require a plus-end directed activity microtubule motor activity, and CENP-E was suggested to fulfill that role.

Similar to prometaphase, chromosome movements in anaphase require the concerted effort of various kinetochore proteins, including motors such as dynein, depolymerases such as the kinesin 13 family members MCAK (34) and Kif2b (35), and CLASPs (36).

2.3 Mitotic checkpoint signaling

Essential for the maintenance of chromosomal stability over many generations is the ability of cells to detect when chromosomes are either unattached, unstably attached, or misattached, and relay that state to inhibition of the cell cycle. For this, cells have evolved the mitotic checkpoint, a.k.a. the spindle assembly checkpoint. Each and every unattached kinetochore is a signal generator that translates its state of attachment to the anaphase-promoting complex/cyclosome (APC/C), a multiprotein E3 ubiquitin ligase that, when activated by its mitosis-specific cofactor Cdc20, controls the transition from metaphase to anaphase (Figure 2). This transition is marked by chromosome segregation and subsequent exit from mitosis. Chromosome segregation is regulated by separase, a cysteine protease related to caspases that cleaves the Scc1 subunit of the cohesin complex that holds the two sister chromatids together (reviewed in (37) and (38)). Separase is inhibited in mitosis by securin until anaphase is allowed to ensue. As soon as APC/C is activated by Cdc20, it targets securin for destruction by the proteasome, allowing activation of separase and chromosome segregation (reviewed in (39)). Mitotic exit is achieved by simultaneous destruction of cyclin B that leaves cdk1 inactive, allowing spindle disassembly, chromosome decondensation and nuclear envelope re-assembly.

The mitotic checkpoint inhibits Cdc20-dependent activation of the APC/C until all kinetochores are properly attached (40, 41). This ensures an extension of mitosis to allow sufficient time to achieve proper attachments and biorientation. Classic experiments by Rieder and colleagues have shown that a single unaligned chromosome with one unattached kinetochore is sufficient to delay anaphase onset in PtK1 cells until that unattached kinetochore is destroyed by laser-ablation (42). This suggested that the checkpoint signal that is generated from that single unattached kinetochore is sufficiently strong to inhibit all cellular APC/C activity.

There is some debate as to what feature of an unattached or mono-oriented chromosome is activating checkpoint signaling. It is clear that lack of attachment is sufficient to activate the checkpoint, but lack of attachment equals lack of tension between sister centromeres. It has thus been argued that the checkpoint

Table 1. Proteins suggested to contribute to mitotic checkpoint signaling

Protein	Criterion 1 localized to or activated by improperly attached kinetochores specifically	Criterion 2 contributes to formation of APC/C inhibitor	Criterion 3 Depletion/inhibition inactivates the checkpoint
Definitely			
Mad2	YES	YES, part of MCC	YES
Mad1	YES	YES, activates Mad2	YES
Bub3	YES	YES, part of MCC	YES
BuBR1	YES	YES, part of MCC	YES
RZZ	YES	YES, localizes Mad1/Mad2	YES
Mps1	YES	YES, localizes Mad1/Mad3	YES
PICH	YES [(1)]	YES, required for Mad1/Mad2 interaction	YES
Bub1	YES	YES, phosporylates Cdc20	YES
Undecided			
USp44	ND	YES, prevents MCC instability	YES
TAO1/MARKK	Localization unclear	YES, required for Mad1/Mad2 interaction	YES
Rae1	ND	ND	YES
Chk1	ND	YES, required for BubR1 localization	YES/NO [(4)]
Unlikely			
CENPE	YES	maybe [(2)]	NO
Hec1/Ndc80	NO	maybe [(3)]	maybe (Conflicting data)
Zwint	NO	YES, required for RZZ function	YES
Aurora B	YES	YES, required for BubR1 localization	YES/NO [(4)]
Rae1	ND	ND	YES
Plk1	YES [(5)]	NO	NO
MAPK	maybe (conflicting data)	ND	ND
NEK2A	maybe [(6)]	YES, required for Mad1/Mad2 interaction	NO [(7)]
p38	ND	ND	NO

Meeting the criteria is based solely on data from mammalian systems. See text for details. [1] relocalizes from kinetochores to threads in between sister centromeres, [2] depends on whether BubR1 kinase activity is required, [3] Required to maintain Mad1 and Mad2 at kinetochores, [4] required for checkpoint response to lack-of-tension but not essential for response to lack-of-attachment, [5] enriched at kinetochores that are not under tension, [6] overexpression of GFP-Kinase-Dead reduces accumulation upon nocodazole, [7] sustained response to nocodazole is diminished but not abrogated. ND; not determined

senses lack of tension. This would make sense, as tension suggests the existence of not only attachment per se, but productive, force-producing attachments, the only kind that should be tolerated by the checkpoint. This hypothesis has received substantial support from elegant micromanipulation experiments on univalent sex chromosomes in grasshopper spermatocytes that cause an infinite delay in meiosis. Applying tension to that already attached chromosome caused the cell to override the arrest and enter anaphase, suggesting that the checkpoint responded to lack-of tension (43). Detailed molecular studies in yeast, however, have now shown that, at least in mitosis, it is in fact most likely the other way around (44, 45). Lack-of-tension is sensed, but is converted to lack-of-attachment by the Aurora B kinase complex, thus ensuring a mitotic checkpoint response to lack-of-tension. Nevertheless, it is completely unclear how lack of tension is sensed and how this activates Aurora B, but it may involve factors like Sgo1 and PICH (46, 47).

