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How Do so Few Control so Many?

Review

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The separation of sister chromatids at the metaphase-to-anaphase transition is triggered by a protease called separase that is activated by the destruction of an inhibitory chaperone (securin). This process is mediated by a ubiquitin protein ligase called the anaphase-promoting complex or cyclosome (APC/C), along with a protein called Cdc20. It is vital that separase not be activated before every single chromosome has been aligned on the mitotic spindle. Kinetochores that have not yet attached to microtubules catalyze the sequestration of Cdc20 by an inhibitor called Mad2. Recent experiments shed important insight into how Mad2 molecules bound to centromeres through their association with a protein called Mad1 might be transferred to Cdc20 and thereby inhibit securin's destruction.

The logic of chromosome segregation during mitosis has been well understood for nearly 80 years, and its description therefore adorns the early chapters of most biology textbooks. The aim is to segregate sister chromatids produced during the duplication of chromosomes to opposite poles of the cell prior to its division. When successful, this process ensures that daughter cells inherit the exact same set of chromosomes possessed by their parents. This is the foundation for the capacity of unicellular organisms to proliferate indefinitely, a property also shared by special stem cells in multicellular organisms. The logic of mitosis is beguilingly simple. It makes such sense that it is usually forgotten that the molecular mechanisms underlying it are about as complicated as any in the vast repertoire of mechanical systems found in living organisms. How indeed do cells ensure that sister DNAs move to opposite poles of the cell with an accuracy that permits error in less than one in 10^5 events?

The mechanical force responsible for chromosome movement is provided by highly dynamic polar filaments created by the oligomerization of tubulin. During mitosis, the positive ends of microtubules are embedded into specific structures on each chromatid known as kinetochores while their negative ends are embedded in large structures at opposite ends of the cells called centrosomes. The traction of chromosomes toward centrosomes arises either from depolymerization of the minus ends of kinetochore-attached microtubules while they remain embedded within centrosomes or by depolymerization of kinetochore-attached microtubules at their positive ends, a process that must occur without their detachment from kinetochores. Movement along microtubules of motor proteins that are simultaneously attached to kinetochores is also thought to contribute to chromosome movement. If the kinetochores of sister chromatids attach to microtubules emanating from opposing poles (known as amphitelic attachment or biorientation), then the forces exerted by microtubules will pull sister DNAs in opposite directions and thereby to opposite sides of the cell prior to its division.

A crucial feature of this mechanism is that cells must somehow avoid the attachment of sister chromatids to microtubules emanating from the same pole (known as syntelic attachment), which would cause sisters to be pulled to the same pole and would thereby cause the production of aneuploid daughter cells. The solution to this problem is to ensure that sister DNAs remain connected with each other even after chromosomal DNA has been fully replicated, a phenomenon known as sister chromatid cohesion (Miyazaki and Orr-Weaver, 1994). Because of sister chromatid cohesion, amphitelic but not syntelic attachment creates tension at kinetochores and along the microtubules attached to them. In the absence of such tension, a poorly understood mechanism dependent on the Aurora B protein kinase actively destabilizes kinetochore microtubules (Nicklas and Ward, 1994; Tanaka et al., 2002). Syntelically attached chromosomes, which do not give rise to tension, are thereby selectively eliminated.

If the first phases of mitosis, known as prophase, prometaphase, and metaphase, are concerned with ensuring that all chromosomes attain amphitelic attachment, the second part of mitosis is about destroying simultaneously the connections that link sister chromatids, which permits their traction to opposite poles of the cell during anaphase. Sister chromatid cohesion is mediated by a multisubunit complex called cohesin, which forms a gigantic ring structure whose integrity is required for holding sister chromatids together (Haering and Nasmyth, 2003). It has therefore been suggested that cohesin might hold sister DNAs together by trapping them inside its ring. When (and only when) all chromosomes have attached amphitelically and thereby come under tension, the cell activates a site-specific protease called separase. This cleaves cohesin's kleisin subunit (Scc1), severs the cohesin ring, and destroys the link between sister DNAs, which permits their traction finally toward opposite poles of the cell (Figure 1).

It is clearly vital that separase not become active before every single chromosome has come under tension. Separase is kept inhibited during the first part of mitosis by two mechanisms: first by its association with an inhibitory chaperone called securin and second due to its phosphorylation by the Cdk1/cyclin B protein kinase. The protease activity of separase is activated by the simultaneous destruction of both securin and cyclin B at the hands of the 26S proteosome, which is preceeded by their ubiquitinylation at the hands of a huge multisubunit ubiquitin protein ligase called the anaphase-promoting complex or cyclosome (APC/C), whose

