The Mad1/Mad2 Complex as a Template for Mad2 Activation in the Spindle Assembly Checkpoint

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Summary

Background: The spindle assembly checkpoint (SAC) imparts fidelity to chromosome segregation by delaying anaphase until all sister chromatid pairs have become bipolarly attached. Mad2 is a component of the SAC effector complex that sequesters Cdc20 to halt anaphase. In prometaphase, Mad2 is recruited to kinetochores with the help of Mad1, and it is activated to bind Cdc20. These events are linked to the existence of two distinct conformers of Mad2: a closed conformer bound to its kinetochore receptor Mad1 or its target in the checkpoint Cdc20 and an open conformer unbound to these ligands.

Results: We investigated the mechanism of Mad2 recruitment to the kinetochore during checkpoint activation and subsequent transfer to Cdc20. We report that a closed conformer of Mad2 constitutively bound to Mad1, rather than Mad1 itself, is the kinetochore receptor for cytosolic open Mad2 and show that the interaction of open and closed Mad2 conformers is essential to sustain the SAC.

Conclusions: We propose that closed Mad2 bound to Mad1 represents a template for the conversion of open Mad2 into closed Mad2 bound to Cdc20. This simple model, which we have named the “Mad2 template” model, predicts a mechanism for cytosolic propagation of the spindle checkpoint signal away from kinetochores.

Introduction

Before being divided into equal complements, sister chromatids attach to microtubules originating from opposite poles of the mitotic spindle (bipolar attachment).

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$\phi$ is aliphatic, and $X$ is any residue) that bind Mad2 in the same pocket [22, 29]. Second, Mad2 adopts two conformations, open Mad2 (O-Mad2, also known as N1-Mad2) and closed Mad2 (C-Mad2, or N2-Mad2), differing for a structural change in the “safety belt,” the 50 residue C-terminal segment of Mad2 (Supplemental Figure S1) [22, 29, 31, 32]. Mad2 adopts the closed conformation when bound to Cdc20 or Mad1 and the open conformation when unbound to these ligands [22, 29, 31–33]. Because a large energy barrier separates the O- and C-Mad2 conformers [29, 32], the conformational transition may be rate limiting for the ability of Mad2 to bind Cdc20 and ultimately for SAC activation. Thus, it is essential to understand the role of Mad1 in this transition.

We investigated how Mad1 recruits Mad2 from the cytosol to the kinetochore and discovered that C-Mad2 stably bound to Mad1, rather than Mad1, constitutes the kinetochore receptor of O-Mad2. After being recruited, O-Mad2 is converted into C-Mad2 bound to Cdc20. Although we do not provide direct insight into how Mad1 bound C-Mad2 favors the conversion of O-Mad2 into Cdc20 bound C-Mad2, we show that the interaction between Mad1 bound C-Mad2 and O-Mad2 is essential to maintain the SAC. In our interpretation, Mad1/Mad2 is a template for the formation of a structurally equivalent Cdc20/Mad2 copy that amplifies the SAC signal away from kinetochores.

Results

Testing the “Mad2 Exchange” Model

In the recently proposed “Mad2 exchange” model [32], Mad1 lowers the energy barrier for the transition of O-Mad2 into C-Mad2 by recruiting O-Mad2 to kinetochores, changing its conformation to C-Mad2 and releasing it as C-Mad2 for Cdc20 binding (Figure 1A). We decided to test the assumption of this model that Mad1/Mad2 functions as a source of Mad2 in the presence of Cdc20. In preliminary control experiments, we studied the interaction of Mad2 with Cdc20 in the absence of Mad1. Although Mad2 binding to Cdc20 requires Mad1 in vivo, it spontaneously occurs in vitro [22–24, 28]. We compared the elution profiles of Mad2 from a Superdex-200 PC 3.2/30 size-exclusion chromatography (SEC) column with that of an incubation of Mad2 with Cdc20 (Figures 1B and 1C). In agreement with previous reports, the elution volume ($V_e$) of Mad2 (1.55 ml) was typical of an oligomer [22, 28, 31, 34]. As explained below, Mad2 forms oligomers thanks to the interaction of its two conformers, O-Mad2 and C-Mad2. Bacterially expressed Mad2 dimerizes because a fraction of O-Mad2 spontaneously converts into empty C-Mad2 (i.e., devoid of Mad1 or Cdc20), which binds the residual fraction of O-Mad2 [32, 33].

Mad2 (40 $\mu$M) was incubated for 1 hr with a 10-fold excess of a synthetic peptide encompassing residues 111–138 of Cdc20 [Cdc20$_{111-138}$], a stronger Mad2 ligand than full-length Cdc20 [9, 35], to generate Mad2/Cdc20. After SEC, a single Mad2 peak was present whose $V_e$ (1.65 ml, Figure 1C) indicated a 1:1 Mad2/Cdc20$_{111-138}$ complex. The shift in $V_e$ relative to apo-Mad2 (the Cdc20 peptide does not significantly contribute to the Stokes radius of the complex) demonstrates that Cdc20$_{111-138}$ binds Mad2 and that pure C-Mad2 created by Cdc20$_{111-138}$ does not form Mad2 dimers, as shown previously [22, 28].

Next, we asked if Cdc20$_{111-138}$ caused the release of Mad2 from Mad1/Mad2. The isolated Mad1$_{485-718}$/Mad2$_{1-110}$ complex eluted as a single peak with an apparent molecular weight (MW) of 180 kDa (Figure 1D). The elongated shape of Mad1/Mad2 likely explains deviation from the expected MW (~110 kDa) because the 2:2 stoichiometry is known from structural analysis [29] and analytical ultracentrifugation (not shown). When Mad1/Mad2 (20 $\mu$M, tetramer concentration) was incubated with GST-Cdc20$_{111-138}$, as already reported [28, 29]. Thus, it cannot be assumed that Mad2 dissociates from Mad1 to bind Cdc20. Despite similar Mad2 binding affinities of the Mad1 and Cdc20 motifs (Supplemental Table S1 and [29]), Mad1/Mad2 is strongly stabilized by tetramerization, explaining why Cdc20 does not remove Mad2 from Mad1/Mad2 [28, 29].