The following sections will elaborate on the molecular aspects of mitotic checkpoint function, the signals that activate it, and its relation to pathological conditions associated with chromosomal instability.

3. COMPONENTS OF THE MITOTIC CHECKPOINT

Soon after the initial discovery of seven genes needed for the spindle checkpoint in *S. cerevisiae* (48-50), the human orthologues of six of these were identified (51-56). These genes, encoding Mad1, Mad2, BubR1 (Mad3-like with the addition of a C-terminal kinase domain), Bub1, Bub3 and Mps1, are all essential for the mitotic checkpoint in human cells, and are considered bona fide mitotic checkpoint proteins. Since their discovery, several other checkpoint proteins for which no obvious orthologues can be found in yeast have been identified in higher eukaryotes. These include ZW10, Rod and Zwilch (RZZ) (57, 58). Many more proteins have been suggested to participate in mitotic checkpoint signaling and have been referred to as mitotic checkpoint genes. Some indeed could be, but most likely aren't. Many kinetochore and/or mitotic proteins have been implicated in checkpoint control based on the observation that depletion or inhibition caused mitotic checkpoint defects. However, a protein that alters the structure of the kinetochore or centromere may affect checkpoint activity as an indirect consequence of these structural alterations. So what defines a bona fide component of the mitotic checkpoint machinery? I suggest three criteria that a candidate mitotic checkpoint protein should meet in order to be classified as one. First, its subcellular location and/or activity should be sensitive to the state of attachment of kinetochores. For instance, Mad2 only localizes to kinetochores that are unattached (59). Second, the protein should contribute to relaying the status of attachment to the cell cycle machinery. For instance, Mad2 is part of a complex that maintains the cell-cycle arrest in response to unattachment (40, 41). Third, depletion or inhibition of the protein should inactivate the mitotic checkpoint. Again, Mad2 RNAi leaves cells unable to mount a checkpoint response and causes severe chromosome missegregations in human cells (60, 61). Based on these criteria, a list of bona fide checkpoint proteins is displayed in tables 1 and 2. Table 1 also includes

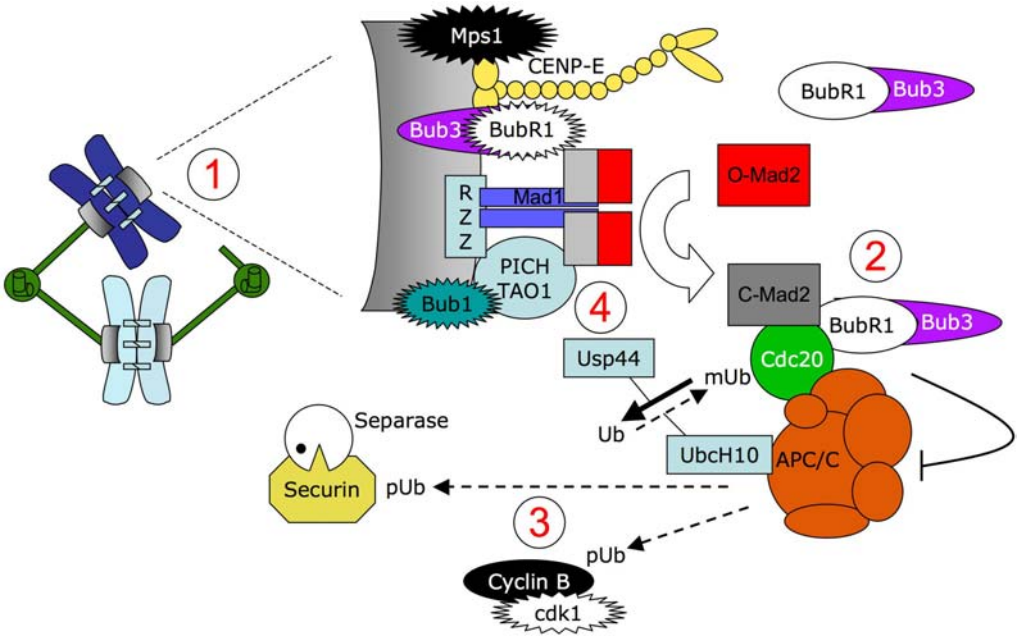

Figure 3. Schematic model of molecular aspects of mitotic checkpoint signaling. Unattached kinetochores (1) specifically recruit proteins that participate in production of the Mitotic Checkpoint Complex (MCC) (2), an inhibitory complex that prevents poly-ubiquitination (pUb) and subsequent degradation of cyclin B and Securin (3) by the APC/C. Integrity of the MCC is further maintained by Usp44, a de-ubiquitinating enzyme that counteracts Cdc20 multi-ubiquitination (mUb) by the APC/C and its E2 co-enzyme UbcH10 (4).

a list of suggested but unlikely checkpoint proteins and the criteria that they do and do not meet.