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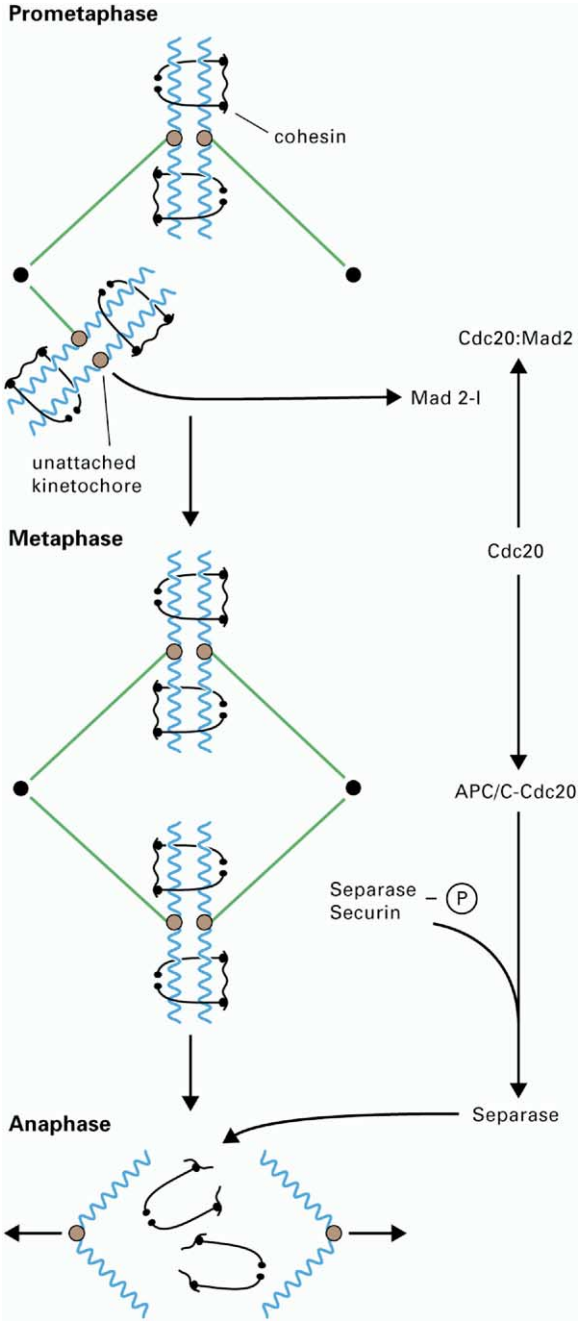

Figure 1. Control of Sister Chromatid Segregation in Mitosis

Sister kinetochores attach to microtubules (green) that face opposite directions (amphitelic attachment) during prometaphase and metaphase. The metaphase-to-anaphase transition is initiated by activation of separase. Until metaphase, separase is kept inactive by the binding of its inhibitory chaperone securin and by CDk1/cyclin B-dependent phosphorylation. Activation of Cdc20 and the anaphase-promoting complex/cyclosome (APC/C) causes destruction of both securin and cyclin B, which leads to cleavage by separase of the Scc1 subunit of cohesin (black ring). Loss of sister chromatid cohesion is accompanied by traction of sister chromatids to opposite poles during anaphase. Unattached kinetochores in prometaphase promote the production of a form of Mad2 (Mad2-I) that sequesters Cdc20 and prevents it from promoting securin and cyclin B ubiquitinylation.

activity depends on an accessory protein called Cdc20 (Zachariae and Nasmyth, 1999) (Figure 1). Cdc20 is thought to recruit substrates to the APC/C by binding to both partners. Cdc20's C-terminal domain is composed of WD40 repeats that form a β propeller that binds both the APC/C and its substrates and a less structured but nevertheless highly important N-terminal domain that is essential for controlling Cdc20's activity.

Video microscopy has shown that the time taken for all chromosomes to biorient during the first part of mitosis can vary considerably between cells but that the interval between biorientation of the last chromosome and sister chromatid separation is relatively invariant and lasts about 20 min in mammalian tissue culture cells (Rieder et al., 1994). Crucially, cells rarely if ever trigger sister chromatid separation while there still exist chromosomes that have not bioriented. Furthermore, disassembly of microtubules by the addition of poisons such as colchicine and nocodazole delays sister chromatid separation as well as exit from the mitotic state for many hours. The suggestion is that cells are capable of monitoring the attachment of kinetochores to microtubules and prevent activation of the APC/C and hence separase when kinetochores are unoccupied by microtubules. This surveillance mechanism is known as the spindle assembly checkpoint or SAC (Hoyt et al., 1991; Li and Murray, 1991). It has been suggested but never fully proven that cells can also sense the lack of tension at kinetochores and block APC/C activity also under these circumstances (Stern and Murray, 2001). Importantly, work on yeast showed that cells carrying specific mutations in Cdc20's N-terminal domain were incapable of delaying destruction of securin and cyclin B in the presence of spindle poisons, which suggested that the SAC prevents separase activation and mitotic exit by inhibiting the ability of Cdc20 to bind either its substrates or the APC/C (Kim et al., 1998; Hwang et al., 1998). The SAC is a remarkable control mechanism because a single unattached kinetochore is capable of preventing the activity of all Cdc20 molecules in a mitotic cell. Activation of the SAC can induce cells to arrest almost indefinitely in a metaphase-like state.

