Recruitment of Mad2 to the Kinetochore Requires the Rod/Zw10 Complex

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Summary

Compromising the activity of the spindle checkpoint permits mitotic exit in the presence of unattached kinetochores and, consequently, greatly increases the rate of aneuploidy in the daughter cells [1–3]. The metazoan checkpoint mechanism is more complex than in yeast in that it requires additional proteins and activities besides the classical Mads and Bubs. Among these are Rod, Zw10, and Zwilch, components of a 700 Kdal complex (Rod/Zw10) [4–6] that is required for recruitment of dynein/dynactin to kinetochores [7, 8] but whose role in the checkpoint is poorly understood. The dynamics of Rod and Mad2, examined in different organisms, show intriguing similarities as well as apparent differences [7, 9] . Here we simultaneously follow GFP-Mad2 and RFP-Rod and find they are in fact closely associated throughout early mitosis. They accumulate simultaneously on kinetochores and are shed together along microtubule fibers after attachment. Their behavior and position within attached kinetochores is distinct from that of BubR1; Mad2 and Rod colocalize to the outermost kinetochore region (the corona), whereas BubR1 is slightly more interior. Moreover, Mad2, but not BubR1, Bub1, Bub3, or Mps1, requires Rod/Zw10 for its accumulation on unattached kinetochores. Rod/Zw10 thus contributes to checkpoint activation by promoting Mad2 recruitment and to checkpoint inactivation by recruiting dynein/dynactin that subsequently removes Mad2 from attached kinetochores.

Results and Discussion

Rod and Mad2 Colocalize throughout Prometaphase and Early Metaphase, but BubR1 Does Not

To gain insight into the role of Rod/Zw10 relative to other checkpoint proteins, we undertook a study of fluorescently tagged (GFP and mRFP1 [10]) Rod (CG1569), Mad2 (CG17498), and BubR1 (CG7838) in a single cell type, the *Drosophila* larval neuroblast. All three fusion proteins are controlled by their natural pro-

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motors, and all three retain their biological activity ([7]; Figure S1 and Table S1 in the Supplemental Data available with this article online; data not shown).

Consistent with earlier reports, Rod and BubR1 are cytoplasmic in interphase [7, 11, 12], whereas Mad2 is associated with the nucleoplasm and nuclear envelope [11, 13, 14] (Figure 1A). In fly neuroblasts, as in Hela cells [15] but unlike in PtK cells [11], BubR1 is the first to accumulate on kinetochores during prophase (at very low levels initially); it precedes Mad2 and Rod by 2–5 min (Movie S1). Mad2 and Rod begin to label kinetochores only during nuclear-envelope breakdown (NEB), easily recognized by the invasion of Rod into the nucleoplasm. The first kinetochore-associated Mad2 signals above the nucleoplasmic background are seen simultaneously with the first Rod signal (Figure 1A and Movie S2).

In prometaphase, the kinetochores brightly label with all three proteins (Figure 1B). Because cytoplasmic Mad2 signal is consistently higher than either BubR1 or Rod, Mad2 kinetochore labeling appears relatively less prominent. As the kinetochores capture MTs, Mad2 and Rod both are transported poleward (Figures 1B–1D; Movies S3 and S4; and Figures S2 and S4), again consistent with previous reports [7, 9]. This process, called "shedding," requires dynein/dynactin and may be important for shutting off the checkpoint once MTs are properly attached [7, 16–18].

These live images reveal a robustness that was not evident for Mad2 transport in earlier studies in PtK cells and *Drosophila* cells [9, 16, 19], although it can be seen sometimes even by immunostaining (Figure S5). It is difficult to quantify these signals, but the films clearly show that new cytosolic Mad2 is continuously recruited to kinetochores even after MT capture and replaces that lost to shedding; the total Mad2 signal on KMTs over the duration of prometaphase and metaphase is far greater than the original kinetochore-associated signal. This is particularly evident in Movie S4, where metaphase is prolonged. Thus Mad2, like Rod, establishes a flux of recruitment to and shedding from attached kinetochores.