4. THE TARGET OF THE CHECKPOINT IN CELL-CYCLE CONTROL

As mentioned, the goal of the checkpoint is to inhibit chromosome segregation and exit from mitosis by inhibiting the APC/C when as little as one chromosome is not attached. How is the APC/C inhibited by these checkpoint proteins? Studies in HeLa cells that attempted to identify an APC/C-inhibitory complex (also known as the Mitotic Checkpoint Complex or MCC) showed that a mitotic APC/C inhibitory activity was present in a subcellular fraction containing complexes of 300-600 kDa. (62). This fraction contained at least Mad2, BubR1, Bub3 and Cdc20. Of these, Mad2 and BubR1 directly interact with Cdc20 and can independently inhibit the APC/C in *in vitro* APC/C activity assays (40, 63, 64). It is unclear, however, how binding of the MCC to Cdc20 inhibits APC/C activity. The most straightforward model poses that MCC binding results in sequestration of Cdc20 away from the APC/C. Indeed, the identification of the MCC showed that it contained stoichiometric amounts of Cdc20 (62). However, TAP-purifications have found a very stable MCC-Cdc20-APC/C complex (GJPLK, unpublished), similar to what has been reported for immunoprecipitated APC/C (40, 62). Possibly, the MCC inhibits Cdc20-dependent activation of the APC/C in a manner other than sequestration of Cdc20, such as substrate recruitment and or product release and may in addition have an impact on the APC/C itself. Conversely, the presence of this mega-complex may reflect a regulatory step that controls Cdc20-ubiquitination, which will be discussed more extensively in section 7.1. I suspect that the identification of the mode of inhibition of the APC/C by the MCC will first require full understanding of how Cdc20 activates the APC/C (see (65) for more thoughts on this).

In summary, the checkpoint responds to improper attachments by catalyzing the production of an inhibitory complex for the APC/C.

5. ACTIVATING THE MITOTIC CHECKPOINT

Kinetochores can engage interactions with microtubules once the nuclear envelope has broken down at the onset of prometaphase. As of that moment, mitotic checkpoint proteins start to localize to the unattached kinetochores (Figure 3). Many attempts have been made to investigate which proteins regulate the localization of the checkpoint proteins in human cells but this has resulted in much confusion as it appears that such dependencies cannot simply be linearized into an assembly pathway. However, some dependencies are crystal-clear. Mad2 cannot bind kinetochores without Mad1 (66), and Mad1 localization depends on the RZZ complex (13, 67). Similarly, BubR1 and Bub1 cannot bind kinetochores without binding to Bub3 (55). Other than that, many dependencies have been suggested, and I refer you to several papers that have made attempts to uncover these (68-70).

Various checkpoint proteins localize to unattached kinetochores in a highly dynamic fashion that is

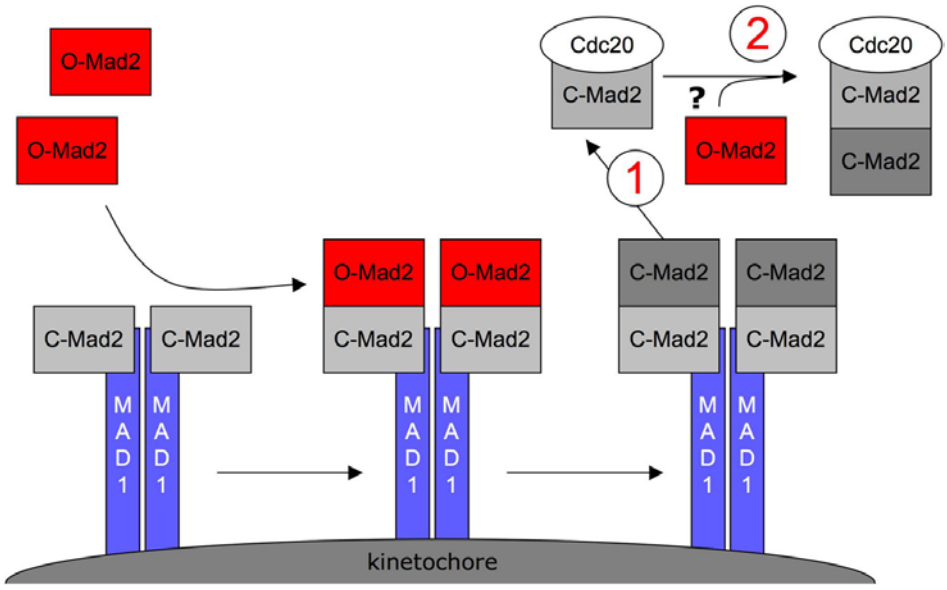

Figure 4. Conversion of Mad2 to a potent Cdc20 inhibitor by the unattached kinetochore. C-Mad2, bound to Mad1, recruits monomeric O-Mad2 molecules from the cytosol. Once dimerized to C-Mad2, O-Mad2 is converted to C-Mad2, released, and able to interact with Cdc20 (1). In addition, it has been suggested that the released C-Mad2 could act as a cytoplasmic amplifier of the checkpoint signal by dimerizing with O-Mad2 and catalyzing its conversion to C-Mad2 that could subsequently proceed to do the same (2). Grey C-Mad2 represents Mad1- or Cdc20-bound C-Mad2 (light grey) or newly activated C-Mad2 (dark grey).

thought to reflect an unattached-kinetochore-dependent activation of these proteins and/or a localized assembly of the MCC-Cdc20-APC/C complex. Interestingly, all components of the MCC, with the addition of Mps1, have a very short half-life at the kinetochore, but non-MCC components such as Bub1 and Mad1 do not (71, 72). The simultaneous recruitment and release of Mad2, Cdc20 and BubR1 to unattached kinetochores therefore likely reflects assembly of the MCC at the kinetochore.