The SAC is essential in mammalian cells but surprisingly not so in budding yeast, where chromosomes can be segregated reasonably accurately without the SAC. The relative unimportance of the SAC during mitosis in yeast was fortunate, as it enabled the identification of proteins necessary for the SAC by isolating mutants that failed to prevent exit from mitosis upon disassembly of microtubules (Hoyt et al., 1991; Li and Murray, 1991). Orthologs of four of these proteins are encoded in most if not all eukaryotic genomes. In mammalian cells, these proteins are known as Mad1, Mad2, BubR1, and Bub3. Though all four proteins are equally essential for the SAC in vivo, Mad2 appears to have the most direct role in inhibiting Cdc20's activity. Mad2 binds directly to Cdc20 when the SAC is active, and, in vitro, this binding is sufficient to block the APC/C's ability to ubiquitinylate securin or cyclin B (Li et al., 1997; Fang et al., 1998). Mad2 is thought therefore to sequester Cdc20 in an inactive state when the SAC is activated (Figure 1). Indeed, production of excess Mad2 is sufficient to provoke cells to arrest in metaphase even when all chromosomes have bioriented successfully.

The levels of Mad2 do not normally vary much during mitosis nor are they greatly elevated when the SAC has been activated. The suggestion is that the bulk of Mad2 protein is normally inactive and not capable of binding stably to Cdc20 but that Mad2 is stimulated to do so by kinetochores that are unoccupied by microtubules. Mad2's association with other checkpoint proteins appears to greatly augment its ability to inhibit the APC/C (Sudakin et al., 2001). This phenomenon is as remarkable as any described in eukaryotic cells. How does a discrete and relatively small structure, namely a single unoccupied kinetochore, catalyze the production of Mad2 proteins capable of binding and inhibiting Cdc20? Moreover, how does an unoccupied kinetochore produce enough "inhibitory" Mad2 to sequester most if not all of the Cdc20 protein in the cell? This is all the more remarkable when one considers that yeast kinetochores bind only a single microtubule and that vacancy of just one such site is capable of preventing almost permanently the metaphase-to-anaphase transition.

How then do unoccupied kinetochores trigger Mad2 to adopt a form capable of inhibiting Cdc20? The finding that Mad2 oligomerizes to form what are now thought to be dimers when produced in *E. coli* raised the possibility that oligomeric Mad2 might be inhibitory, while monomeric Mad2 might be incapable of inhibiting Cdc20 (Fang et al., 1998). If true, unoccupied kinetochores might have the ability to catalyze the formation of inhibitory Mad2 oligomers. However, there has never been any indication that Mad2 oligomerizes only in cells with an active SAC. It has even been claimed, erroneously as it turns out, that oligomerization can be eliminated by specific mutations without compromising the SAC (Sironi et al., 2001). Mad2's ability to dimerize is indeed vital to the SAC, but the role of unoccupied kinetochores is not simply to catalyze the formation of oligomeric inhibitory Mad2.

Another crucial finding was the observation that a fraction of Cdc20 and SAC proteins including Mad1and Mad2 are concentrated at kinetochores of chromosomes that have not yet bioriented (Waters et al., 1998). Might the Mad2 detected at such kinetochores be in the process of being converted to an inhibitory form capable of binding Cdc20? Might the sequestration of Cdc20 by an inhibitory form of Mad2 occur exclusively at such kinetochores? Importantly, is the flux of both Mad2 and Cdc20 at unattached kinetochores sufficient to inactivate all Cdc20 protein in the cell within a few minutes? As far as I know, the actual fluxes of these proteins at kinetochores have not yet been determined. Any rigorous understanding of the SAC will require this information. Nevertheless, photobleaching of fluorescent variants of Mad1 and Mad2 has revealed that most Mad1 associated with unattached kinetochores does not turn over rapidly, whereas a large fraction of Mad2 turns over rather rapidly (Howell et al., 2004). Other studies also suggest that there exists a second population of Mad2 at kinetochores that turns over more slowly (Shah et al., 2004). Note that, despite the slow turnover of Mad1 at the kinetochores of lagging chromosomes, it nevertheless disappears abruptly from kinetochores once chromosomes biorient successfully.

The recruitment of Mad2 to kinetochores depends on Mad1 but not vice versa (Chen et al., 1998). It seems that one of the first events in activating the SAC is the recruitment of Mad1 to kinetochores, which in turn recruits Mad2. Mad1 is a long protein (718 amino acid residues) composed largely of coiled coils. Like Mad2, Mad1 oligomerizes to form Mad1:Mad1 dimers. Mad2 binds directly to Mad1, and it is presumably via this interaction that Mad2 is recruited to kinetochores. Thus, Mad2 forms complexes both with Mad1 and with Cdc20. This has led to the notion that Mad1 not only recruits Mad2 to kinetochores, but, once it has done so, it induces a change in Mad2's conformation that permits Mad2 now to bind Cdc20 (Luo et al., 2004). This concept is known as the "Mad2 exchange" model. The Mad2 recruited to unattached kinetochores is postulated to exchange partners from Mad1 to Cdc20.

Clearly, the structure of Mad2's complexes with Mad1 and Cdc20 should provide insight into how Mad2 might be transferred from Mad1 to Cdc20. Such structures have indeed been determined and have shed fascinating insight into how the SAC might function (see below). Crystal and NMR structures show that Mad1 and Cdc20 bind to Mad2 in an apparently identical fashion. Indeed, biochemical studies show that they compete for the very same binding site. The simple notion that the binding of Mad2 to Mad1 alters its conformation so that the very same Mad2 molecule can now bind Cdc20 is therefore implausible without invoking a very complex series of events.