GFP-Rod and RFP-Mad2 show a near-perfect coincidence of signal in prometaphase and early metaphase, not only on kinetochores but also along the KMTs (Figures 1C and 1D; Movies S3 and S4). The overall patterns of the two proteins are superimposable (Figures 1C and 1D, Figure S4). Where discrete particles of GFP-Mad2 could be followed, they always contained RFP-Rod (Figure 1D and Figure S4). These results suggest that Mad2 and Rod/Zw10 remain associated as they leave the kinetochore along the KMTs.

By late metaphase, Mad2 signal has essentially disappeared from kinetochores and is only faintly visible on the spindle above the cytoplasmic Mad2 background, whereas Rod shedding continues robustly up to anaphase onset (Figure 1C and Movie S3). In larval neuroblasts, the timing of NEB to anaphase onset is typically 7–12 min, of which metaphase lasts 2–8 min (see also [20]). There does not appear to be much delay

between Mad2 disappearance from the spindle and anaphase onset. On average, Mad2 is gone less than 1 min prior to anaphase (average is 35 s, range 0–2 min, n = 16), and sometimes just seconds before (compare Movies S3 and S5). This contrasts with the situation in PtK cells [9], where anaphase occurs on average 10 min after the disappearance of the last detectable Mad2 signal. The significance of this difference is for now unclear. It may reflect simply an adaptation to the very rapid mitosis in flies (7–12 min NEB-anaphase, compared to 25 min after alignment of the last chromosome for Ptk cells). Alternatively, it may reflect a more fundamental difference in the way the spindle checkpoint is turned off.

The behavior of Mad2 and Rod was distinguishable from that of BubR1 in several ways. BubR1 remained tightly associated with kinetochores and was not detectable along the spindle after MT capture (compare Mad2 and BubR1 in Figure 1B and Movies S1 and S2). Although in PtK cells BubR1 may be transported from kinetochores to poles after energy depletion [16], in normal fly neuroblasts shedding does not appear to be a major route by which BubR1 levels are reduced on attached kinetochores. Moreover, close inspection of in vivo double-labeled cells revealed that, as the metaphase plate develops, BubR1 becomes enriched in a kinetochore domain slightly internal to that of Rod and Mad2 (Figure 1E; Movie S1; see also Figure S3 and Table S2).

Rod/Zw10, dynein/dynactin, Mad2 and BubR1, and all the transient kinetochore proteins are normally classified as outer-domain kinetochore components [12, 18, 21], and indeed they all form enlarged crescents around the MT-free kinetochores [7, 18, 21, 22]. The outer domain can be further subdivided into a more interior "outer plate" which appears to be the MT attachment site as well as the location of BubR1 [21], and an outer fibrous corona that is believed to contain Rod/Zw10, dynein/dynactin, and CenpE [7, 23, 24]. The relative locations of the various checkpoint proteins have not been compared in attached kinetochores of living cells. Our observation that Mad2 colocalizes with Rod but not with BubR1 (Figure 1E; Figure S3 and Table S2) is to our knowledge the first demonstration that Mad2 is part of the corona.

The different locations of Mad2 and BubR1 are consistent with certain distinct features of their behavior. For example, Mad2 accumulation is highly sensitive to MT attachment and is depleted from kinetochores by shedding along KMTs. BubR1 by contrast is not depleted significantly by shedding and responds more to changes in tension (for example, [19]). If this correlation holds, perhaps other proteins with robust shedding (for example, CenpF [25]) will prove to colocalize in the corona with Mad2, Rod/Zw10, and dynein.

In summary, Mad2 and Rod/Zw10 behavior on kinetochores and spindles are qualitatively closely linked. They are simultaneously recruited and are shed together during prometaphase and early metaphase. BubR1, by contrast, is independently recruited to a different kinetochore domain and does not undergo detectable shedding.

