Structural activation of Mad2 in the mitotic spindle checkpoint: the two-state Mad2 model versus the Mad2 template model

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The inheritance of a normal assortment of chromosomes during each cell division relies on a cell-cycle surveillance system called the mitotic spindle checkpoint. The existence of sister chromatids that do not achieve proper bipolar attachment to the mitotic spindle in a cell activates this checkpoint, which inhibits the ubiquitin ligase activity of the anaphase-promoting complex or cyclosome (APC/C) and delays the onset of anaphase. The mitotic arrest deficiency 2 (Mad2) spindle checkpoint protein inhibits APC/C through binding to its mitotic-specific activator, Cdc20. Binding of Mad2 to Cdc20 involves a large conformational change of Mad2 and requires the Mad1–Mad2 interaction in vivo. Two related but distinct models of Mad1-assisted activation of Mad2, the "two-state Mad2" and the "Mad2 template" models, have been proposed. I review the recent structural, biochemical, and cell biological data on Mad2, discuss the differences between the two models, and propose experiments that test their key principles.

Introduction

During the mitotic cell division cycle, cells make an exact copy of their chromosomes and physically tether the original and replica chromosomes together to form pairs of sister chromatids (Koshland and Guacci, 2000; Nasmyth, 2002). In mitosis, the two opposing kinetochores (i.e., protein complexes at the centromeres) of a given pair of sister chromatids attach to microtubules originating from the two opposite spindle poles, a process referred to as biorientation (Cleveland et al., 2003; Tanaka et al., 2005). After all of the sister chromatids have achieved biorientation and have aligned at the cell equator, a large ubiquitin ligase called the anaphase-promoting complex or cyclosome (APC/C) catalyzes the ubiquitination of securin, which is an inhibitory chaperone of a protease called separase (Harper et al., 2002; Peters, 2002). The degradation of securin activates separase, which then cleaves a subunit of the cohesin protein complex that is required for the physical linkage of sister chromatids (Nasmyth, 2002). The loss of sister chromatid cohesion allows the two sets of separated chromatids to move to opposite spindle poles through their attachment to microtubules and to be evenly sorted into the two daughter cells.

The kinetochore–microtubule attachment is achieved through a search-and-capture mechanism (Cleveland et al., 2003; Tanaka et al., 2005). Because of the inherent stochastic nature of this process, not all sister chromatids are captured by the mitotic spindle at the same instance. Because premature separation of a single pair of sister chromatids may lead to aneuploidy, cells use a surveillance system called the mitotic spindle checkpoint to delay the onset of anaphase until all of the pairs of sister chromatids have achieved biorientation (Musacchio and Hardwick, 2002; Bharadwaj and Yu, 2004). Kinetochores that have not bioriented are thought to generate diffusible signals to inhibit the cytoplasmic pool of APC/C^{Cdc20} (i.e., the complex between APC/C and its mitotic-specific activator, Cdc20), thus stabilizing securin and cyclin B1 and preventing chromosome segregation and mitotic exit (Fig. 1; Yu, 2002).

Inhibition of APC/C by the mitotic checkpoint complex (MCC)

The nature of the diffusible APC/C inhibitory signals emitted by the unattached/untense kinetochores has not been established. Recent studies have revealed attractive candidates that involve the mitotic arrest deficiency (Mad) and budding uninhibited by benomyl (Bub) proteins, which are key conserved components of the spindle checkpoint in organisms ranging from yeast to man (Fig. 1; Musacchio and Hardwick, 2002; Bharadwaj and Yu, 2004; Yu and Tang, 2005). One candidate is the BubR1–Bub3–Cdc20–Mad2 complex or the MCC (Sudakin et al., 2001; Tang et al., 2001; Fang, 2002). BubR1 and Mad2 inhibit APC/C^{Cdc20} stoichiometrically and synergistically in vitro (Tang et al., 2001; Fang, 2002). Both the BubR1–Cdc20 and Mad2–Cdc20 interactions are enhanced during mitosis (Tang et al., 2001; Chen, 2002; Fang, 2002). Thus, the concentration of MCC is much higher in mitotic cells than in interphase cells

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Abbreviations used in this paper: APC/C, the anaphase-promoting complex or cyclosome; Bub, budding uninhibited by benomyl; Mad, mitotic arrest deficiency; MCC, mitotic checkpoint complex; NMR, nuclear magnetic resonance.