In the “Mad2 exchange” model Mad1 and Cdc20 bind the same Mad2 pocket. Even if under appropriate conditions Mad1/Mad2 dissociated to release Mad2 for Cdc20, Mad1 should be viewed as a competitive inhibitor of Mad2/Cdc20 rather than a catalyst as proposed [32]. To show this, we studied how the Mad2 binding segment of Mad1 (Mad1$_{527-559}$) influenced Mad2 binding to Cdc20. If Mad1 catalyzed this interaction, on a time course, one should observe at least equal but possibly larger amounts of Cdc20 bound Mad2 in the presence of Mad1 than in its absence. In the presence of increasing amounts of Mad1$_{527-559}$ peptide, Mad2 (3 $\mu$M) was incubated with GST-Cdc20$_{111-138}$ (1.4 $\mu$M) preabsorbed onto glutathione-Sepharose (GSH) beads. At different times, the beads were collected, washed, and the amount of bound Mad2 was evaluated (Figure 1F). In all experiments, we observed inhibition of Mad2 binding to Cdc20 that increased with the concentration of Mad1$_{527-559}$. This confirms that direct exchange of Mad2 from Mad1 to Cdc20 implicates Mad1 and Cdc20 as competitors, contradicting the proposition that Mad1 catalyzes formation of Mad2/Cdc20 [32]. Consistently, overexpression of the Mad2 binding region of Mad1 abrogates the SAC likely because Mad2 engages in a complex with Mad1 that prevents it from binding Cdc20 [22, 24, 36]. Although this does not prove the “Mad2 exchange” model wrong, it proves it inadequate to explain the requirements for fast activation of Mad2 for Cdc20 binding (the model is further discussed in Supplemental Figures S2 and S3).

Mad1/Mad2 Binds Mad2 In Vitro

Knowing that Mad1 mediates kinetochore localization of Mad2 and that Mad1/Mad2 is stable, we hypothesized that Mad1/Mad2, rather than Mad1, is the Mad2 receptor. To test this, we labeled Mad2 covalently with Alexa Fluor 488 (abbreviated in Alexa) and compared the SEC elution profiles of Alexa-modified Mad2 (40 $\mu$M) before or after adding stoichiometric amounts of Mad1/Mad2 (20 $\mu$M of divalent tetramer). Alexa-Mad2 eluted as an
Figure 1. Testing the “Mad2 Exchange” Model

(A) The “Mad2 exchange” model predicts that Mad1 binds O-Mad2 (defined N1-Mad2 in [29] and displayed as a red square), generating the Mad1/Mad2 core complex (gray and yellow, respectively). This releases C-Mad2 (N2-Mad2 in [29] and displayed as a yellow circle) for Cdc20, leaving a vacancy on Mad1 filled by O-Mad2.

(B) SEC elution profile of Mad2wt. All 50 fractions spanning from 1.15 to 1.8 ml were separated by SDS-PAGE and stained with Coomassie.

(C) After incubating with Cdc20111–138, Mad2wt is turned into C-Mad2 and elutes as a 1:1 Mad2/Cdc20 complex. The Cdc20 peptide eluted after the 2 ml mark.

(D) Elution profile of purified recombinant Mad1485–718/Mad2wt core complex.

(E) When incubated in vitro with Cdc20111–138, the core complex remains intact and does not release free Mad2/Cdc20.

(F) GST-Cdc20111–138 bound to GSH beads was incubated with Mad2wt with or without Mad1527–554. Beads were washed, and bound proteins were identified by Coomassie staining after SDS-PAGE.
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Mad2R133E-Q134A Fails to Bind Mad1/Mad2

In summary, O-Mad2 binds (1) C-Mad2 in the Mad1/Mad2 complex, (2) C-Mad2 generated with the Mad2 binding motifs of Mad1 or Cdc20 (Supplemental Table S1, Supplemental Figure S5, and data not shown), and (3) C-Mad2 spontaneously forming in bacterial preparations of Mad2, causing its oligomerization [33]. Mutations impairing the O-Mad2/C-Mad2 binding interface should prevent Mad2 oligomerization. If such mutations did not affect the potential of Mad2 to convert from O- to C-Mad2, the ability to bind Mad1 and Cdc20 would be retained. Mad2R133A is a monomeric mutant that binds tightly to Mad1 and Cdc20 and whose bacterial preparations contain both O-Mad2 and C-Mad2 [28, 29, 32]. Confirming our expectation, Alexa-Mad2R133A bound poorly to the Mad1/Mad2 complex [33]. To obtain a more penetrant phenotype, we created the double mutant Mad2R133E-Q134A (the Mad2R133A-Q134A mutant was insoluble). Binding of Alexa-Mad2R133E-Q134A to Mad1/Mad2 was impaired (Figures 2H–2I). In solution and solid phase, Mad2R133E-Q134A bound Mad1 and Cdc20 as effectively as Mad2wt (Supplemental Table S1 and Supplemental Figure S5). R133 and Q134 are invariant in all Mad2 orthologs [7], indicating that the binding function they mediate is conserved in evolution. Mad2SC and Mad2R133E-Q134A have complementary properties. Mad2SC fails to bind Mad1 and Cdc20 but retains the ability to bind C-Mad2 (Figure 2F and Table 1). Mad2R133E-Q134A binds Mad1 and Cdc20, but its open conformer fails to bind C-Mad2 (Figure 2I, Table 1, Supplemental Figure S5, and data not shown).

Mad1/Mad2 Is the Kinetochore Receptor of Mad2

We asked if our results in vitro correlated with the ability of the same Alexa-Mad2 species to bind the endogenous Mad2 receptor at kinetochores. Early prometaphase PtK1 cells were microinjected with 1%–5% cell volume of Alexa-labeled Mad2wt, Mad2SC, Mad2R133E-Q134A, or Mad2SC-R133E-Q134A (typically at needle concentrations of 3 μM) and analyzed by live cell fluorescence microscopy. Imaging was started ~20 min after injection and continued for ~30 min. Out of 28 cells injected with Alexa-Mad2wt, 26 showed strong kinetochore localization at unattached kinetochores of chromosomes that had not congressed to the spindle equator, as previously described [18], and only two showed weak localization (Figure 3A).

As Alexa-Mad2wt, also Alexa-Mad2SC displayed kinetochoore localization at unattached kinetochores in 29 out of 31 injected cells (Figure 3B). The Alexa-Mad2R133E-Q134A double mutant, on the other hand, failed to localize to the kinetochore in 22 of 23 injected prometaphase PtK1 cells with only one dimly positive cell (Figure 3C) in agreement with its inability to interact with the Mad1/Mad2 complex in vitro. To show that Mad2SC uses the interface containing Arg133 and Gln134 to localize to kinetochores, we injected Alexa-labeled Mad2R133E-Q134A-SC triple mutant in PtK1 cells. As expected, Alexa-Mad2R133E-Q134A-SC was unable to localize to the kinetochore in all 17 injected cells (Figure 3D), confirming that O-Mad2 (elicited by the ΔC deletion) requires Arg133 and Gln134 to bind its endogenous kinetochore receptor. The coincidence of results with Mad2wt, Mad2SC, Mad2R133E-Q134A, and Mad2R133E-Q134A-SC in vitro and in living demonstrates that the Mad1/Mad2 core complex, rather than Mad1, is the kinetochore receptor for Mad2. Our results confirm that Mad2 is recruited to kinetochores as O-Mad2. Furthermore, lack of kinetochore recruitment of an excellent Mad1 ligand such as Mad2R133E-Q134A suggests that there are no Mad2 vacancies on kinetochore Mad1 and that Mad1/Mad2 is stable during checkpoint activation.