Before considering alternatives to the exchange model and before considering the structural implications of Mad1's mode of interaction with Mad2, let us summarize the key facts that any model will have to explain. (1) Mad1 binds unoccupied kinetochores and is stably bound to them. (2) Mad2 is subsequently recruited to kinetochores by binding Mad1 that has previously bound there. (3) There are potentially two populations of Mad2 at kineotochores, one turning over rapidly and one relatively stable. (4) Cdc20 is also recruited to unoccupied kinetochores and forms complexes with Mad2 whose mode of interaction may be identical to that between Mad1 and Mad2. (5) Mad2 forms dimers. (6) Mutation of the amino acid residues within Cdc20 that bind Mad2 abrogates the SAC. The whole purpose of the SAC is presumably therefore to catalyze the formation of complexes between Mad2 and Cdc20. (7) Bub3 and BubR1 also form complexes with Cdc20. Their function is at the moment obscure, but it might also be to facilitate complex formation between Mad2 and Cdc20. To these facts, we need to add an additional assumption that is not supported by hard facts but instead by intuition. Because the amount of Mad1 at kinetochores is much less than the amount of Cdc20 in the cell that must eventually be sequestered by Mad2, the SAC cannot work merely by recruiting Cdc20 to kinetochores where it binds to Mad2 recruited there by Mad1.

What then is the structure of Mad2, and does this shed insight into how Mad1 facilitates Mad2's sequestration of Cdc20? The structure of Mad2 has been determined by NMR (Luo et al., 2000) as well as by X-ray crystallography (Sironi et al., 2002). By mutating Mad2's N terminus, Luo et al. (2000) managed to create a version of Mad2 that formed mainly monomers and whose

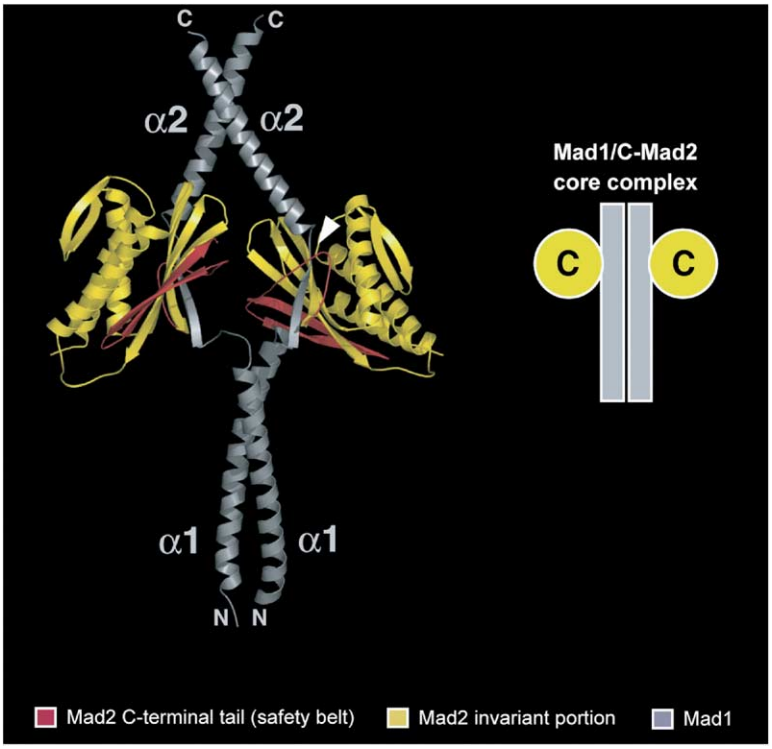

Figure 2. The Crystal Structure of Mad1: Mad2 Complexes

structure could therefore be solved by NMR. This revealed a novel fold composed of α helices and β sheets. Sequence analyses suggest that this fold, known as the HORMA domain, is shared by a regulatory subunit of DNA polymerase ζ called Rev7 and by the meiosis-specific recombination protein Hop1 (Aravind and Koonin, 1998). The fold consists of three layers: a central layer formed by three α helices, a large six-stranded β sheet on one side, and a short β hairpin on the other side (Figure 3A). The structure was, however, not particularly informative per se. More interesting was the finding that addition of a Cdc20 peptide sufficient to bind Mad2 caused an extensive change in Mad2's structure and that Mad2's very C-terminal sequences were essential for its interaction with Cdc20 (Luo et al., 2000).

A key breakthrough in the field was the elucidation by Sironi et al. of a crystal structure formed between Mad2 and a fragment of Mad1 (Mad1$^{485–584}$) that contained its Mad2 binding sequences and some but not all of its coiled coils (Sironi et al., 2002). This segment of Mad1 contained two α helices (α1 and α2) that surrounded the short segment of Mad1 that actually binds Mad2. The key to growing these crystals was the elimination of Mad2's ability to oligomerize, which otherwise creates polydisperse complexes. Because oligomerization was a property shared by Mad2 proteins from many different organisms, Sironi et al. (2002) mutated to alanine all conserved residues on Mad2 that were predicted to be exposed to solvent and hence available for interaction with equivalent residues on another Mad2 molecule. It is important to point out that this exercise was only possible because the existing NMR structure was available to guide their mutagenesis studies. They discovered that mutation of an arginine at position 133 to alanine had the desired properties, namely that Mad2^{R133A} still bound to both Mad1 and Cdc20 peptides but no longer formed complexes with itself. Contrary to their initial claims (Sironi et al., 2001), Mad2^{R133A} is not biologically functional, and the reasons for this are interesting and germane to our story (see below).