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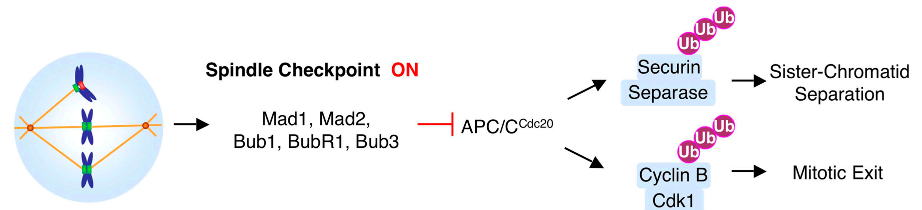
Figure 1. **The mitotic spindle checkpoint.** In response to kinetochores that have not achieved biorientation, this checkpoint blocks the activation of APC/C, preventing chromosome segregation and mitotic exit.



(Hardwick et al., 2000; Chen, 2002; Millband and Hardwick, 2002). Moreover, all four components of MCC are enriched at unattached kinetochores during mitosis, and the kinetochore-bound pools of these proteins exchange rapidly with their cytosolic pools (Howell et al., 2000, 2004; Kallio et al., 2002; Shah et al., 2004). These findings suggest that the unattached kinetochores facilitate the formation of MCC. On the other hand, the formation of MCC does not strictly require the kinetochores (Fraschini et al., 2001; Sudakin et al., 2001; Poddar et al., 2005), suggesting that there are kinetochore-independent mechanisms for MCC formation (Chan et al., 2005).

The conformational change of Mad2 and MCC assembly

It is unclear how the formation of MCC is stimulated during mitosis and how MCC is disassembled after the checkpoint is inactivated. However, it is clear that the formation of MCC and, more specifically, the binding of Mad2 to Cdc20 involves a large conformational change of Mad2 (Luo et al., 2000, 2002, 2004; Musacchio and Hardwick, 2002; Sironi et al., 2002; Yu, 2002). Free Mad2 exists in two folded conformations; one is a less stable monomeric form with native fold 1 (N1) and the other is a more stable homodimeric form with native fold 2 (N2; Fig. 2, note that the structure of dimeric Mad2 has not been determined and that the structure of the monomeric N2–Mad2^{R133A} mutant is shown; Luo et al., 2004). The two conformers of Mad2 interconvert very slowly, with an in vitro half-life on the order of hours (Luo et al., 2004). The homodimeric N2–N2 form of Mad2, but not its monomeric N1 form, is active in inhibiting APC/C^{Cdc20} in mitotic *Xenopus laevis* egg extracts (Fang et al., 1998; Luo et al., 2004). Furthermore, Mad2 mutants that can only adopt the N1 conformation not only fail to interact with Cdc20 but also block the activity of the exogenous N2–Mad2 in *X. laevis* egg extract and the function of endogenous Mad2 in HeLa cells (Fang et al., 1998; Luo et al., 2004). These findings are nicely explained by the observation that the N1 and N2 forms of Mad2 can "heterodimerize" (the quotation marks indicate the fact that the two Mad2 molecules in this dimer are only different in conformation, but are identical in composition) to form an N1–N2 dimer with mixed conformations, which is incapable of APC/C inhibition in *X. laevis* egg extracts or in HeLa cells (Luo et al., 2004). Thus, Mad2 is a two-state protein, with the N2 state being the more active species for Cdc20 binding that inhibits its activation of APC/C toward mitotic substrates. N2–Mad2 has preformed vacant Cdc20-binding sites and resembles the Cdc20-bound form of Mad2 (referred to as N2'), whereas this site is blocked in N1–Mad2 by strands β7 and β8 (Fig. 2). I speculate that N2–Mad2 has a faster on-rate and, thus, higher affinity toward Cdc20 by readily forming an edge-on interaction with the Mad2-binding region of Cdc20, providing a possible explanation for why N2–Mad2 is more active in APC/C^{Cdc20} inhibition. Depending on