Functional Analysis of Mad2SC

Mad2SC has a dominant-negative (DN) effect on the SAC [12, 32, 36, 37]. As shown in Figure 4A, this effect can be explained by the binding of Mad2SC to Mad1/Mad2, which creates an unproductive complex antagonizing the transfer of endogenous O-Mad2 to Cdc20. Consistent with this idea, Cdc20111–138 releases Mad2wt pre-loaded onto the Mad1/Mad2 core complex (Figure 2D), but not Mad2SC (Figure 2G). For confirmation of the previous observations, HeLa cells were transfected with pcMV vectors expressing myc-tagged Mad2wt or Mad2SC to test the effects of their overexpression on the SAC (Figure 4B). 28–30 hr after transfection, half of the culture was incubated with nocodazole to activate the SAC. 18 hr later, the cells were harvested and
Figure 2. The Mad1/Mad2 Core Complex Binds O-Mad2

SDS-PAGE and Coomassie staining of the content of thirteen 50 μl fractions eluting between 1.15 and 1.8 ml. Traces recorded at 280 nm and 495 nm are black and green, respectively. A green bulb on Mad2 squares and circles indicates Alexa modification. (A) Elution profile of the Mad1485–718/Mad2wt core complex, already shown in Figure 1B. (B) Profile of Alexa-Mad2wt. (C) SEC profile of Alexa-Mad2wt mixed with stoichiometric amounts of Mad1485–718/Mad2wt core. Mad2wt is made of O-Mad2/C-Mad2 dimers, but only O-Mad2wt binds Mad1/Mad2. C-Mad2 and O-Mad2 interconvert rather rapidly. During a 1 hr incubation, most C-Mad2 is converted into O-Mad2, which then binds Mad1/Mad2, explaining why the majority of Mad2wt is incorporated onto the Mad1/Mad2 complex. (D) As in (C), after addition of Cdc20111–138. The majority of Alexa-Mad2wt is released in the presence of Cdc20111–138. (E) Profile of Alexa-Mad2wt incubated stoichiometrically with Cdc20111–138. (F) Profile of Alexa-Mad2wt incubated stoichiometrically with Cdc20111–138. (G) Addition of Cdc20111–138 to Alexa-Mad2wt bound to Mad1/Mad2 core complex. Alexa-Mad2wt does not dissociate in the presence of Cdc20111–138. (H) Elution profile of Alexa-Mad2R133E-Q134A. (I) Profile of Alexa-Mad2R133E-Q134A incubated stoichiometrically with the Mad1/Mad2 core complex.
### Table 1. Properties of Mad2 and Mad2 Mutants

<table>
<thead>
<tr>
<th>Mad2 Allele</th>
<th>Mutation</th>
<th>Effect</th>
<th>Conformation Allowed</th>
<th>Mad1 or Cdc20 Binding</th>
<th>Binding to Core Complex</th>
<th>Most Relevant References for Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mad2&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>None</td>
<td>Not applicable</td>
<td>O-Mad2</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Mad2&lt;sup&gt;LIC&lt;/sup&gt;</td>
<td>10 residue C-terminal deletion</td>
<td>Prevents conversion of C-terminal tail into closed position.</td>
<td>O-Mad2</td>
<td>Yes</td>
<td>Yes</td>
<td>[28, 31, 32, 34, 36]</td>
</tr>
<tr>
<td>Mad2&lt;sup&gt;R133A&lt;/sup&gt;</td>
<td>Arg133 to Ala</td>
<td>Prevents interaction of open form with closed form in a pure preparation.</td>
<td>O-Mad2</td>
<td>Yes</td>
<td>Weak</td>
<td>[28, 32, 40]</td>
</tr>
<tr>
<td>Mad2&lt;sup&gt;R133E-Q134A&lt;/sup&gt;</td>
<td>Arg133 to Glu Gin134 to Ala</td>
<td>Prevents interaction of open form with closed form with increased penetrance relative to Mad2&lt;sup&gt;R133A&lt;/sup&gt;</td>
<td>O-Mad2</td>
<td>Yes</td>
<td>No</td>
<td>This study</td>
</tr>
<tr>
<td>Mad2&lt;sup&gt;R133E-Q134A-DC&lt;/sup&gt;</td>
<td>Arg133 to Glu Gin134 to Ala 10 residue C-terminal deletion</td>
<td>Prevents interaction of open form with closed form and conversion of C-terminal tail into closed position.</td>
<td>O-Mad2</td>
<td>No</td>
<td>No</td>
<td>This study</td>
</tr>
</tbody>
</table>

As it was an essential precondition for this experiment, we tested the ability of the RNAi-insensitive alleles of Mad2 to bind Mad1. HA-Mad2<sup>wt</sup>, HA-Mad2<sup>R133A</sup>, and HA-Mad2<sup>R133E-Q134A</sup>, but not Mad2<sup>LIC</sup>, bound Mad1 after depletion of endogenous Mad2 (Figure 5C).

Being expressed at relatively low levels, none of the Mad2 alleles caused cell cycle arrest in the absence of nocodazole (Figure 5D, left). In the presence of nocodazole, control cells depleted of Mad2 failed to arrest in mitosis, showing that loss of Mad2 abrogates the SAC (Figure 5D, right, yellow bars). Complementation with RNAi-insensitive Mad2<sup>wt</sup> reestablished a significant level of mitotic arrest (red bars). As for analogous complementation experiments with BubR1 [39], we did not observe full recovery of checkpoint competence in cells expressing the RNAi-insensitive Mad2<sup>wt</sup> allele. A possible explanation is that the expression levels of HA-Mad2 complementing endogenous Mad2 are not completely stable, peaking at 40 hr after transfection and rapidly decreasing thereafter (not shown).