5.1. Mechanism of action: the two states of Mad2

The kinetochore doesn't merely act as a scaffold to concentrate components of the MCC in order to facilitate MCC assembly. Studies on the Mad2 molecule show this best. Free cytosolic Mad2 (referred to by two studies as O-Mad2 (Open-Mad2) or N1-Mad2, and I shall hereafter use the term O-Mad2 (73, 74)) uses dimerization with the Mad2 molecule within Mad1-Mad2 heterodimers that stably localize to unattached kinetochores to catalyze a conformational change in the O-Mad2 molecule (Figure 4). This conformational change in free Mad2 resembles the conformation Mad2 takes on when it's bound to Mad1 or Cdc20, and is referred to as C-Mad2 (Closed-Mad2) or N2-Mad2 (73-75). There is some debate as to what is the actual conversion that happens at the kinetochore. One study suggests that O-Mad2 is primed by the unattached kinetochore for subsequent interaction with Cdc20 (73). Binding to Cdc20 then converts O-Mad2 into C-Mad2. Since Mad2 in this C-Mad2-Cdc20 complex is identical to C-Mad2 bound to Mad1, the C-Mad2-Cdc20 can then function as a kinetochore-independent catalyst for the conversion of more free Mad2 molecules, leading to signal amplification. On the other hand, another study has suggested that O-Mad2 is converted by the unattached kinetochore to C-Mad2 (74). C–Mad2 is then either directly passed on to Cdc20 from Mad1 or dissociates from Mad1 to form a transient dimeric intermediate that then binds to Cdc20. Both models basically propose a very similar way in which Mad2 is activated: Mad2 dimerizes to Mad1-Mad2 heterodimers and is converted to a form that is able to tightly bind and inhibit Cdc20 (Figure 4). For a good review on these models and the differences between them, see (76).

The MCC contains a second direct inhibitor of Cdc20: BubR1, that binds Cdc20 and inhibits APC/C activity *in vitro* (64, 76). BubR1 seems even better at inhibiting Cdc20 than Mad2 is (64, 76), although it was not taken into account that only a fraction of Mad2 purified from bacteria is in the active (C-Mad2) form. Importantly, careful *in vitro* studies have shown that Mad2 and BubR1 act synergistically to inhibit the APC/C (63). Whereas either protein can inhibit the APC/C quite efficiently *in vitro*, 10-20-fold lower levels of both proteins combined reached similar inhibition efficiencies (63). It has not yet been investigated whether BubR1 needs to undergo conformational changes at the kinetochore in order to efficiently bind Cdc20, but it seems likely that BubR1, like Mad2, is modified at the kinetochore. If this is the case, such a modification will likely not involve enhancement of its kinase activity, as this was shown to be dispensible for the role of BubR1 in the MCC complex (64). It thus seems that the conserved Mad3-like N-terminus of BubR1 is sufficient for APC/C inhibition and checkpoint function, although careful studies on chromosome segregation and checkpoint control in cells expressing BubR1 molecules without kinase activity have yet to be done.

In short, although it is still unresolved what the exact APC/C-Cdc20 inhibitor is and how this inhibitor is assembled at, and modified by, the unattached kinetochore, several very striking principles of how an unattached kinetochore creates a potent inhibitor of the APC/C have become apparent.

5.2 Post-translational modifications in checkpoint signaling

The original screens for alleviators of mitotic arrest in yeast identified two kinases as regulators of the checkpoint: Bub1 and Mps1 (48, 50). Searches for orthologues in higher eukaryotes have revealed widespread conservation of these proteins, as expected from the essential contributions of these kinases to mitosis.

5.2.1 Bub kinases

The search for the human Bub1 orthologues unveiled a Bub1-related kinase, termed BubR1 that appears to be the closest human homologue of yeast Mad3 with the addition of a Bub1-like kinase domain at its C-terminus (55). There has been much confusion about the exact roles of these kinases in the checkpoint. For instance, doubt has been raised whether Bub1 is in fact a checkpoint protein in human cells. Studies using a dominant-interfering mutant of Bub1 that contained the N-terminal kinetochore-targeting domain but lacked the kinase domain showed mitotic checkpoint defects (56). With the introduction of RNAi, however, proper removal of Bub1 caused checkpoint defects in one study (77) while causing the opposite, mitotic arrest, in two others (68, 78). The Taylor group subsequently suggested that perhaps the DN-Bub1 used in their previous studies also dominantly interfered with BubR1, which would explain the checkpoint defects seen with that mutant (68). One could argue that depletion of Bub1 needs to be almost 100% for a checkpoint defect to surface while partial depletion is sufficient to expose its role in kinetochore-microtubule interactions (see section 6). A very recent study has conclusively settled the issue whether Bub1 is a true checkpoint component: mouse embryonic fibroblasts in which the Bub1 alleles were conditionally removed had no functional checkpoint (79). It is thus clear that Bub1 protein is essential for the checkpoint, but is its kinase activity as well? While required in yeast (80, 81), it is dispensible for the checkpoint per se in *Xenopus* egg extracts but instead contributes to full signal strength when few kinetochores are signalling (82). Bub1 phosphorylates Cdc20 on multiple residues both *in vitro* and *in vivo* in human cells, and this phosphorylation causes inhibition of the ability of Cdc20 to activate the APC/C (83). Both sets of data are in agreement with a model in which Bub1 kinase activity contributes to amplification or strengthening of the signal per kinetochore and is hence essential to continue the checkpoint-dependent arrest when few kinetochores are unattached. Re-expression of a kinase-dead allele in the Bub1 conditional knock-out MEFs will resolve this.