**Mad2 Recruitment Is Severely Impaired
in *rod* and *zw10* Mutants**

To further probe the relationship of Mad2 and Rod/Zw10, we examined the behavior of GFP-Mad2 in *rod* and *zw10* null-mutant cells. Given the importance of dynein-dynactin for shedding [16, 17] and the role of Rod/Zw10 in dynein recruitment [8], we had anticipated that *rod* or *zw10* mutants would show abnormal retention of Mad2 on kinetochores. In fact, however, in these cells kinetochore-associated GFP-Mad2 was significantly reduced (Figure 2A, frames 3–5; Movie S6), although Mad2 was still prominent on interphase *rod* nuclei (Figure 2A, frame 9). The reduction of kinetochore-associated Mad2 was evident in every *rod* or zw10 mutant cell examined, although the extent of reduction was somewhat variable. In three of 15 *rod* cells (20%) filmed from NEB to anaphase onset, no kinetochore-associated Mad2 was detectable above the cytoplasmic background at any stage. In the rest, a weak signal was briefly detectable on some kinetochores during prometaphase. Quantitation of these signals revealed that the kinetochore intensity in *rod* cells was only about 20% above the cytoplasmic level, (range 0%–50%, n = 15) at their maximum, whereas in wild-type cells kinetochore Mad2 signals averaged 4.4-fold higher than cytoplasmic signals (range 2.5–8, n = 19). Depolymerizing microtubules with colchicine, which normally elevates kinetochore levels of checkpoint proteins, including Mad2 [22], did not increase Mad2 kinetochore signals in *rod* cells, (Figure 2A, frame 6). These observations indicated that Mad2 requires the Rod/Zw10 complex to achieve its normal levels on kinetochores. An earlier report did not find that inactivating Rod by antibody injection of Hela cells had any effect on Mad2 recruitment [5], although the antibody did block Rod recruitment at the kinetochore and did lead to premature mitotic exit. The discrepancy with our results may be due to the different methodologies employed.

We also examined several other checkpoint proteins in *rod* and *zw10* mutants (Figure 2B). BubR1 and Bub3 were still present, as had been reported before [26]. Mps1 and Bub1 were also unaffected by *rod* mutants (Figure 2B). Thus, the requirement for Rod/Zw10 seems to be specific to Mad2. By contrast, treatments that remove Mad2 from kinetochores in vertebrate cells have no effect on Rod/Zw10 [27].

It was possible that the failure of *rod* and *zw10* mutant cells to recruit Mad2 was caused by the premature degradation of cyclin B in these checkpoint-defective cells [4]; perhaps Mad2 cannot bind kinetochores when cyclinB/cdc2 kinase activity is low. To test this possibility, we examined Mad2 behavior in cells doubly mutant for *rod* and *ida*, the gene encoding APC5, a component of the APC/C. *ida* cells arrest in M phase with consistently elevated cyclin B [28]. The *ida* phenotype is epistatic to *rod*: i.e., *ida rod* double mutants do not exit mitosis, and they retain elevated cyclin B (Figure S6).