Mad2<sup>R133A</sup> and Mad2<sup>R133E-Q134A</sup> were completely impaired in complementing loss of Mad2 in nocodazole (Figure 5D, 5E, and 5F, in blue and violet bars). These alleles caused even lower counts of mitotic cells than in the absence of rescue constructs, suggesting an exacerbation of the checkpoint incompetence caused by loss of endogenous Mad2. Despite the imperfect levels of recovery obtained with Mad2<sup>wt</sup>, these results confirm that the binding interface between O-Mad2 and C-Mad2 is essential to maintain the SAC at physiological protein concentrations. Of note, pCMV-driven overexpression of Mad2<sup>R133A</sup> effectively arrested cells in mitosis to an extent similar to that obtained with Mad2<sup>wt</sup> (not shown). High levels of Mad2 may drive formation of sufficient Mad2/Cdc20 by mass action, rendering the interaction of O-Mad2 with C-Mad2 dispensable. This interaction, however, is absolutely required at low Mad2 concentrations. Consistent with this, when moderately overex-
Discussion

Here, we propose a new model to describe the roles of Mad1 and Mad2 in the SAC, which we have named the "Mad2 template" model (Figure 6). This model can be broken down as follows: (1) two distinct conformers of Mad2 exist: O-Mad2, which predominates in the cytosol and accounts for the majority of Mad2, and C-Mad2, the conformation Mad2 adopts when bound to Mad1 or Cdc20; (2) Mad2 is recruited to kinetochores as O-Mad2, with the exception of that contained in the Mad1/Mad2 complex; (3) the O-Mad2 receptor at the kinetochore is Mad1/Mad2→Mad1→C-Mad2, which accounts for the majority of Mad2, and C-Mad2 contains the critical surface of the O-Mad2 receptor; (4) O-Mad2 and Mad1 bound C-Mad2 are distinct and nonexchanging within the timeframe of SAC activation; (5) the interaction with C-Mad2 facilitates binding of O-Mad2 to Cdc20; and (6) the Cdc20/C-Mad2 complex represents a structural copy of Mad1/C-Mad2 acting to promote further transformation of O-Mad2 into Cdc20 bound C-Mad2 away from kinetochores.

There is good evidence that C-Mad2 and O-Mad2 are stable conformations of Mad2 (point 1) [22, 28, 29, 31, 32]. O-Mad2 is the conformation of cytosolic Mad2 [32, 33]. It is possible that the O-Mad2 conformation is actively maintained in the cytoplasm to revert spontaneous formation of ligand-free C-Mad2. In HeLa cells, Mad2 exists predominantly as O-Mad2, but bacterially expressed Mad2 populates both conformations in the absence of Mad1 or Cdc20 [32, 33]. Spontaneous formation of empty C-Mad2 in vitro is not necessarily significant and not surprising when considering that the safety belt is designed to convert reversibly from O- to C-Mad2 and that empty C-Mad2 is stabilized by O-Mad2 in the Mad2 dimer.

Mad2 is recruited to kinetochores as O-Mad2 by the Mad1/C-Mad2 complex, and O-Mad2 and Mad1 bound C-Mad2 are distinct and nonexchanging (points 2–4). In this perspective, Mad1 and Cdc20 do not compete for Mad2 binding despite their related Mad2 binding motifs. The existence of Mad1-free and Mad1 bound pools of Mad2, corresponding to ~70% and ~30% of total Mad2, respectively, is well established, but it was unclear whether these pools exchanged Mad2 subunits at relevant rates [8, 9, 14, 15, 22, 24, 26, 32]. Several indications suggest that the two pools remain distinct: (1) recombinant Mad1/Mad2 is stable and does not release Mad2 in the presence of Cdc20 in vitro (Figure 1); (2) Mad2R133E-Q134A, which binds Mad1, but not C-Mad2, fails to be recruited to kinetochores, suggesting that there are no C-Mad2 vacancies to be filled (Figure 3)—given the high turnover rate of Mad2 at the kinetochore [18,
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Figure 4. Functional Analysis of Mad2<sup>2C</sup> in HeLa Cells

(A) Model for dominant-negative effect of Mad2<sup>2C</sup>. After binding Mad1/Mad2, Mad2<sup>2C</sup> prevents kinetochore recruitment of O-Mad2<sup>wt</sup> and formation of Mad2/Cdc20.

(B) Western blotting with an anti-Mad2 Ab of lysates of HeLa cells transfected with empty pCMV (lane 1) or pCMV expressing myc-Mad2<sup>wt</sup> (lane 2), myc-Mad2<sup>2C</sup> (lane 3), and myc-Mad2<sup>R133A-2C</sup> (lane 4). Like Mad2<sup>R133E-Q134A-2C</sup>, this mutant does not bind the Mad1/Mad2 core [33].

(C) Left, 44–46 hr post transfection of the indicated constructs, HeLa cells were harvested, and DNA content and Ser10 phosphorylation of Histone H3 (H3P, a mitotic marker) was analyzed for myc-positive cells by three-color flow cytometry. Right, 26–28 hr post transfection of the indicated constructs, nocodazole was added to trigger the checkpoint. Cells were harvested after 18 hr and analyzed as above. Error bars represent standard deviations.

20, 21], if C-Mad2 core subunits were exchanging, we would expect significant kinetochore recruitment of Mad2<sup>R133E-Q134A</sup> (3) an immobile pool of Mad2 at mitotic kinetochores coexists with a high turnover mobile fraction of equal intensity [20]. Most likely, visualization of this immobile fraction was made possible by longer times of incorporation of fluorescent Mad2 into the Mad1/Mad2 core complex relative to other analyses [18, 21]. Given that Mad1 is immobile [20, 21], the nonrecovering fraction of Mad2 likely represents C-Mad2 in the Mad1/Mad2 complex.

We did not address how the interaction of O-Mad2 with Mad1/C-Mad2 facilitates binding of Mad2 to Cdc20 (point 5). All genetic and biochemical evidence shows that Mad2 binding to Cdc20 is Mad1 dependent [15, 22–25]. Because the role of Mad1 is to localize C-Mad2 at kinetochores, we propose that Mad1 bound C-Mad2 is also required to form Mad2/Cdc20. Indeed, the interaction of O-Mad2 with C-Mad2 is essential to maintain the checkpoint (Figures 5 and 6). The structural conversion of O-Mad2 into C-Mad2 bound to Cdc20 involves relatively large activation energies [22, 29, 32]. We suspect that the O-Mad2/C-Mad2 interaction accelerates this conversion during SAC activation. A structural investigation of the O-Mad2/C-Mad2 complex is underway and will hopefully shed light on how this conversion takes place. We are also investigating if and how the Mad1/Mad2 core complex directly affects the kinetics of Mad2 binding to Cdc20. Because the Mad2/Cdc20 interaction depends on Mad1 in living cells, appropriate conditions recreating this dependency in vitro must be identified. Furthermore, Cdc20 segments such as Cdc20<sup>111–138</sup> bind Mad2 more tightly than full-length Cdc20, and Cdc20 is also expected to undergo defined rearrangements to bind Mad2 [9, 35, 41]. These events may take place on a unique platform at the kinetochore, whose exact composition needs now to be unveiled. Addressing the role of the O-Mad2/C-Mad2 interaction directly with purified components is unlikely to yield insightful responses before more sophisticated in vitro assays are developed.