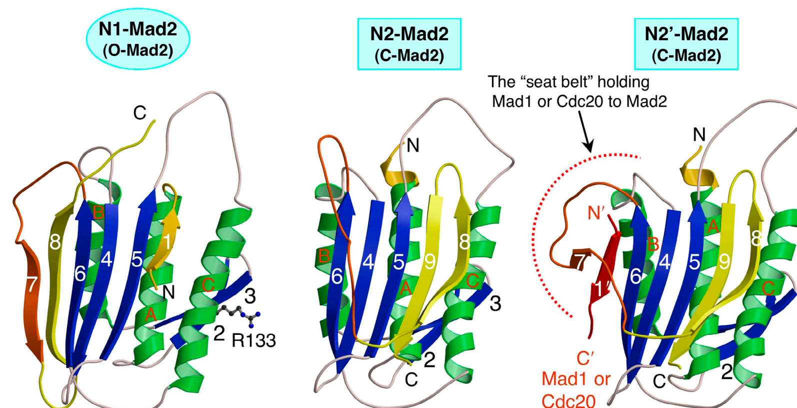


Figure 2. **The multiple conformations of Mad2.** Ribbon drawings of the structures of N1–Mad2, N2–Mad2^{R133A}, and Mad2 bound to the Mad2-binding motifs of Cdc20 or Mad1 (N2'–Mad2); the corresponding structural elements that undergo large conformational changes are colored orange or yellow. The structure of the wild-type dimeric Mad2 is unknown, but it is very likely a homodimer with each monomer resembling the conformation of N2–Mad2^{R133A}. Because the R133A mutation disrupts Mad2 dimerization, the αC helix is likely a part of the dimerization interface. As pointed out by Sironi et al. (2002), the COOH-terminal region (orange and yellow) in N2'–Mad2 topologically traps its ligands (MBP1, Cdc20, or Mad1) in a manner analogous to the seat belt of an automobile. For this reason, they referred to N2– and N2'–Mad2 as closed Mad2 (C-Mad2) and N1–Mad2 as open Mad2 (O-Mad2; De Antoni et al., 2005). However, it is somewhat misleading to name N1–Mad2 open Mad2 simply because strand β9 of the "seat belt" in N2– and N2'–Mad2 is absent in N1–Mad2 (a large portion of β9 in N2–Mad2 corresponds to the COOH-terminal tail in N1–Mad2) and because strands β7 and β8 in N1–Mad2 occupy its ligand-binding site.

whether Cdc20 (alone or bound to BubR1) exists as monomers or oligomers, the dimeric nature of N2–Mad2 might provide additional advantage for its binding to Cdc20.

Regulation of Mad2 by Mad1: the two-state Mad2 model

Mad2 binds to Mad1 throughout the cell cycle (Chen et al., 1999; Chung and Chen, 2002). The mechanism by which Mad1 regulates Mad2 has been somewhat of a mystery. Mad1 is a positive regulator of the spindle checkpoint and is expected to facilitate Mad2 to carry out its function in vivo, i.e., binding to Cdc20 (Chen et al., 1999; Chung and Chen, 2002). On the other hand, binding of Mad1 to Mad2 triggers a similar conformational change of Mad2, as does Cdc20 binding (Luo et al., 2002). Mad1 and Cdc20 bind to similar binding sites on Mad2, with similar affinities. Thus, binding of Mad1 to Mad2 should block Cdc20 from binding to Mad2. Indeed, high levels of Mad1 inhibit Mad2 binding to Cdc20 in vitro and in vivo (Luo et al., 2002). To explain this apparent Mad1 paradox, we have proposed a two-state Mad2 model (Luo et al., 2004). The essence of the two-state Mad2 model is that Mad2 exists in two distinct conformations: a latent N1 conformation and an active N2 conformation. Mad1 facilitates the formation of the active conformation of Mad2 in a manner that is reminiscent of the role of guanine-nucleotide exchange factors in the activation of G proteins. As N1–Mad2 is thermodynamically less stable than N2–Mad2 (Luo et al., 2004), Mad1-catalyzed N1–N2 structural transition of Mad2 is energetically favorable and does not require ATP.