In *ida* cells, chromosomes are frequently found unattached to spindles [28], and Mad2 accumulation on kinetochores is therefore prominent even without colchicine (Figure 2A, frame 7). Significantly, in *ida rod* or *ida zw10* double mutants, Mad2 signal on kinetochores was greatly reduced, just as in *rod* or *zw10* mutants alone (Figure 2A, frame 8). This result argues that the Rod/Zw10 complex is physically required, directly or in-
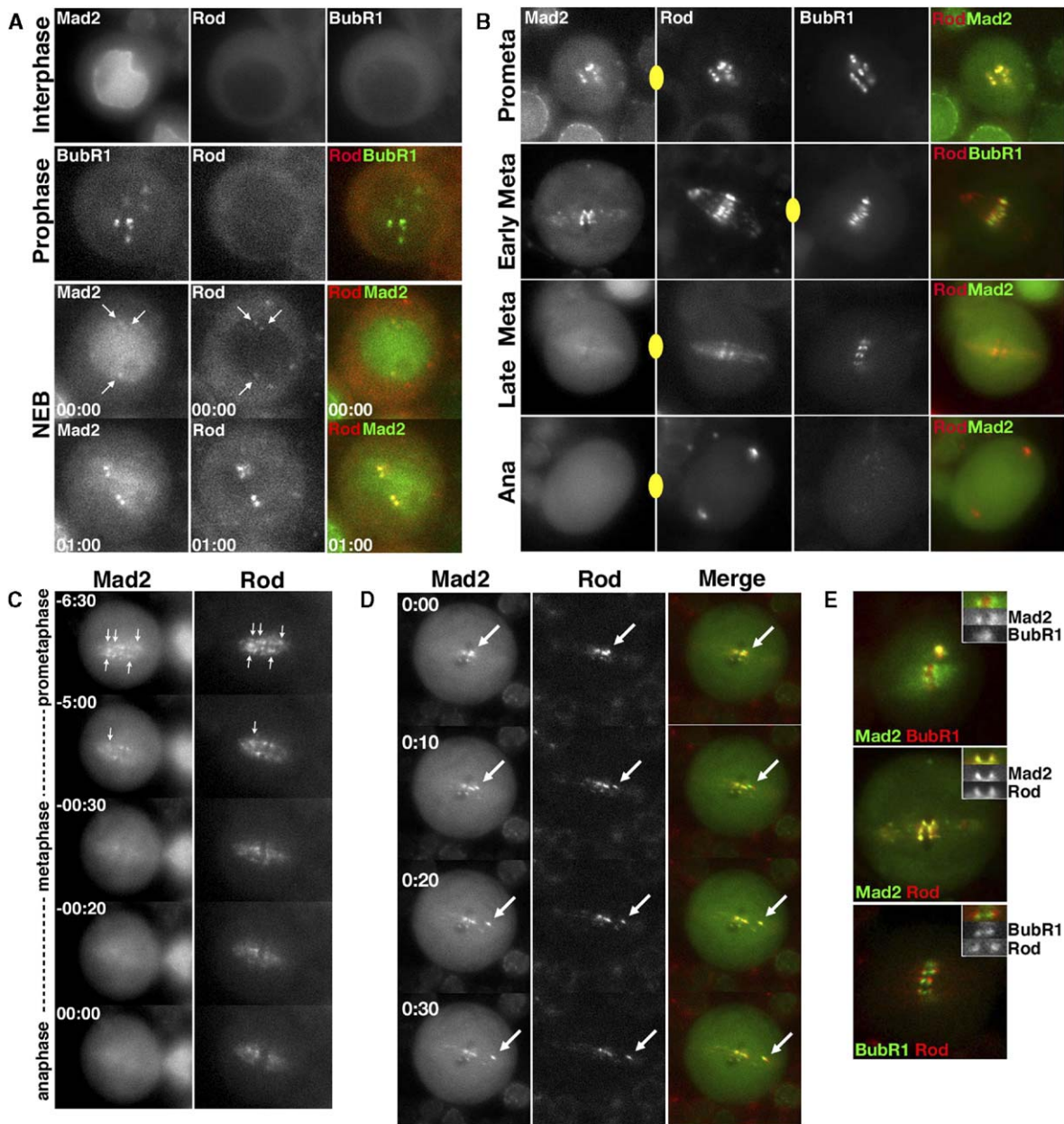
Figure 1. Comparison of In Vivo Behavior of Mad2, Rod, and BubR1

(A) Mad2 and Rod arrive nearly simultaneously on kinetochores, but after BubR1. *Top row*: BubR1 and Rod are cytoplasmic in interphase, whereas Mad2 is associated with the nuclear envelope. *Second row*: Recruitment of BubR1 to kinetochores precedes that of Rod. A single doubly labeled cell is shown in prophase. The first Rod kinetochore signal appears 2 min after BubR1 (see Movie S1). *Third and fourth rows*: Mad2 and Rod are simultaneously recruited to kinetochores. In the third row, the first time kinetochores are visibly associated with mRFP-Rod, they are also faintly labeled with GFP-Mad2 above the background of the nucleus (arrows). In the fourth row, the same cell is shown 1 min later, and four kinetochores now brightly label with both proteins. See Movie S2.

(B) The mitotic cycle of Mad2, Rod, and BubR1. In each set, two of the three images are of the same doubly labeled cell (frames connected by a yellow dot). See Movies S1, S2, and S3. *Prometaphase*: GFP-Mad2, RFP-Rod, and GFP-BubR1 all brightly label kinetochores. *Early metaphase*: Chromosomes have aligned (note the neat row of kinetochores). Mad2 and Rod are both shed from the kinetochores and travel along KMTs to the poles. Both proteins still have significant kinetochore association. Mad2 has a relatively high cytoplasmic component as well. BubR1 brightly labels kinetochores and does not display detectable shedding along the KMTs. *Late metaphase*: Mad2 is barely visible on the spindle above the level of cytoplasmic signal. Rod, by contrast, is still abundant on kinetochores and still sheds along the KMTs. BubR1 remains as discrete dots on the aligned kinetochores. *Anaphase*: As sister chromatids separate, Mad2 is now undetectable on kinetochores; BubR1 signal rapidly declines; and Rod remains bright.