Although there is general agreement that BubR1 is an essential component of the checkpoint machinery, the contribution of it's kinase activity is less certain. BubR1 kinase is activated in humans and frogs by binding to the tail of CENP-E (84-87). This is direct, as mixing the two recombinant proteins causes BubR1 kinase activation *in vitro* (84, 86). Add-back of kinase-dead BubR1 to checkpoint-competent frog extracts has produced mixed results as to whether BubR1 kinase activity is required for the checkpoint (84, 88), and no thorough investigation into this question in humans has been reported to date. Model organisms like yeast and *C. elegans* have Mad3-like molecules that lack a kinase domain. Intriguingly, Mad3-like proteins with a kinase domain seem to have co-evolved with the presence of CENP-E, suggesting an intricate connection between the two activities. Although CENP-E is not required for the checkpoint per se in human cells (89), CENP-E-negative mouse embryo fibroblasts enter anaphase with misaligned chromosomes that recruit half the BubR1 protein normally recruited in prometaphase (86). Possibly therefore, the argument made for Bub1 kinase activity applies to BubR1 as well: kinase activity is required for signal amplification when few kinetochores are signaling. Another intriguing explanation for co-evolution between BubR1 and CENP-E is that the two control attachments and congression (see section 6).

5.2.2 Mps1 and TAO1 kinases

Mps1 is the only undebated checkpoint kinase. Its activity is required for the checkpoint in frog extracts (52), yeast (90-92) and humans (93). Of all true checkpoint proteins (see table 1), Mps1 is most mysterious with regards to its contributions to MCC formation. All that's known is that Mad1 and Mad2 are absent from unattached kinetochores in Mps1-depleted cells, but not BubR1, Bub1 or any other kinetochore protein investigated (69). In yeast, Mps1 is required for Mad2 activation by directly phosphorylating Mad1 (94), but no such function has been reported yet in mammals, where Mad1 does not appear to be phosphorylated to the same extent.

TAO1/MARKK was recently found to be a novel checkpoint kinase, whose kinase activity is essential for the checkpoint (95). Although investigated, localization of TAO1/MARKK to unattached kinetochores was inconclusive. Like a bona fide checkpoint kinase, TAO1/MARKK activity was enhanced in mitosis, although this was not investigated with the resolution to distinguish whether this depended specifically on improperly attached kinetochores (95). RNAi of TAO1/MARKK inhibited checkpoint signaling in a kinase-dependent manner and Mad2 no longer localized to unattached kinetochores, whereas Mad1 did. Thus, TAO1/MARKK could be a novel checkpoint kinase that is required for Mad1/Mad2 interaction.

5.3. Contributions by other mitotic proteins

5.3.1 PICH, Rae1 and the CPC

Various proteins that are not part of the core checkpoint machinery have been implicated in checkpoint control. A recently identified bona fide member of the checkpoint family is PICH, a DNA helicase and Plk1 substrate whose depletion by RNAi rendered the checkpoint inactive (Table 2) (46). PICH is required for Mad2 but not Mad1 localization to kinetochores, suggesting that PICH may be required for checkpoint activity by regulating Mad1/2 interactions. Surprisingly,

Table 2. Characteristics of mitotic checkpoint proteins

Protein	Type	Size	Interaction partners	Kinetochore localization depends on[1]
Bub1	S/T Kinase	122 kDa	Bub3	Bub3
BubR1	S/T Kinase	120 kDa	Bub3, Cdc20, CENPE	Bub3
Bub3	WD40 repeats	37 kDa	Bub1, BubR1	NI
Mad1	coiled coil	83 kDa	Mad2	Rod, ZW10, Zwilch
Mad2	HORMA-domain	23 kDa	Mad1, Cdc20, p31comet	Mad1
Mps1	Y/S/T kinase	97 kDa	NI	Ndc80/Hec1
Rod	NI	251 kDa	Zwilch, ZW10	Zwint-1
Zwilch	NI	67 kDa	Rod, ZW10	Zwint-1
ZW10	NI	89 kDa	Zwilch, Rod	Zwint-1
PICH	Helicase	141 kDa	Plk1	Plk1

[1]most direct link known, NI: not identified

PICH localizes to kinetochores at prometaphase and to DNA threads in between sister centromeres that are under tension in metaphase. These threads could reflect a removal of PICH from kinetochores that are under tension, hence inhibiting production of the active Mad2 conformer.