The Mad1$^{485–584}$:Mad2^{R133A} complex was a tetramer, not due to interaction between two Mad2 subunits, which had been eliminated by the Mad2^{R133A} mutation, but because the Mad1$^{485–584}$peptides were tightly held together by a parallel intermolecular coiled coil formed between α1 helices from different Mad1 molecules (Figure 2). The binding of Mad1 causes a remarkable reorganization of Mad2's C-terminal domain compared with the apo-Mad2 structure determined by NMR. While the first 160 residues of Mad2 are structurally invariant in apo and Mad1 bound Mad2, including all three of its α helices (from left to right, α3, α1, and α2), the C-terminal region containing β strands 7 and 8 as well as the loop connecting β strand 7 with 6 is displaced from its association with helix α2 at the right side of Mad2 and binds instead to helix α3 on its left side (Figures 2 and 3). In apo-Mad2, β7 and β8 augment on its right-hand side a β sheet composed of β5, β4, and β6, while, in Mad1-Mad2 complexes, β7 and β8 augment this same sheet through association with β5 on its left-hand edge. In the course of this huge rearrangement, β1 is displaced as β5's left-hand neighbor and the register of both β7 and β8 altered so that sequences at the extreme C terminus of Mad2 are now included within β8. The latter neatly explains the previous finding that deletion of Mad2's C-terminal amino acid sequences pre-

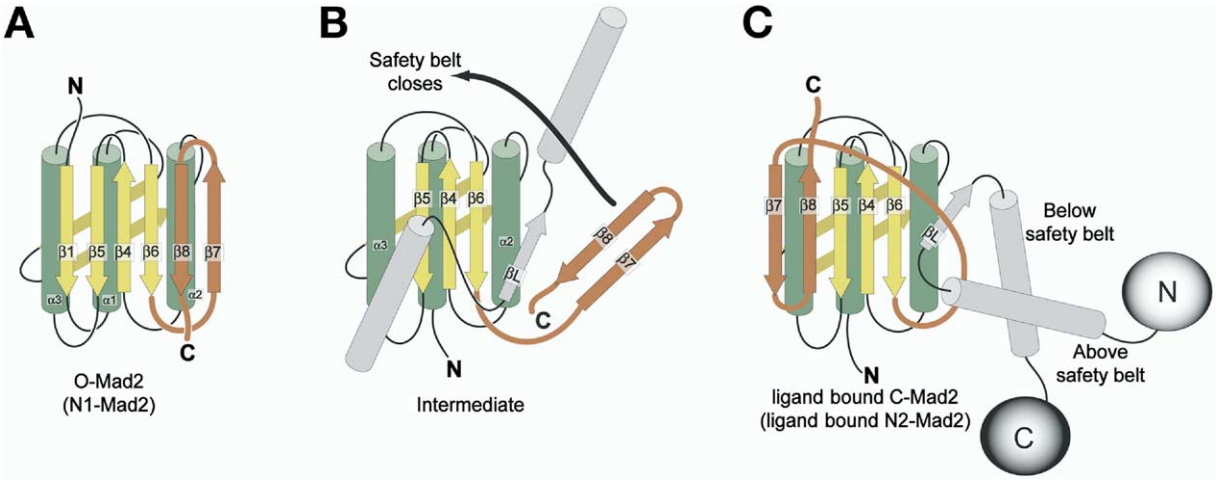

Figure 3. The Conformation of Mad2 before and after Binding Mad1 or Cdc20 Polypeptides

vents Mad2 from binding either Mad1 or Cdc20 (Luo et al., 2000). This mutation (Mad2$^{\Delta C}$) eliminates amino acids that are included in the β8 of Mad1:Mad2 complexes but does not substantially affect the β8 found in apo-Mad2. Similar changes in Mad2's structure were also observed upon its association with an artificial peptide known to compete for the binding of Mad1 or Cdc20 (Luo et al., 2002).

What is remarkable about the rearrangement of Mad2 is that the Mad1 polypeptide chain is threaded through the long loop that now connects Mad2's β6 and its new β7 (Figures 2 and 3). Because Mad1 contains extensive peptide sequences on both sides of the section enclosed by Mad2's new loop, Mad1 becomes topologically trapped by Mad2 in a manner that resembles the trapping of a car passenger by their safety seat belt. Because Mad1 and Cdc20 contain similar Mad2 binding motifs (Luo et al., 2002; Sironi et al., 2002), Cdc20 is presumably sequestered by Mad2 in a similar manner. Thus, Mad2 blocks Cdc20 function by trapping Cdc20's N-terminal peptide sequences in the same manner as it traps Mad1 at kinetochores. The key mystery is therefore how the locking of Mad1 within Mad2's safety belt at an unattached kinetochore facilitates the locking of Cdc20 within Mad2 in a similar if not identical manner. Both structural and biochemical data suggest that a single molecule of Mad2 can only trap a single polypeptide, be it Mad1 or Cdc20. Thus, a single Mad2 safety belt can accommodate only a single passenger at any one time. Furthermore, due to the topological nature of the association between Mad1 and Mad2, Mad1 passengers cannot readily be released from Mad2. They cannot therefore easily be replaced by Cdc20 polypeptide sequences.