We postulate that the SAC starts when Mad1/Mad2 and O-Mad2 meet at the kinetochore near nuclear envelope breakdown. This enhances the transformation of O-Mad2 into Cdc20 bound C-Mad2 (Figure 6A). In the absence of stimulatory kinetochore function, the rate of formation of the MCC complex is insufficient to avoid anaphase prior to completion of kinetochore attachment. In this model, kinetochores accelerate the formation of the MCC, whereas its rate of disassembly remains relatively fast to allow rapid anaphase onset upon completion of bipolar attachment. This implies faster dissociation of Cdc20/Mad2 relative to Mad1/Mad2. For reasons discussed above, this is reasonable but will need to be re-assessed with the MCC of which Cdc20/Mad2 is only a subcomplex.

Although we do not have a molecular description of this mechanism, our data indicate that C-Mad2 bound...
Figure 5. Functional Analysis of Mad2^{R133A} and Mad2^{R133E-Q134A} in HeLa Cells

(A) Mad2^{R133A} and Mad2^{R133E-Q134A} mutants are expected to be unable to sustain the SAC in the absence of Mad2^{wt}. Both mutants bind Mad1 as tightly as Mad2^{wt}. Because both mutants are impaired in O-Mad2/C-Mad2 binding, they cannot sustain the SAC.

(B) Mad2 expression in HeLa cells was silenced by RNAi (lane 1) with pSUPER-Mad2 [38]. Mad2^{wt} (lane 2), Mad2^{R133A} (lane 3), Mad2^{R133E-Q134A} (lane 4), and Mad2^{H9004/C} (lane 5) alleles made insensitive to RNAi with silent point mutations were expressed as HA-tag fusions from a pBabe vector cotransfected with pSUPER. HA-tagged Mad2 expressed from pBabe was detected with an anti-Mad2 antibody.

(C) HA-tagged Mad2 proteins enter a complex with Mad1, as shown by immunoprecipitation. Mad2^{H9004} was the only allele that did not interact with Mad1 in the absence of endogenous Mad2.

(D) Left, flow cytometry of exponentially growing HeLa cells transfected with the indicated vectors. Right, cell cycle profiles of cells harvested 18 hr after adding nocodazole. Error bars represent standard deviations.

We show that Mad1/Mad2 is the kinetochore receptor to Mad1 favors the transformation of O-Mad2 into C-Mad2 bound to Cdc20. This is reminiscent of the template-assisted conversion of prion proteins [42]. For sake of clarity, we are not proposing that Mad2 is a prion because differences with prions are heavier than similarities in this comparison. For instance, the transformation of Mad2 may be completely reversible and protein misfolding is unlikely to play a role in the regulation of Mad2. Furthermore, prions instate an inheritable state, whereas there is no evidence that Mad2 has such properties.

From a structural perspective, however, the parallel holds as it emphasizes a mechanism of homodimerization based on the selective interaction of different conformers of the same protein, ultimately resulting in the conversion of one into the other. The parallel is also conceptually useful as it suggests a mechanism for signal amplification in the SAC (point 6). Mad1 and Cdc20 share a similar Mad2 binding motif, and Mad2 adopts the C-Mad2 conformation within both complexes [22, 29]. In this respect, the C-Mad2/Mad1 and C-Mad2/Cdc20 complexes are structurally equivalent. Their relationship is one of template (C-Mad2/Mad1 core) and copy (C-Mad2/Cdc20). As a functional copy of C-Mad2/Mad1, C-Mad2/Cdc20 released in the cytoplasm might cause further conversion of O-Mad2 into C-Mad2/Cdc20. This speculation, which is contained in point 6 of the model, suggests that signal amplification in the SAC is caused by the C-Mad2-generating activities of kinetochore C-Mad2/Mad1 core and cytoplasmic C-Mad2/Cdc20 (Figure 6).

The characterization of p31comet (previously known as CMT2) as a Mad2 ligand required to exit mitosis provides a strong indication in favor of this model [43]. As O-Mad2, also p31comet recognizes selectively C-Mad2 in the Mad1/Mad2 and Cdc20/Mad2 complexes and fails to bind O-Mad2 [44] (R. Hagan et al., submitted). The “Mad2 template” model suggests that p31comet hampers the transformation of O-Mad2 into C-Mad2 by competing with O-Mad2 for binding to C-Mad2/Mad1 and C-Mad2/Cdc20 (Figure 6B), a prediction that we are currently testing.

Conclusions
We show that Mad1/Mad2 is the kinetochore receptor for Mad2. The interaction of distinct Mad2 conformers is the core of the “Mad2 template” model and allows...
understanding the behavior of Mad2 in the SAC. The novelty of the model presented here is that it includes a mechanism for SAC signal amplification away from the kinetochore. Attempts to validate this model and to understand the interaction of Mad2 with other components of the SAC, such as Bub1, BubR1, and Bub3, are undergoing in our laboratories.

Experimental Procedures

Plasmids

pGEX-Mad1 (505-505), pGEX-CDC20 (175-178), and pCMV-mycMad2 were described [28]. pET43-Mad2/6His-Mad1 (565-716) contains the coding sequence of human Mad2 separated from human 6His-Mad1 (565-716) by a ribosome binding site. We generated pET43-6His-Mad2, pET43-6His-Mad2 (565-716), and pET43-6His-Mad2 (565-716) ligated by Ndel/EcoRI sites of pET43. The Mad2 coding sequence was ligated into BamHI of pBabe-HA-puro to obtain pBabe-HA-Mad2. Mutants were generated with QuikChange (Stratagene).

Expression, Purification, and Alexa Labeling of Proteins

Mad2/6His-Mad1 (565-716) was expressed in E.coli BL21-c41(DE3). Mad2 was expressed at limiting levels with respect to 6His-Mad1. A stoichiometric core forms because excess 6His-Mad1 unbound to Mad2 is insoluble in E. coli. After affinity chromatography on Ni-NTA-agarose (Qiagen), the protein was purified by ion exchange and SEC on Superdex-200 in PBS. Mad2 proteins were expressed in E. coli BL21(DE3) and purified as described [28]. Proteins were labeled with Alexa-488 succimidyl ester dye (Molecular Probes, Inc.) as described [18] with final dye-to-protein ratio of ~2:1.