(C) Rod and Mad2 colocalize along KMTs during prometaphase and metaphase. Time-lapse series of a GFP-Mad2 and mRFP1-Rod doubly labeled neuroblast from prophase through anaphase. Times are indicated as minutes:seconds from anaphase onset (00:00). There is a near perfect correspondence of signal between Mad2 and Rod along the KMTs (arrows), even as the relative level of Mad2 declines in late metaphase. See Movies S3 and S4 and Figure S4.
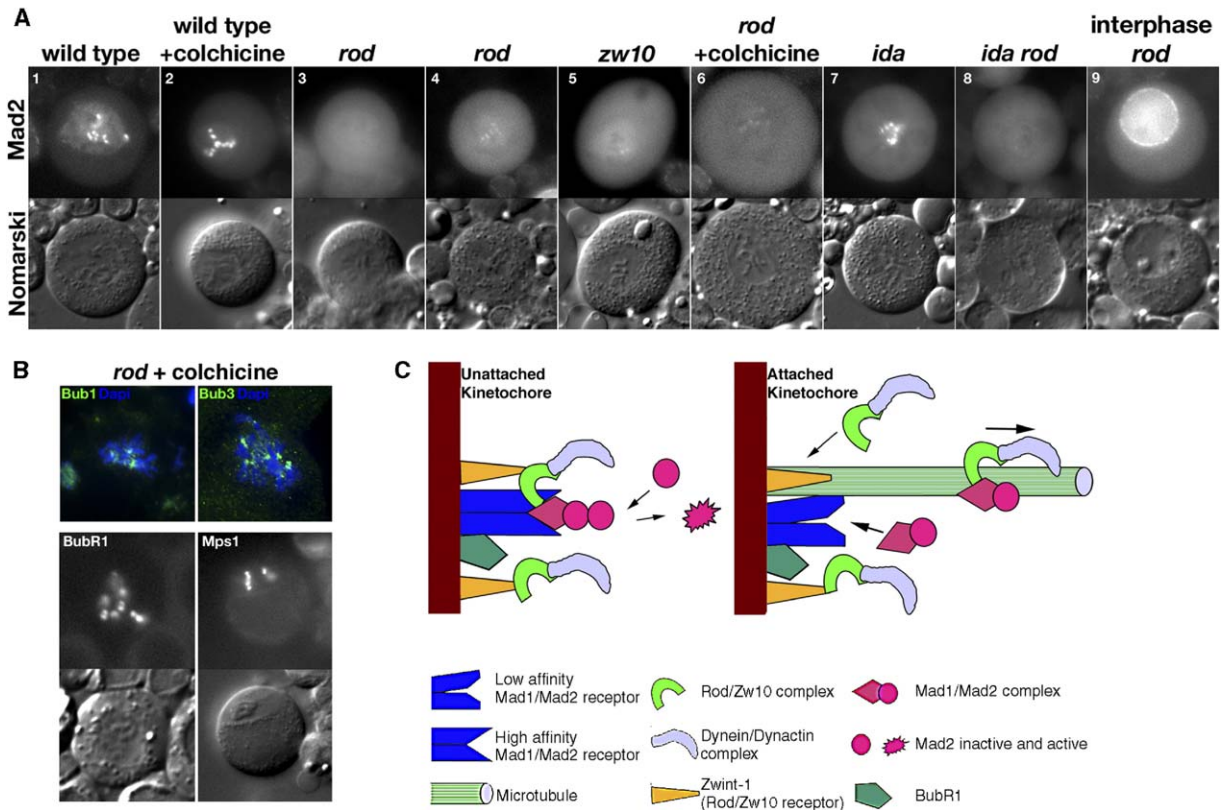
Figure 2. Rod/Zw10 Is Specifically Required for Mad2 Accumulation on Kinetochores