To envision how Mad1:Mad2 complexes might facilitate the formation of equivalent Cdc20:Mad2 complexes, it is necessary to recall that Mad2 forms complexes with itself as well as with Mad1 or Cdc20. Could not Mad1 bind to one Mad2 subunit and thereby influence the probability that a second Mad2 subunit adopts a conformation compatible with Cdc20 binding? Note that a "Mad2 allosteric" model of this type would only work if Mad1 was considerably less abundant than Mad2 and therefore incapable of binding to Mad2's second subunit as well as its first. The simplest version of the allosteric model would suppose that Mad2 subunits adopt two different conformations, one capable of binding Mad1 and Cdc20 (the R state) and another that is not (the T state), and that Mad2 dimers composed of subunits with different conformations (Mad2R/Mad2T) are energetically unfavorable. Under these circumstances, binding of a single Mad1 subunit would favor the formation of Mad2R/Mad2R dimers, one of whose binding sites would be occupied by Mad1 while the other would be free for Cdc20 to bind. The fundamental problem with this model is that the Mad1:Mad2R/Mad2R:Cdc20 complexes formed through this conventional allosteric mechanism would remain stuck at kinetochores. Mad2 would not turn over at kinetochores and would never manage to sequester the entire population of Cdc20 in the cell. Mad1 at kinetochores must act catalytically and not stoichiometrically as proposed by the allosteric model.

Though a simple allosteric model seems implausible, the ability of Mad2 to oligomerize might still be crucial. The actual structure of Mad2 dimers would presumably be informative, but this has so far eluded both NMR and X-ray crystallography. Nevertheless, a recent series of biochemical experiments involving the analysis of Mad1:Mad2 complexes by gel filtration has provided key insights into the peculiar nature of Mad2 oligomers (De Antoni et al., 2005). The results imply that Mad2 dimers only form between Mad2 subunits that are in different conformations; that is, they form between a Mad2 subunit whose safety belt has "closed" (with β7 and β8 adjacent to the left side of β5), with or without a Mad1 or Cdc20 polypeptide topologically enclosed, and a Mad2 subunit whose safety belt is in a state ready to receive a passenger (with β7 and β8 adjacent to the right side of β6). These two states are referred to as C- and O-Mad2, respectively, though note that the safety belt is not strictly open in the O state but rather

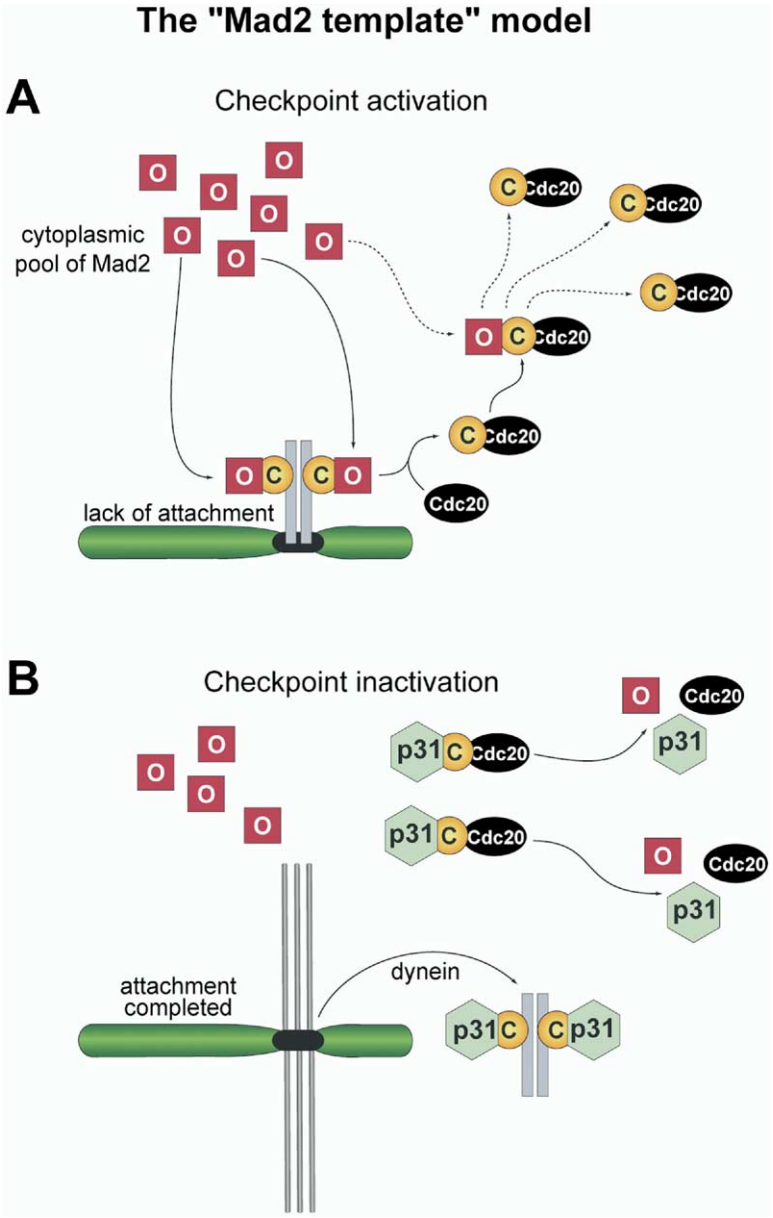

Figure 4. The Mad2 Template Model

(A) C-Mad2 that has trapped Mad1 associated with an unattached kinetochore binds O-Mad2, which in turn can trap Cdc20 and thereby generate free Cdc20:C-Mad2 complexes. The latter have the potential to catalyze their own production via a mechanism similar to their catalysis by Mad1:C-Mad2 complexes.