Microscopy

Culture of PtK1 cells, microinjection, digital imaging microscopy, and image analysis were previously described [18]. Early prometaphase cells were microinjected with about 1%–5% cell volume of Alexa-labeled Mad2 proteins at about 3 μM needle concentration. Cells were imaged on a Nikon TE300 inverted microscope equipped with a Nikon 100×/NA 1.4 Plan Apo phase objective and an Orca II ER cooled-CCD camera (Hamamatsu). Fluorescence was detected by using 2×2 binned images with 250 msec exposures.

Analytical SEC

Analytical SEC was carried out on a SMART device (Amersham-Pharmacia Biotech) with a Superdex-200 PC 3.2/30 column in PBS. 20 μM Mad2/6His-Mad1 (565-716) complex was incubated for 1 hr at 20°C with 40 μM Alexa-Mad2 or Alexa-Mad2 mutants. The reactions were separated by SEC. Elution was carried out at 40 μl/min.

Cell Culture and Transfection

HeLa cells were grown in Dulbecco’s modified Eagle medium (DMEM, Euroclone) supplemented with 10% bovine calf serum (Hyclone) and 2 mM L-glutamine. Growing HeLa cells were transfected with either a standard calcium phosphate precipitation procedure or lipofectamine reagent (Gibco-BRL). Nocodazole (Sigma) was used at 100 ng/ml. PtK1 cells were grown as described [18].

Antibodies

Anti-Mad2 (clone AS55-A12) and anti-Mad1 (clone BB3-8) monoclonals were obtained by immunizing Balb/C mice with the Mad2/Mad1 (565-716) complex and produced at the IFOM-IEO campus monoclonal antibody facility; AC-40 anti-c-Myc monoclonal was from Onco- gene; rabbit polyclonal anti-phospho-histone-H3 was from Upstate and anti-c-Myc monoclonal was from Onco- gene; rabbit polyclonal anti-phospho-histone-H3 was from Upstate Biotechnology. Donkey anti-mouse or anti-rabbit IgG secondary antibodies conjugated to FITC or Cy5 were from Jackson Laboratories, Inc.

Immunoprecipitations

HeLa cells were harvested by trypsinization and lysed in 50 mM Hepes (pH 7.5), 150 mM NaCl, 0.5% NP40, 1% glycerol, 5 mM EDTA, 10 mM Na2VO4, 50 mM NaF, 20 mM Na3-pyrophosphate, 100 ng/ml leupeptin, 100 ng/ml Aprotinin, and 1 mM PMFS for 20 min on ice. Cell lysates were centrifuged for 15 min at 13,000 rpm at 4°C in an Eppendorf microcentrifuge. Protein amounts were measured with protein assay reagent (Bio-Rad Laboratories) as specified by the manufacturer, and equivalent amounts of total protein were used for immunoprecipitation. 0.4 mg of each cell lysate were incubated with 2 μg of rabbit polyclonal anti-HA antibody (BABC0) for 1 hr at 20°C followed by incubation with protein A Sepharose beads for 2 hr. The beads were washed three times in lysis buffer, eluted in SDS sample buffer, and analyzed by SDS-PAGE and Western blotting.

RNAi and Overexpression

HeLa cells (50% confluence) were cotransfected with calcium phosphate twice at 24 hr intervals with 8 μg pSUPER-Mad2 [38] and 10
µg of rescue constructs pBABE-HA-Mad2, pBABE-HA-Mad2Δ134, or pBABE-HA-Mad2Δ134 containing silent mutations GAA169 and TCG170 in place of Mad2 codons GAG169 and TCG170. 20–24 hr after second transfection, cells were split 1:3 to maintain log phase. 30–40 hr after second transfection, nocodazole was added (100 ng/ml). Cells were harvested 18–22 hr after adding nocodazole and treated for FACS. Samples were also lysed and analyzed by Western blotting. For overexpression, HeLa cells were grown to 70% confluence and transfected with 10 µg pCMV-mycMad2 (or Mad2 mutants) by using lipofectamine, and after 18–18 hr, cells were split 1:3. 26–28 hr after transfection, nocodazole was added. Cells were harvested 18 hr after nocodazole addition and treated for FACS analysis or WB.

Flow Cytometry
Cells were harvested by trypsinization and fixed with 1% formaldehyde and 75% ethanol. After fixation, cells were permeabilized with 0.1% Triton X-100 and stained with anti-phospho-histone-H3, anti-myc, or anti-HA antibodies. For DNA content, cells were treated with 5 µg/ml propidium iodide and 250 µg/ml RNase A. Data acquisition and analysis were performed by using a FACSCalibur flow cytometer (Becton Dickinson) with CellQuest 3.3 software.

Supplemental Data
Supplemental Data include four figures and a table and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/3/214/DC1/.

Acknowledgments
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The Mad1/Mad2 Complex as a Template for Mad2 Activation in the Spindle Assembly Checkpoint

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Supplemental Experimental Procedures

GST-Mad1253-550 and GST-Cdc20111-138 were expressed in *E. coli* BL21-c41(DE3). After lysis by sonication in buffer A (10 mM Hepes-HCl [pH 7.5], 100 mM NaCl, 1 mM DTT, and 0.5 mM EDTA), 1% Triton X-100 was added. The GST proteins were purified with glutathione-agarose (Amersham). 9262 g of GST, GST-Mad1523–550 or GST-Cdc20111–138 on beads were incubated for 1 hr at room temperature (RT) with 20262 g of Mad2wt or Mad2R133E in 0.3 ml of buffer A (final concentrations of GST fusion protein and Mad2 were 1 M and 3 M, respectively). Beads were washed two times with 0.4 ml buffer A supplemented with 1% Triton X-100 and separated by SDS-PAGE. For competition, GST-Cdc20 (1.4 M) and Mad2 (3 M) were incubated with competing Mad1 peptide at concentrations of 0.10 M, 0.7 M, 3.5 M, and 14 M.