(A) Prometaphase (frame 1) or colchicine-treated (frame 2) wild-type neuroblasts have bright GFP-Mad2 signals on their kinetochores. In *rod* or *zw10* mutant cells (frames 3–5), little or no Mad2 is seen on the kinetochores or spindle, although Mad2 is still present in interphase nuclei (frame 9). Colchicine treatment does not increase Mad2 kinetochore accumulation (frame 6). Mad2 is unaffected in *ida* mutants alone (frame 7). In double mutants of *rod* (or *zw10,* not shown) and *ida* (which maintains elevated cyclin B levels, see Figure S6), Mad2 still fails to accumulate on kinetochores. Thus, premature degradation of cyclin B cannot explain the failure to recruit Mad2.

(B) Bub1, Bub3, BubR1, and Mps1 all bind to kinetochores in *rod* mutant cells. Bub1 and Bub3 are immunostained *rod* cells; BubR1 and Mps1 are GFP-tagged transgenes expressed in *rod* cells.

(C) A model for the behavior of Rod/Zw10 and Mad2 at the kinetochore before and after MT capture. Before attachment (left), Rod/Zw10 binds to its receptor (Zwint-1 [42]) and brings the dynein/dynactin complex with it. Mad1/Mad2 arrives independently to kinetochores, but Rod/Zw10 binds (directly or indirectly) to it and promotes its stable association with its kinetochore receptor (possibly composed of Ndc80, Bub1, and other components). The bound Mad1/Mad2 complex then promotes the activation of free Mad2 [14], which is capable of inhibiting the APC/C. Once MTs attach (right), the affinity of the Mad1/Mad2 receptor is reduced. Rod/Zw10 and the Mad1/Mad2 are simultaneously shed, as dynein/dynactin pulls them off together along the KMTs. At the same time, new Rod/Zw10 and new Mad1/Mad2 arrive at the attached kinetochore, but Mad1/Mad2 is immediately shed again as long as proper KMT linkages are maintained. If a kinetochore partially detaches, the continuous recruitment of Mad1/Mad2 assures that the anaphase inhibitor can be rapidly regenerated. Mad1/Mad2 levels decline to zero on properly attached kinetochores, whereas Rod/Zw10 levels do not because they use different receptors, with different responses to KMT binding.

directly, for normal Mad2 accumulation on kinetochores.

We have shown that many aspects of Mad2 behavior are intimately associated with the Rod/Zw10 complex. Rod/Zw10 accompanies Mad2 as it accumulates on unattached kinetochores and as it leaves kinetochores after MT attachment, and in the absence of Rod/Zw10, little or no Mad2 accumulates on kinetochores. Given that Rod/Zw10 is also required for dynein/dynactin recruitment, which removes Mad2 from attached kinetochores, one can say that the entire kinetochore cycle of Mad2 depends, directly or indirectly, on Rod/Zw10. The checkpoint defect of *rod* and *zw10* mutants is now presumably explained by this failure to recruit Mad2.

(D) Mad2 and Rod comigrate in individual particles along the KMTs. A single particle containing both mRFP-Rod and GFP-Mad2 is shed from an aligned chromosome and migrates toward the pole (arrow). The speed (approximately 8 μm/min) is similar to that measured for Rod in embryonic spindles [7]. The slight separation in green and red signals (evident in the second and fourth frames of the merged image) probably reflects the 2.5 s time delay in acquisition of the two signals. See Figure S4.

(E) BubR1 in attached kinetochores is spatially distinct from Mad2 and Rod. The image shows doubly labeled cells (as indicated) during metaphase. Insets: Higher magnification of the topmost kinetochore pair in each cell. BubR1 is consistently internal to the Mad2 and Rod signals. See also Figure S3 and Table S2.