The exact mechanistic steps with which Mad1 catalyzes the formation of N2–Mad2 are unknown. I present a very speculative model of how I envision Mad1 accomplishes this goal (Fig. 3 A). In this model, Mad1 recruits N1–Mad2 (the predominant form of free Mad2 in the cytosol) to the kinetochores by forming the Mad1–N2’–Mad2 complex, which can then recruit another copy of N1–Mad2 through N1–N2 Mad2 heterodimerization. The N1–N2 Mad2 heterodimers are converted to N2–N2 Mad2 homodimers, which either are directly passed on to Cdc20 or dissociate from Mad1 in three possible pathways (Fig. 3 A). Using FRAP, Shah et al. (2004) showed that only half of the kinetochore-bound pool of YFP-Mad2 was dynamic in Ptk cells. They proposed that Mad1–Mad2 acts as a stable template to recruit another copy of Mad2 to kinetochores, which exchanges rapidly (Shah et al., 2004). Consistently, a similar methodology measuring only the exchange of Mad2 bound to kinetochores after initial recruitment of the stably bound Mad1–Mad2 demonstrated rapid, complete exchange (Howell et al., 2004). Only pathway I in my model (Fig. 3 A) is consistent with the finding that only ~50% of Mad2 turns over rapidly at the kinetochores (Shah et al., 2004).

Mad1 interacts with Mad2 constitutively throughout the cell cycle and facilitates the formation of the Mad2–Cdc20 interaction, which is enhanced in mitosis. For Mad1-assisted structural transition of Mad2 to play an important regulatory role in the formation of Mad2–Cdc20–containing checkpoint complexes, the mode of the Mad1–Mad2 interaction has to be regulated differently across mitosis. The p31^{comet} protein selectively binds to the N2– and N2’–Mad2, but not the N1–Mad2 (Fig. 3 B; Habu et al., 2002; Xia et al., 2004). Binding of p31^{comet} to Mad2–Cdc20–containing complexes inhibits the activity of Mad2 (Habu et al., 2002; Xia et al., 2004). Binding of p31^{comet} might also prevent the Mad1-assisted conformational change of Mad2 by hindering further binding of N1–Mad2 to the Mad1–Mad2 core. Unfortunately, the concentration of the Mad1–Mad2–p31^{comet} ternary complex does not decrease appreciably in mitosis (unpublished data). Thus, it is possible that modifications of Mad1 and/or binding of other proteins at the kinetochores positively regulate the Mad1-assisted structural transition of Mad2.

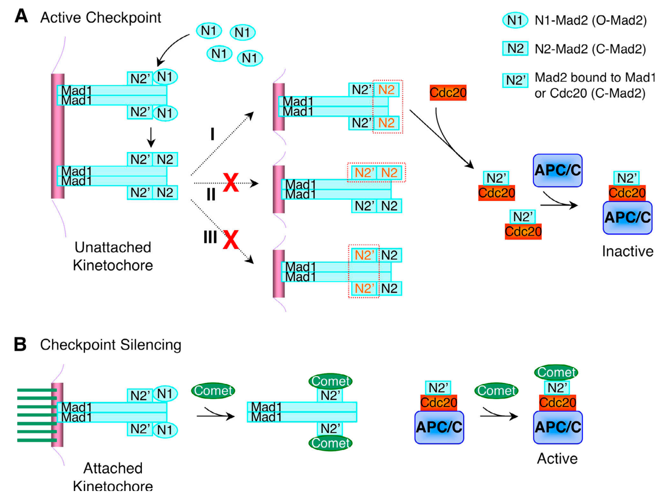


Figure 3. **The two-state Mad2 model.** (A) Mad2 binds to Mad1 and adopts the N2′ conformation. Upon checkpoint activation, the Mad1–Mad2 complex is recruited to the unattached kinetochores. Another copy of N1–Mad2 is recruited to Mad1, mainly through its binding to N2′–Mad2, and is then converted to N2–Mad2. N2–Mad2 is either directly passed on to Cdc20 from Mad1 or dissociates from Mad1 to form a transient dimeric intermediate that then binds to Cdc20. Because Mad1 exists as a homodimer, three pathways can be envisioned for the transfer of N2–Mad2 from Mad1 to Cdc20: (I) the two loosely bound N2–Mad2 molecules dissociate from Mad1 as a unit; (II) one tightly bound N2′–Mad2 and one loosely bound N2–Mad2 dissociate as a unit; (III) the two tightly bound N2′–Mad2 molecules dissociate as a unit. Pathways I and III require the dimerization of Mad1. Pathway III is equivalent to the so-called “exchange” model (De Antoni et al., 2005). Only pathway I is consistent with FRAP studies in Ptk cells. (B) Upon microtubule attachment, the Mad1–Mad2 complex is depleted from kinetochores. Binding of p31^{comet} prevents further Mad2 turnover on Mad1 and neutralizes the inhibitory activity of Cdc20-bound Mad2, leading to activation of APC/C, degradation of securin and cyclin B, and exit from mitosis.