Supplemental References


Table S1. Determination of Binding Affinities for Mad2 Interactions by Using ITC

<table>
<thead>
<tr>
<th>Interaction</th>
<th>( K_a ) (10^6 M(^{-1}))</th>
<th>( K_d ) (10^-4 M(^{-1}))</th>
<th>( \Delta H^{\text{obs}} ) (kcal/mol)</th>
<th>( TDS ) (kcal/mol)</th>
<th>( \Delta G^{\text{obs}} ) (kcal/mol)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mad2wt + Mad1253-550</td>
<td>3.77</td>
<td>0.27</td>
<td>-18.27</td>
<td>-9.27</td>
<td>-9</td>
<td>0.73</td>
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<tr>
<td>Mad2wt + Cdc20111-138</td>
<td>5.05</td>
<td>0.20</td>
<td>-12.51</td>
<td>-3.32</td>
<td>-9.19</td>
<td>0.68</td>
</tr>
<tr>
<td>Mad2wt + MBP1</td>
<td>10.8</td>
<td>0.09</td>
<td>-15.09</td>
<td>-5.44</td>
<td>-9.65</td>
<td>0.74</td>
</tr>
<tr>
<td>Mad2wt + Cdc20111-138 + Mad1253-550</td>
<td>1.1</td>
<td>0.91</td>
<td>-29.89</td>
<td>-21.62</td>
<td>-7.37</td>
<td>0.80</td>
</tr>
<tr>
<td>Mad2wt + MBP1</td>
<td>7.6</td>
<td>0.13</td>
<td>-25.81</td>
<td>-16.39</td>
<td>-8.41</td>
<td>0.61</td>
</tr>
<tr>
<td>Mad2R133E + MBP1</td>
<td>5.17</td>
<td>0.19</td>
<td>-19.41</td>
<td>-9.21</td>
<td>-9.19</td>
<td>0.79</td>
</tr>
<tr>
<td>Mad2wt/MBP1 + Mad2wt</td>
<td>NBD</td>
<td>NBD</td>
<td>2.6</td>
<td>11.31</td>
<td>-8.71</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Thermodynamic parameters for the interaction of wt-Mad2 with synthetic peptides corresponding to the Mad2 binding sites of Mad1, Cdc20, and MBP1 (a Mad2 binding peptide described in [S5]) or the binding of O-Mad2 to C-Mad2. ITC measurements were carried out using a VP-ITC titration calorimeter (MicroCal, Inc.). The interactions of Mad2wt with Mad1253-550, Cdc20111-138, and MBP1 peptides were studied by titrating aliquots of peptide (50 M) into 5 M Mad2wt, Mad2R133E, or Mad2wt/MBP1. The interactions of open Mad2 (Mad2wt) with C-Mad2 bound to MBP1 was studied through the titration of aliquots of 100 M wild-type Mad2 into 10 M wild-type Mad2 previously incubated for 1 hr at 26°C with 15 M MBP1. All proteins were in PBS and titrations were done at 26°C. Binding isotherms were corrected for dilution heats by subtraction of blank titration, and the corrected data were fitted with the Origin software package. \( K_a \), binding constant; \( K_d \), dissociation constant; \( \Delta H^{\text{obs}} \), observed binding enthalpy; \( TDS \), entropy; \( \Delta G^{\text{obs}} \), observed Gibbs’ free energy; N, binding stoichiometry. NBD, no binding detected.
Figure S1. Structure of O-Mad2, C-Mad2, and the Mad1/Mad2 Core Complex

(A) Rotated views of O-Mad2 [S1]. The C-terminal tail of Mad2 (the safety belt) is red, and the invariant part of Mad2 is yellow. R133 and Q134 are shown. In schemes, we display O-Mad2 as a red square and C-Mad2 as a yellow circle.

(B) C-Mad2 with bound Mad1 [S2] displayed in the same orientation as in panel A. Mad1 is gray. The cartoon displays only half of the Mad1/Mad2 tetramer. The white arrowhead shows that the polypeptide chain of C-Mad2 (red, the “safety belt”) runs above the Mad1 chain. The Mad2 β7-β8 segment is positioned below the Mad1 α2 helix. The only way in which Mad1 can be released from its complex with Mad2 is via unfolding of the β7-β8 segment from the rest of the β sheet (see Supplemental Figure S2E).

(C) Mad1<sup>1485–584</sup>/Mad2 is a 2:2 tetramer [S2]. Mad1 dimerizes using a coiled coil, and each Mad1 protomer contains a Mad2 binding site. (A) through (C) were created with PyMOL (http://pymol.sourceforge.net/).

(D) Comparison of secondary structure in the C-terminal tail of Mad2 in O-Mad2 and C-Mad2. The C-terminal deletion of Mad2<sup>1485–584</sup> does not affect the structure of O-Mad2 but impinges on the stability of C-Mad2 as the deletion removes part of the β8 strand. This is why Mad2<sup>1485–584</sup> is unable to turn into C-Mad2 and fails to bind Mad1 and Cdc20.
Figure S2. Implications of the Safety Belt, Part I

A schematic diagram of Mad2 in the O-Mad2 conformation is shown in (A). Luo and collaborators (2004) [S3] named this conformer of Mad2 as N1-Mad2. The orientation of this diagram is approximately the same shown for the ribbon model on the left of Figure 1A. In this diagram, α helices are in green, and β strands are in yellow, except for β7, β8, and the loops connecting them, which are red. The red segment is the −60 residue safety belt [S2]. For accommodation of the ligand (gray) as shown in (D), the safety belt has to unfold (B and C), so that the β6 strand is now ready to pair with β strand from the incoming ligand, a mechanism known as β augmentation. In this case, the β7 and β8 strands have to be removed first, but they form again (in a different frame, as explained in Figure S1D) at the opposite end of the β sheet, pairing with β5 (D). This must be accompanied by the removal of β1, which in the O-Mad2 structure (A), pairs with β5 and which extends the N-terminal helix α1 in C-Mad2. The structures in (A) and (D) correspond respectively to O-Mad2 and C-Mad2. The latter is defined as N2-Mad2 by Luo and collaborators (2004) [S3]. In both cases, there is a six-stranded, exposed, continuous β sheet, but the order of strands is different. From left to right, the order of strands in O-Mad2 is β1, β5, β4, β6, β8, and β7. Also from left to right, the order of strands in C-Mad2 is β7, β8, β5, β4, β6, and the ligand’s β strand βL. The arrow in (D) indicates the same point indicated by the equivalent arrow in Figure S1B. From a careful analysis of (D), it is clear that the safety belt must unfold to release the ligand. To make this concept clearer, we modified the orientation of the ligand in (E) to show that the ligand is topologically locked by the safety belt. Two bulky domains were added at the N and C termini of the ligand to clarify that the ligand can’t be unthreaded out of the safety belt “needle.” For instance, if the ligand were Mad1, there would be ~500 residues N-terminal to βL and ~200 C-terminal to it. Similarly, Cdc20 would have ~150 residues N-terminal to βL and ~400 C-terminal to it. From this drawing, it will become clear that a passage through a state similar to that shown in (C) is required for the ligand to be released. This is why we state that if Mad2 were released from Mad1 (which we don’t think it is), it would not be released in the C-Mad2 conformation as suggested [S3].
Figure S3. Implications of the Safety Belt, Part II

In the absence of a ligand, Mad2 may still undergo the transition from O-Mad2 (Supplemental Figure S2A) to C-Mad2 through presumably similar intermediates to those displayed in (Supplemental Figure S2F) and (A), which entail the unfolding of the safety belt and its refolding in the closed conformation. This will result in the creation of empty C-Mad2, shown in (B). (C) clarifies that any ligand wishing to bind C-Mad2 will have to wait for the unfolding of the safety belt (compare this view with that shown in Supplemental Figure S2E), as shown in (D)–(F).