(B) p31^{comet} is postulated to inactivate the SAC by competing with O-Mad2 for binding to C-Mad2.

ready to be opened and subsequently shut (Figure 3). Thus, the addition to Mad2 dimers of a Cdc20 peptide causes the formation of Cdc20:Mad2 complexes whose Mad2 is exclusively in the C-Mad2 conformation and cannot therefore oligomerize due to Mad2:Mad2 interactions. C-Mad2 cannot bind to a second Mad2 molecule in the same closed state. Likewise, Mad2$^{\Delta C}$, which is incapable of adopting the closed conformation and is therefore locked in the O form, is incapable of forming oligomers and only forms Mad2 monomers. The implication is that O-Mad2 can only bind a second Mad2 molecule if the latter is in a closed conformation.

According to this "template model" (Figure 4A), a Mad1 polypeptide bound to an unattached kinetochore is trapped by a Mad2 protamer that will be in its C state and therefore capable of associating with a second Mad2 protamer as long as the latter is in its O state. Crucially, the O-Mad2 bound to Mad1:C-Mad2 complexes in this manner would be in a state capable of receiving Cdc20. To detect formation of such complexes in vitro, fluorescently labeled Mad2 (Alexa Mad2) was added to preassembled unlabeled Mad1:C-Mad2 complexes (De Antoni et al., 2005). After gel filtration, much of the Alexa Mad2 comigrated with Mad1:Mad2 complexes. The explanation is that the preassembled Mad1:Mad2 complexes contained C-Mad2 bound to Mad1 and O-Mad2 bound to the C-Mad2 and that the Alexa Mad2 displaced the unlabeled O-Mad2 but not the C-Mad2 bound to Mad1. Crucially, addition of a Cdc20 peptide to preassembled Mad1:Mad2 complexes completely failed to disrupt them; that is, the Cdc20 peptide could not displace Mad1 trapped by Mad2. However, the same peptide nevertheless caused the dissociation of most Alexa Mad2 from Mad1:

C-Mad2 complexes and led to the formation of Cdc20:Alexa C-Mad2 complexes. When the same experiment was repeated with Alexa Mad2$^{\Delta C}$ that is locked in the O conformation, then this O-Mad2 protein was also incorporated into Mad1:Mad2 complexes, but, unlike wild-type Mad2, the Alexa Mad2$^{\Delta C}$ protein could not be displaced from the Mad1:C-Mad2 complexes by Cdc20 peptides.

The implication is that Mad1:Mad2 complexes contain two types of Mad2 protamer: one that has trapped Mad1 and is in the C form and a second that is bound to the first but is in an O conformation capable of trapping a Cdc20 peptide. Importantly, once this second Mad2 traps Cdc20 and now adopts a C conformation, it drops off the Mad1:Mad2 complex. Mad2 bound to Mad1 is therefore capable of providing a landing pad for Mad2 protamers in their O conformation, which are in turn primed to bind Cdc20, but, once they do so, the Cdc20:C-Mad2 complex dissociates from the preexisting Mad1:Mad2 landing pad (Figure 4A). The C-Mad2 bound to Mad1 provides as it were a "template" that catalyzes the formation of what are essentially equivalent Cdc20:C-Mad2 complexes.

This template model appears consistent with most if not all the facts, but it is far from proven. What is most important is that the model provides an explanation for how Mad1 bound to kinetochores seeds the formation of Cdc20:Mad2 complexes in a manner that is catalytic and not merely stoichiometric. The model also explains why there are both slow- and fast-turning-over populations of Mad2 at unattached kinetochores. The former would be C-Mad2 that is interlocked with Mad1, while the latter would be the O-Mad2 that binds to the C-Mad2 and is subsequently released from the Mad1 bound to kinetochores due to Mad2's association with Cdc20. Note also that O-Mad2 may bind to C-Mad2 much less tightly than Mad1 trapped by a C-Mad2. The real beauty of the template model is that it no longer invokes mysterious unknown forces. The next step must be to solve the structure of Mad2 oligomers and thereby describe the mechanism by which O-Mad2 binds to C-Mad2. This will be necessary to explain why neither C-Mad2 nor O-Mad2 can bind to themselves.