Regulation of Mad2 by Mad1: the Mad2 template model

Recently, De Antoni et al. (2005) confirmed the earlier finding (Luo et al., 2004) that N1–Mad2 and N2–Mad2 (which are referred to as open and closed Mad2, respectively, by DeAntoni et al. [2005]) can heterodimerize. They also confirmed that Mad2 mutants that are locked into the N1 conformation dominant-negatively inhibit the function of the endogenous Mad2 and that the dominant-negative effects of these mutants depend on their ability to heterodimerize with N2–Mad2 (Luo et al., 2004; De Antoni et al., 2005). A novel and important extension of DeAntoni et al. (2005) was that the kinetochore localization of

fluorescently labeled recombinant Mad2 injected into Ptk cells depends on its ability to dimerize with N2'–Mad2 already bound to Mad1 at the kinetochores. $Mad2^{R133A/Q134E}$, a Mad2 mutant that does not form N1–N2' heterodimers, was not recruited to kinetochores, suggesting that the fast-exchanging pool of Mad2 is recruited to the kinetochores through an N1–N2' Mad2 heterodimerization event (De Antoni et al., 2005). Based largely on this finding, they proposed an alternative model, referred to as the Mad2 template model, to explain the regulation of Mad2 by Mad1 (Fig. 4; De Antoni et al., 2005; Hagan and Sorger, 2005; Hardwick, 2005; Nasmyth, 2005). In this model, the N2'–Mad2 molecule tightly bound to Mad1 recruits another N1–Mad2 molecule to the kinetochores through a Mad2–Mad2 interaction. The loosely bound N1–Mad2 molecule is passed on to Cdc20. The Cdc20-bound Mad2 adopts the N2' conformation and can presumably recruit another N1–Mad2 through N1–N2' heterodimerization. In this way, the N2'–Mad2–Cdc20 complex can amplify itself by self-propagation away from the kinetochores and is proposed to account for the sensitivity of the spindle checkpoint.

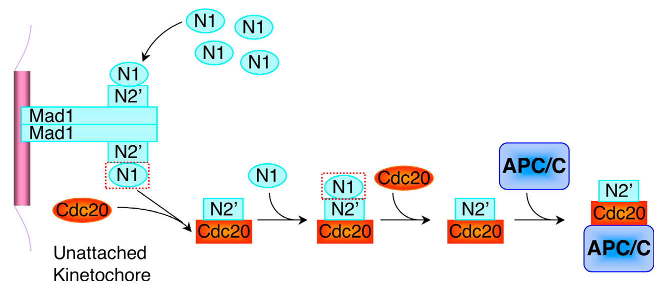


Figure 4. **The Mad2 template model.** The symbols used are the same as in Fig. 3. In this model, N2'–Mad2 bound to Mad1 recruits N1–Mad2, which is passed on to Cdc20. The Cdc20-bound N2'–Mad2 recruits another N1–Mad2. In this way, the N2'–Mad2–Cdc20 can self-propagate away from the kinetochores.

The active conformation of Mad2

It is apparent that the two models for Mad1-assisted Mad2 activation are variations of the same major theme. The key difference of the two-state Mad2 and Mad2 template models is the nature of the active conformation of Mad2. In the two-state Mad2 model, N2-Mad2 is the active Mad2 species (Fig. 3 A). In the Mad2 template model, the N1–Mad2 molecule in the N1–N2' heterodimer is the active Mad2 species (Fig. 4). Moreover, the two-state Mad2 model requires that Mad1 exists as a homodimer (an important known property of Mad1; Fig. 3 A), but Mad1 dimerization is not required by the Mad2 template model (Fig. 4).

The whole Mad2 story started with the demonstration by Fang et al. (1998) that dimeric and oligomeric Mad2, but not monomeric Mad2, inhibited APC/C in *X. laevis* egg extracts. The monomeric Mad2 adopts the N1 conformation, as revealed by structure determination using nuclear magnetic resonance (NMR) spectroscopy (Luo et al., 2000). The atomic structure of dimeric Mad2 has not been determined so far. However, NMR experiments are consistent with the observation that the dimeric wild-type Mad2 is a homodimer of N2–N2 Mad2 (Luo et al., 2004). Importantly, monomeric $N2–Mad2^{R133A}$ (whose structure has been determined by NMR) is more active in APC/C inhibition in *X. laevis* egg extracts than $N1–Mad2^{R133A}$ (Luo et al., 2004). Therefore, consistent with the two-state Mad2 model, N2–Mad2 is most likely the active species of Mad2.