Thus, both O-Mad2 and C-Mad2 can be regarded as starting points for ligand binding, but in both cases, the safety belt will have to undergo an unfolding transition to be able to bind the ligand. This is why we think it is very unlikely that C-Mad2 is the active Mad2 species as postulated by Luo and collaborators (2004) [S3]. We suggest that the results of Luo et al. (2004) [S3] on the Mad1-catalyzed formation of empty C-Mad2 have a more straightforward explanation. We suggest that two different chemical reactions were monitored in the absence or in the presence of Mad1: in the first case, the reaction consisted in the slow transformation of O-Mad2 into C-Mad2. In the second case, the event monitored was the formation of Mad1/C-Mad2 from O-Mad2 and Mad1, a reaction in which Mad1 is a reagent not a catalyst and whose faster rate relative to the generation of empty C-Mad2 is likely to be explained by Mad1-mediated stabilization of the transition from O-Mad2 to C-Mad2. It is clear from Supplemental Figure S1, however, that once bound to Mad1, Mad2 will be topologically linked to this molecule, and its release will entail the unfolding of C-Mad2.
Figure S4. Circular Dichroism and Thermal Unfolding

(A) Circular Dichroism (CD) spectrum of Mad2wt. Molar ellipticity ($\theta$) is presented as a function of wavelength. CD data were recorded at 37°C with a Jasco J810 spectropolarimeter at a protein concentration of ~11 μM in 100 mM NaF.

(B) CD spectrum of Mad2ΔC.

(C) CD spectrum of Mad2R133E-Q134A.

(D) CD spectrum of Mad2R133E-Q134A-ΔC.

(E) The thermal denaturation curve of Mad2wt is expressed as ellipticity as a function of temperature. The curve is a sigmoid, indicative of a simple unfolding transition. The melting point of the thermal denaturation curve ($T_m$) is slightly above 70°C. This curve was measured at 208 nm at a protein concentration of ~11 μM in 100 mM NaF, gradually increasing the temperature to 90°C.

(F) The thermal denaturation curve of Mad2R133E-Q134A-ΔC measured under the same conditions and at a similar protein concentration is essentially identical to that shown in (E) for Mad2wt. This Mad2 mutant contains all the mutations described in our study, confirming that these mutations do not significantly affect the folding nor the stability of Mad2.
(A) To clear any doubts about the actual inability of Mad2\textsuperscript{C} to bind Mad1 or Cdc20, we tested Mad2\textsuperscript{C} binding to GST fusions of the Mad2 binding sites of Mad1 or Cdc20 immobilized on solid phase. GST-Mad\textsubscript{1}\textsuperscript{523-550} and GST-Cdc20\textsubscript{111-138} preabsorbed to glutathione beads were incubated with 3 \( \mu \)M Mad2\textsuperscript{C}, Mad2\textsuperscript{wt}, or both. Bound proteins were separated by SDS-PAGE and revealed by Coomassie or Western blotting with an anti-Mad2 antibody. The asterisk marks an unspecific band generated by proteolysis or premature termination of GST-Mad\textsubscript{1}\textsuperscript{527-555}. No binding of Mad2\textsuperscript{C} to GST-Mad\textsubscript{1}\textsuperscript{527-555} or GST-Cdc20\textsubscript{111-138} was observed while Mad2\textsuperscript{wt} bound well (lanes 6 and 10). Similarly, Mad2\textsuperscript{C} did not display any binding to Mad1\textsuperscript{527-555} and Cdc20\textsuperscript{111-138} in ITC experiments (Supplemental Table S1). We also generated C-Mad2 by incubating Mad2\textsuperscript{wt} with GST fusions of Mad1\textsuperscript{527-555} and Cdc20\textsuperscript{111-138}. Being short and mostly buried within Mad2, these motifs are essential to generate C-Mad2 but are otherwise unlikely to contribute significantly to the interaction with O-Mad2 [S4, S2]. As above, immobilized GST-Mad\textsubscript{1}\textsuperscript{527-555} and GST-Cdc20\textsuperscript{111-138} were incubated with Mad2\textsuperscript{wt} for 1 hr, after which unbound Mad2\textsuperscript{wt} was removed, the beads washed, and Mad2\textsuperscript{C} added (3 \( \mu \)M) for a second 1 hr incubation. Mad2\textsuperscript{C}, which is unable to bind GST-Mad1\textsuperscript{527-555} and GST-Cdc20\textsuperscript{111-138} (lanes 6 and 10), remained bound in the presence of Mad2\textsuperscript{wt} (lanes 8 and 12). The \( k_{\text{off}} \) of the interaction of O-Mad2 with C-Mad2 is relatively fast (approximately 0.06 s\textsuperscript{-1}) [M. Vink, A.D.A., M.F., and A.M., unpublished data], explaining why the binding of O-Mad2\textsuperscript{C} to C-Mad2 was not prevented by O-Mad2\textsuperscript{wt} that might have remained bound to C-Mad2 from the previous incubation with Mad2\textsuperscript{wt}.

(B) In Figure 3I, we show that the open conformer of Mad2\textsuperscript{R133E-Q134A} is unable to bind C-Mad2. We also wished to know whether the closed form of Mad2\textsuperscript{R133E-Q134A} was capable of binding open Mad2-like wild-type Mad2. For this, we generated the closed forms of wild-type Mad2 and Mad2\textsuperscript{R133E-Q134A} with GST-Cdc20 and tested binding by the open conformer Mad2\textsuperscript{C}. Although Mad2\textsuperscript{R133E-Q134A} bound to the closed wild-type Mad2 (lane 8), it failed to bind Mad2\textsuperscript{R133E-Q134A} (lane 11). Thus, both the open and the closed conformers of Mad2\textsuperscript{R133E-Q134A} are unable to bind a functional form of the opposite conformer (compare with Figure 3I). This mutation has more penetrant effects relative to the single R133A mutation, which completely eliminates the interaction of the open and closed conformer only when present on both interacting conformers [S4].