These results suggest that Rod/Zw10 is physically interacting with a complex containing Mad2 (or Mad1, see below) throughout mitosis. However, two-hybrid screening, immunoaffinity columns [6, 8], and coimmunoprecipitation experiments (our data not shown) have not revealed any interaction between Rod/Zw10 and Mad1 or Mad2. Thus, unlike dynein/dynactin, Mad1/Mad2 may be binding only indirectly to Rod/Zw10, perhaps via an unknown protein. Alternatively, there may be direct interactions between Rod/Zw10 and Mad1/Mad2, but only under native conditions on intact kinetochores. We have summarized our findings and some speculations in a model shown in Figure 2C; Mad1/Mad2 binding sites are depicted as comprising multiple components whose affinity for Mad1/Mad2 can be enhanced by the Rod/Zw10 complex and reduced by MT capture during spindle assembly. MT capture also leads to depletion of Mad1/Mad2 by another route, as it is dragged off the kinetochores (along with Rod/Zw10) by dynein-mediated transport.

Kinetochore recruitment of Mad2 initially occurs as part of a complex with Mad1, to which it is tightly bound even in interphase [29, 30]. The Mad1/Mad2 complex is relatively stable at unattached kinetochores [11, 14], but a second Mad2 population, which depends on the first, turns over rapidly and presumably becomes an activated form, the "wait anaphase" signal [14, 31]. Once MTs have attached, however, the Mad1/Mad2 complex is rapidly depleted, at least partially by dynein-mediated shedding along KMTs [11, 14], and this is believed to be part of the mechanism that extinguishes the checkpoint signal [16, 17]. It is therefore likely that the Rod/Zw10 complex is exerting its effect on the Mad1/Mad2 complex and not on Mad2 alone. Recent work in Hela cells supports this contention by showing that depletion of Zw10 by RNAi reduces both Mad1 and Mad2 recruitment to unattached kinetochores [32].

It is unclear what kinetochore components constitute the Mad1/Mad2 "binding site." The hierarchy of kinetochore assembly has been studied in several model systems, not always with consistent results. However, it appears that the Ndc80 complex [33–38], Bub1 [39, 40], and Mps1 kinase activity [27, 41] are required for the subsequent assembly of Mad1/Mad2 on kinetochores. Conversely, interfering with Mps1 [27] or the Ndc80 complex in Hela cells has no effect on Rod or dynein recruitment [33, 34, 36, 37], and *rod* and *zw10* mutants have no effect on BubR1, Bub3 [26], Cenp-Meta (the fly homolog of CenpE) [6], Bub1, or Mps1 (this study), nor in all likelihood on the Ndc80 complex (in *rod* mutants, chromosomes are efficiently captured by MTs and congress). Thus Rod/Zw10, with Ndc80 complex, Bub1, and Mps1, all contribute to Mad2 kinetochore recruitment. The role of Rod/Zw10 may be to enhance the affinity of Mad1/Mad2 for its binding site (because some Mad2 binds even in *rod* mutants), increasing its stability on kinetochores prior to MT capture, perhaps by interacting with Ndc80 complex.

Our results also demonstrate that, just like Rod/Zw10 [7], Mad1/Mad2 is continuously recruited to and then released from kinetochores, even following MT capture (Figure 1C and Movies S3 and S4), and only disappears from spindles just prior to anaphase onset. This differs significantly from the behavior reported in vertebrate cells, in which MT capture appears to shut off new Mad2 recruitment [16]. The difference need not conflict with the basic model in which kinetochore Mad2 generates the anaphase inhibitor. In both cases, there is a rapid decline, perhaps below a critical threshold, in the net steady-state abundance of Mad2 on attached kinetochores. Alternatively, MT capture may render the remaining kinetochore-associated Mad2 inactive. Perhaps the difference is in the rate of Mad2 recruitment in the two cell types. Even in PtK cells there is some evidence that Mad2 is capable of recruitment to attached kinetochores: If dynein activity (and therefore shedding) is blocked after chromosome alignment, Mad2 eventually reaccumulates at attached kinetochores [16], suggesting that prior to dynein inhibition, Mad2 was being recruited and immediately shed from these kinetochores. This continuous recruitment of Mad1/Mad2 to attached kinetochores may ensure that it will always be available to begin generating anaphase inhibitor should one or more MTs inadvertently detach. At the same time, the continued presence of Rod/Zw10 ensures the dynein levels required both to remove unneeded Mad1/Mad2 and, later, to power anaphase movement.

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