Based on indirect biochemical experiments, De Antoni et al. (2005) concluded that the dimeric wild-type Mad2 is a conformationally mixed heterodimer of N1–N2 Mad2 and that this may be the active species of Mad2 both at kinetochores and in solution. There is, however, no evidence to suggest that N1–Mad2 bound to N2'–Mad2 as a heterodimer is more active in Cdc20 binding or APC/C inhibition than free N1–Mad2. Moreover, based on the Mad2 template model, the Mad2 molecules that exchange at the kinetochores have the same conformation as the cytosolic free N1–Mad2, it is unclear how this copy of Mad2 is more suitable for binding to Cdc20. An unattractive argument of subtle allostery has to be invoked to explain this phenomenon (De Antoni et al., 2005; Nasmyth, 2005). Furthermore, in the absence of kinetochores, the wild-type N1–Mad2 monomer dominant-negatively blocks the APC/C-inhibitory function of the Mad2 dimer in *X. laevis* egg extracts (Fang et al., 1998), a finding inconsistent with the prediction of the amplification step of the Mad2 template model (Fig. 4). In contrast, consistent with this finding, after the transient dissociation of N2–N2 Mad2 dimers, the free N1–Mad2 in the two-state Mad2 model would drive the formation of N1–N2 heterodimers, which are inactive in APC/C inhibition. Although it can be argued that the in vitro findings on the APC/C-inhibitory activity of Mad2 in *X. laevis* egg extracts are not physiologically relevant, I stress that, to date, this remains to be the only assay that can differentiate the biochemical activities of various Mad2 conformers.

There remains no evidence supporting self-propagation of the N2'–Mad2–Cdc20 complex, which is a key aspect of the Mad2 template model. Furthermore, mathematical modeling by Doncic et al. (2005) has argued that a self-propagation model (which is the category that the Mad2 template model belongs to) is insufficient to explain the behavior of the spindle checkpoint, as this model predicts a self-sustained cellular state that contains high concentrations of APC/C-inhibitory signals, regardless of the attachment status of the kinetochores. On the other hand, the same modeling study revealed that an emitted inhibition model (the category that the two-state Mad2 model belongs to) can explain key aspects of spindle checkpoint signaling (Doncic et al., 2005). Thus, both experimental evidence and mathematical modeling are more consistent with the two-state Mad2 model.

Perspective

The fundamental difference between the two models of Mad1-assisted activation of Mad2 is the nature of the activated conformation of Mad2; N2–Mad2 in the two-state Mad2 model and N1–Mad2 when bound to N2'–Mad2 in the Mad2 template model. Obviously, this issue can be best addressed by additional structural studies. For example, the determination of the atomic structure of the dimeric wild-type Mad2 will go a long way in testing the two models. The monomeric Mad2 (inactive in *X. laevis* egg extracts) adopts the N1 conformation (Luo et al., 2000).

If the structure of dimeric Mad2 is indeed an N2–N2 Mad2 homodimer, it will prove that N2–Mad2 is the active species (at least in *X. laevis* egg extracts) and lend strong support to the two-state Mad2 model. In addition, structures of the Mad1–Mad2–p31^{comet} ternary complex and the Mad1–Mad2 complex with each Mad1 molecule bound to two Mad2 molecules will shed light on how Mad1 facilitates the conformational change of Mad2 and how p31^{comet} blocks this process.

Another way to distinguish between the two models is to construct Mad2 mutants that are locked in the N2 conformation and to examine their biochemical activities in vitro and in vivo. The Mad2 template model predicts that such Mad2 mutants will be inactive in the absence of the endogenous Mad2, as N1–Mad2 is the active species in this model and the N2-specific mutants cannot adopt the N1 conformation. In contrast, based on the two-state Mad2 model, these N2-specific Mad2 mutants will not only be active but also bypass the requirement for Mad1 in the spindle checkpoint. Future biochemical, structural, and cell biological experiments aimed at testing both models will undoubtedly lead to a better understanding of this fascinating problem.

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