Rae1 is a highly conserved nuclear transport factor that is involved in the pathway for mRNA export in interphase (96). Rae1 shares sequence homology with Bub3 and both bind to GLEBS-domain containing proteins, including Bub1 (97). Cells from mice in which one Rae1 allele is deleted have a compromised checkpoint and are aneuploid, and this is enhanced when an additional Bub3 allele is removed (98). Recently, a Rae1-Nup98 complex was shown to inhibit an APC/C complex that is activated by the Cdc20-like protein Cdh1 and that is required for mitotic progression after anaphase onset (99). Rae1-Nup98 therefore acts on APC/C-Cdh1 in a similar fashion as the MCC does on APC/C-Cdc20. Regulation of the Rae1-Nup98 complex by the attachment status of kinetochores, however, has not yet been reported.

Finally, the CPC contributes to checkpoint signaling in specific circumstances. Inhibition of Aurora B abrogates the checkpoint response to lack-of-tension, for instance when taxol is used to reduce tension between sister centromeres by inhibiting microtubule dynamics (45, 100-102). As discussed in section 2.3, this is likely due to the fact that Aurora B destabilizes attachments when tension is absent. In agreements with this, Aurora B is dispensible when kinetochores are unattached to begin with, for instance when cells are treated with nocodazole. Nevertheless, based on the observation that the duration of the arrest in response to nocodazole is shortened when Aurora B is inactive, direct, microtubule-independent involvement of Aurora B in checkpoint function has been suggested (103, 104). Importantly, Aurora B activity is required for BubR1 to bind kinetochores under all circumstances (100, 100, 105). This begs the question what the role of BubR1 at kinetochores is. Perhaps the pool of BubR1 that can be visualized at kinetochores is part of the mechanism that senses tension and converts it to unattachment, while another pool of BubR1 is part of the MCC and required for the responses to all kinds of improper attachment. It is unknown how Aurora B regulates BubR1 kinetochore localization. Recent data from budding yeast suggest that it may be direct through phosphorylation (106), but this remains to be investigated in mammalian systems.

5.3.2 MAPK family members

Members of the MAPK family have been reported to contribute to checkpoint activity in mammalian cells. Chemical inhibition of p38 caused checkpoint inhibition in mouse cells (107), suggesting that p38 activity is required for the mitotic checkpoint. These findings, however, have not been confirmed, and recent evidence obtained by RNAi suggests that in fact p38 is dispensible for cells to mount a checkpoint response (e.g. (95)). MAPK itself was suggested as a checkpoint kinase in frog extract (108), and in that system MAPK can directly phosphorylate Mps1 and Cdc20 to control kinetochore localization and MCC formation, respectively (109, 110). At least one MAPK-dependent phosphorylation site in Cdc20 is the same as one identified as phosphorylated by Bub1 (83). It is thus currently unclear how these phosphorylations are regulated by the different kinases in space and time, and how the different phosphorylations combine to control Cdc20 in prometaphase. Active MAPK was shown to localize to prometaphase kinetochores of various cell lines including human (111, 112) and to interact with CENP-E (112). Contradictory findings, however, have been reported on whether MAPK is active at all in mitosis (107, 113). MAPKK/MEK, the direct activator of MAPK, does not localize to kinetochores, although it is activated in mitosis (111). To add to the confusion, MAPKKK/Raf1, the direct activator of MAPKK/MEK, is active in mitosis (114), but the small GTPase Ras, the direct activator of MAPKKK/Raf1, is inactive (115). Possibly, only tiny, kinetochore-localized fractions of the kinases are active and play a role during prometaphase, but more analysis needs to be done in order to verify a role of the MAPK pathway in mitosis. As for the mitotic checkpoint, no evidence of a role for MAPK in human cells has been reported.

6. CHECKPOINT-INDEPENDENT FUNCTIONS OF THE CHECKPOINT PROTEINS

The mitotic checkpoint is necessarily active when chromosomes are in the process of establishing bipolar attachments in order to align. Interestingly, some proteins essential for checkpoint signaling also contribute to attachment processes. For example, generation of stable attachments of kinetochores to spindle microtubules requires BubR1 (100, 116), while Bub1 is essential for the establishment of end-on attachments (68, 77). These functions are likely indirect through regulation, possibly by phopshorylation, of bona fide attachment factors such as those mentioned in section 2.1. An additional function for Bub1, and one that appears to be