Even without a structure of Mad2 dimers, it has nevertheless been possible to identify residues within Mad2 that are essential for its oligomerization but not for its ability to sequester either Mad1 or Cdc20 polypeptides. As already pointed out, mutation of an arginine at position 133 to alanine (Mad2^{R133A}) enabled the formation of Mad1:C-Mad2 crystals by compromising Mad2's ability to oligomerize. However, for clean biochemical experiments, it was necessary to create a Mad2 mutant that was also incapable of forming complexes with otherwise wild-type Mad2 molecules. This was achieved by mutating simultaneously arginine 133 to glutamic acid and an adjacent glutamine 134 to alanine (Mad2$^{R133E\ Q134A}$). Gel filtration experiments confirmed that Alexa-labeled Mad2$^{R133E\ Q134A}$ was both monomeric and incapable of being incorporated into preassembled Mad1:Mad2 complexes (De Antoni et al., 2005). Crucially, the template model predicts that this version of Mad2 should be defective in Mad2 function in vivo. To test this, the endogenous Mad2 of HeLa cells was knocked down by RNA interference (RNAi), and the ability of RNAi-resistant Mad2 variants to restore SAC function was tested. Such experiments suggested that Mad2$^{R133E\ Q134A}$ was indeed nonfunctional. I for one was not fully convinced by these experiments. The techniques for manipulating tissue culture cells using RNAi do not seem to be rigorous enough to establish these sorts of facts unambiguously. However, I am told that the equivalent mutation to Mad2^{R133A} has since been made in yeast (Mad2^{R126A}) and has indeed been found to be nonfunctional.

Another crucial prediction of the template model is that Mad2 incapable of trapping Mad1 or Cdc20, namely Mad2$^{\Delta C}$, should nevertheless be capable of being recruited to mitotic kinetochores that have already assembled Mad1:Mad2 complexes, while Mad2 incapable of forming oligomers, namely Mad2$^{R133E\ Q134A}$, should not be capable of being recruited to such kinetochores. This was tested by injecting Alexa-labeled variants of Mad2 into mitotic HeLa cells (De Antoni et al., 2005). As predicted, wild-type Mad2$^{\Delta C}$ associated with kinetochores of prometaphase cells as well as wild-type Mad2, but neither Mad2$^{R133E\ Q134A}$ nor Mad2$^{\Delta C\ R133E\ Q134A}$ associated with such kinetochores.

A fascinating aspect of the template hypothesis is that there is no reason that the principle by which Mad1:C-Mad2 complexes catalyze the formation of Cdc20:C-Mad2 complexes should not also apply to the generation of Cdc20:C-Mad2 complexes by preexisting Cdc20:C-Mad2 complexes. It is conceivable that the Cdc20:C-Mad2 complexes that drop off the Mad1:C-Mad2 complexes at unattached kinetochores start an autocatalytic reaction in which Cdc20:C-Mad2 complexes "breed" further Cdc20:C-Mad2 complexes. Such a phenomenon could explain one of the great mysteries of the SAC, namely the ability of a single unoccupied kinetochore to cause the sequestration of the entire pool of Cdc20 in inactive complexes with Mad2.

Another attractive feature of the template model is that it provides an explanation for how a protein called p31^{comet} facilitates the escape of cells from cell cycle arrest caused by the SAC. p31^{comet} appears to share several properties with O-Mad2, namely an ability to bind C-Mad2 but not O-Mad2. This raises the possibility that p31^{comet} inactivates the SAC by acting as a competitive inhibitor of O-Mad2. If so, p31^{comet} would bind to Mad1:Mad2 complexes assembled at kinetochores and prevent them from recruiting O-Mad2, thereby preventing their catalysis of Cdc20:C-Mad2 complex formation (Figure 4B). The structure of complexes between p31^{comet} and Mad2-C will clearly be illuminating.

It should be stressed that many features of the SAC remain deeply mysterious, not least the function of proteins like BubR1 and Bub3, which also form complexes with Cdc20. Future experiments will be required to test whether the binding of these proteins to Cdc20 somehow facilitates the ability of O-Mad2 to trap Cdc20. Also completely unexplained at the moment is how Mad1:Mad2 complexes are only recruited to kinetochores that are unoccupied by microtubules and have not come under tension. It is also quite unclear how the cell prevents O-Mad2 in the cytoplasm from sequestering Cdc20 without the intervention of Mad1 bound to unattached kinetochores or how the recruitment of

Cdc20 to kinetochores contributes to its sequestration by O-Mad2 bound to Mad1:C-Mad2 complexes. A hallmark of all important scientific breakthroughs is that they generate more ignorance than they do understanding, a principle that certainly seems to apply to the template model. The key point is that there exists for the first time a clear and testable hypothesis for one important cog in the SAC, namely the mechanism by which Mad1 might catalyze the formation of Cdc20:Mad2 complexes. This represents an important advance and provides a rigorous intellectual platform within which other aspects of the SAC can begin to be incorporated. It will be interesting to find out whether other proteins containing Mad2's HORMA domain will prove to share Mad2's remarkable properties.

In an age when a great deal of cant has been written about the coming of age of systems biology, the experiments from Musacchio's lab reveal the variety of experiments that are essential to understand complex biological control mechanisms in this day and age (De Antoni et al., 2005). Structural biology, genetics, hardcore biochemistry, and sophisticated imaging of tagged proteins in vivo are all required simply to begin to think clearly about sophisticated biological processes, and highly sophisticated experiments are required before one can begin to contemplate the utility of mathematical modeling. Future departments of systems biology might take note. The Mad2 work also demonstrates the futility of structural genomics, namely the solution of crystal structures without a parallel commitment to understanding their biology. No insight into the mechanism of the SAC could ever have emerged from structures of SAC proteins without in parallel a huge investment by the structural lab itself into sophisticated biochemistry and cell biology.

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