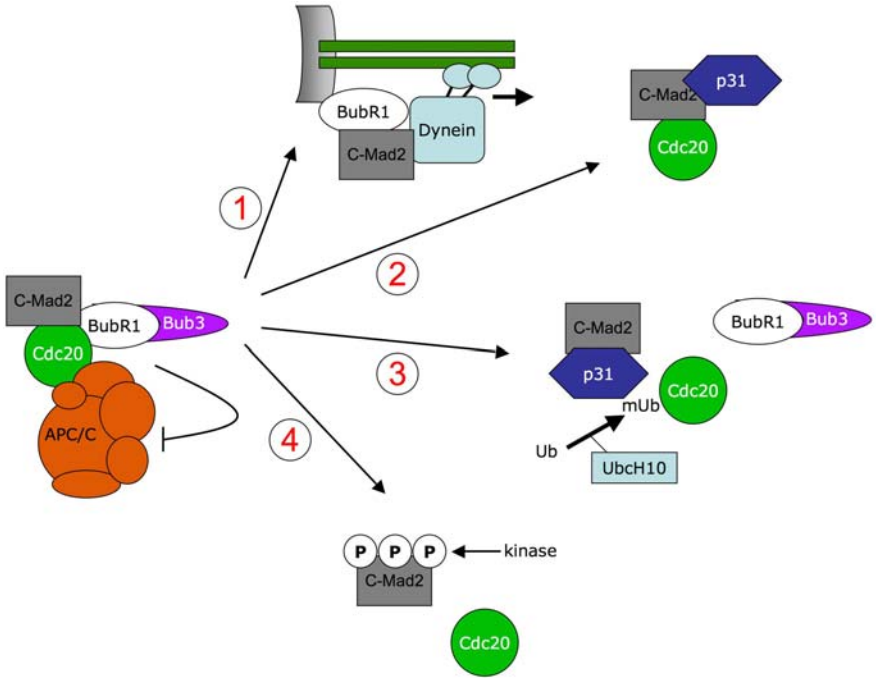

Figure 5. Inhibiting checkpoint signaling. Every kinetochore that attaches properly is depleted of mitotic checkpoint proteins by dynein that transports these proteins along kinetochore fibers to spindle poles, thus inhibiting production of the MCC (1). Upon attachment of the last kinetochore, p31^{comet} binds and inhibits C-Mad2 (2) and contributes to UbcH10-dependent multi-ubiquitination of Cdc20 that causes the MCC to fall apart (3). Additional phosphorylation of Mad2 prevents it from binding Cdc20 (4).

conserved across species, is the control of cohesion by correctly localizing Sgo1 to kinetochores (78). In human cells, Sgo1 protects cohesin from being released from DNA by counteracting Plk1-dependent phosphorylation of the SA2 subunit of cohesin, via its association with the PP2A phosphatase (117, 118). Interestingly, Sgo1 may also play a role in Bub1-dependent control over attachments, as Sgo1 contributes to the establishment of productive kinetochore-spindle microtubule interactions (119). Very recently, Bub1 was reported to be responsible for establishing inner-centromere localization of the CPC complex in frog extracts (120). Whether this function of Bub1 is conserved in mammals is unclear, as several reports have provided conflicting data as to whether Bub1 depletion affects CPC localization in mammalian cells (77, 120).

We have found recently that Mps1, too, is involved in aligning chromosomes on the metaphase plate. Mps1-depleted cells have many misaligned chromosomes due to lack of attachment-correction by Aurora B (N Jelluma and GJPLK, unpublished). In addition to Bub1, BubR1 and Mps1, TAO1/MARKK is also essential for chromosome alignment (95). Although uninvestigated for Bub1 and BubR1, the kinase activities of Mps1 and TAO1/MARKK are required for chromosome congression ((95) and N Jelluma and GJPLK, unpublished). The checkpoint kinases are therefore crucial activities in coordinating various mitotic processes, but direct substrates that exert control over these processes have yet to be identified for any of the kinases. Thus, a general principle is emerging in which kinases that set up the requirements for faithful chromosome segregation also signal to the cell-cycle machinery to halt until those requirements are met.

Of the non-enzymatic components of the mitotic checkpoint machinery only the RZZ complex has clear checkpoint-independent functions. ZW10 interacts directly with p50 dynamitin, a member of the dynein motor complex, and members of the RZZ complex are required to load dynein onto kinetochores (121). Kinetochore-dynein subsequently contributes to chromosome movements in prometaphase and anaphase as well as to the maintenance of stable kinetochore-spindle microtubule interactions (31).

7. SILENCING THE CHECKPOINT

7.1 Ubiquitination of Cdc20 by the APC/C

The APC/C is activated only minutes after the final kinetochore has engaged productive attachments with spindle microtubules (122), suggesting that the checkpoint signal from that last kinetochore is inhibited very rapidly. Two very recent papers have suggested that direct modification of Cdc20 is essential for this. It was found that the APC/C auto-ubiquitinates its Cdc20 subunit, causing the MCC to fall apart (123). Although there is no indication that this is regulated by the attachment state of kinetochores, a second regulatory step in this process, that was identified simultaneously, may be. Cdc20 is actively de-ubiquitinated by the de-ubiquitinating enzyme Usp44, thereby upholding the inhibitory activity of the MCC (124) (Figure 5). Interestingly, Usp44 is a good candidate for regulation by the unattached kinetochore, as it is

specifically phosphorylated in mitotic cells (124). Conceivably, the APC/C is constitutively active towards Cdc20 but is counteracted by Usp44 until the final kinetochore attaches and Usp44 activity is downregulated.

7.2 p31^{comet}

Another protein crucial for checkpoint silencing is p31^{comet} (a.k.a. CMT2, for caught by mad2). p31^{comet} is a Mad2-binding protein, depletion of which chronically activates the checkpoint (125). p31^{comet} binds the C-mad2 conformer specifically (126, 127) (Figure 5), whether bound to Mad1 or Cdc20, but does not displace Cdc20 from the complex (127). Nevertheless, p31^{comet} prevents Mad2 from inhibiting the APC/C *in vitro* (127) and prevents O-Mad2 from binding C-Mad2 (126, 128). Moreover, p31^{comet} facilitates Cdc20 ubiquitination by the APC/C to promote efficient inhibition of the checkpoint signal (123). It is entirely possible that p31^{comet} itself is regulated by the kinetochore in a way reminiscent of Cdc20. In this way, an unattached kinetochore could keep both Cdc20 and p31^{comet} inhibited. Attachment of the final kinetochore could then cause a halt in production of the MCC while simultaneously activating p31^{comet}, allowing rapid disassembly of the MCC and rapid activation of the APC/C. Although this is a very attractive way of rapidly activating the APC/C upon final attachment, it remains highly speculative at this time.

7.3 Preventing production of the MCC by dynein

Yet another suggested mechanism for inhibiting the checkpoint is inherent to the attachment process: the plus-end directed microtubule motor dynein is kinetochore-localized in mitosis and actively transports BubR1, CENPE and Mad2 from kinetochores to the spindle poles along the established kinetochore fibers (129) (Figure 5). This is important in silencing the checkpoint, as inhibition of dynein by antibody injection caused a mitotic arrest with high levels of Mad2 on attached kinetochores (129). A recent report showed that a specific cargo-linker of dynein, DYNLT3, binds to Bub3 and RNAi of this linker specifically arrested cells in prometaphase (130).

7.4 Phosphorylation of Mad2

Finally, phosphorylation of Mad2 may contribute to checkpoint silencing. Mad2 gets phosphorylated on multiple residues during a release from nocodazole, and phosphorylated Mad2 does not interact with APC/C-Cdc20 or Mad1 (131) (Figure 5). Moreover, overexpressing a Mad2 mutant in which constitutive phosphorylation is mimicked caused an inactive checkpoint, suggesting that phosphorylated Mad2 is non-functional in inhibiting the APC/C (131). It is known neither if a kinase phosphorylates Mad2 upon attachment of the last kinetochore or if a phosphatase is inhibited, nor how the phosphorylation of Mad2 contributes to checkpoint silencing. Possibly, phosphorylation interferes directly with Mad2 inhibitory activity, for instance by affecting the interaction with Cdc20. Alternatively, it may contribute to any of the inhibitory principles outlined above. It could, for instance, facilitate p31^{comet} binding, although phosphorylation is not required for the interaction *in vitro*, since Mad2 and p31^{comet} strongly interact when purified from bacteria (127, 128).

Taken together, many modifications and interactions of the MCC combine to quickly and efficiently re-activate the APC/C to allow fast and synchronous chromosome segregation upon attachment of the last kinetochore.

8. CHECKPOINT DEFECTS IN PATHOLOGICAL CONDITIONS

The checkpoint is essential for viability during mammalian development and is essential for viability of tumor cells. Mutations in checkpoint proteins that wipe out checkpoint activity are therefore unlikely to contribute to tumor formation. However, weak mitotic checkpoint activity is prevalent in human tumors (132). Such weak activity can contribute to tumorigenesis, as mice heterozygous for various mitotic checkpoint genes generate aneuploid cells with high frequency and are more prone to tumor development, especially when mitotic checkpoint weakening is combined with common carcinogens or tumor suppressors like *APC* or *BRCA2* (reviewed in (133, 134)). Recently, germline mutations in the gene encoding BubR1 were reported in a recessive condition called mosaic variegated aneuploidy (MVA), a characteristic of which is the development of childhood cancer (135). This finding supports a causal link between mitotic checkpoint defects, aneuploidy and tumor development.

Comprehensive analysis of over 20,000 tumor samples revealed that aneuploidy is the characteristic most commonly shared by all tumors (136). Mitotic checkpoint signaling seems often impaired in cancer cell lines, with cells able to respond with an initial checkpoint arrest but less able to maintain it (132). It is unclear what underlies weakening of the mitotic checkpoint in these cells. Somatic mutations in checkpoint genes have occasionally been found but otherwise seem rare (see (134)). Altered expression of various checkpoint components has been observed in various tumor samples but it is unclear if this promotes checkpoint weakening and chromosomal instability. More likely, molecular changes that affect checkpoint activity exist but no such changes have yet been reported.

It has thus been proposed that a subset of tumors in one way or another has a compromised mitotic checkpoint that facilitates tumor development by causing chromosomal instability. On the other hand, recent studies have shown that fully abolishing mitotic checkpoint signaling is lethal to cells (60, 61). Thus, whereas decreasing mitotic checkpoint signal strength can be beneficial to tumor cells, abolishing it is invariably lethal. The observation that mitotic checkpoint signaling is often impaired in tumor cells may thus prove to be an important factor: non-cancerous cells with a robust checkpoint may be able to cope with a level of checkpoint inhibition that is deadly for tumor cells with a weakened checkpoint. Obviously, this is theory and will need to be tested in animal tumor models.

9. PERSPECTIVES

Recent years has seen tremendous advances in understanding activation and inhibition of the mitotic checkpoint on a molecular level. Nevertheless, several important questions remain: How exactly is inhibition of the APC/C accomplished, and how do Mad2 and BubR1 cooperate to inhibit the APC/C? What is the role of the checkpoint kinases? How are they activated and what substrates do they target that can explain their function in checkpoint signaling? How is the activity of checkpoint inhibitors such as p31^{comet} and the kinase that phosphorylates Mad2 coordinated to cause rapid activation of the APC/C? Are Usp44 or UbcH10 regulated by unattached kinetochores? And with respect to clinical significance: what aspects of checkpoint signaling are misregulated in tumors, and can we utilize this knowledge to develop novel anti-tumor strategies? Judging the speed by which the field has developed in recent years, the near future promises exciting insights into the working principles of this crucial cell-cycle checkpoint.

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