CENP-I and Aurora B act as a molecular switch that ties RZZ/Mad1 recruitment to kinetochore attachment status

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The RZZ (Rod, ZW10, and Zwilch) complex and Mad1 proteins tightly associate with kinetochores to generate the spindle checkpoint signal, but they are released when a kinetochore forms mature microtubule attachments. Here we demonstrate that the centromere protein CENP-I is required to generate a stable association of RZZ and Mad1 with kinetochores. CENP-I also inhibits their removal by dynein stripping. This regulation of Mad1 and RZZ dissociation functions independently of Aurora B, which regulates their association. We show that the microtubule status of each kinetochore independently dictates the recruitment of Aurora B kinase, kinase activity on a kinetochore substrate, and loading of spindle checkpoint proteins. This dynamic regulation of Mad1 association by Aurora B is only uncovered when CENP-I is depleted, consistent with our finding that CENP-I inhibits the dissociation of Mad1. We conclude that the dual activities of Aurora B and CENP-I generate a molecular switch that maintains a robust spindle checkpoint signal at prometaphase kinetochores until they attain mature attachments to microtubules.

Introduction

The faithful segregation of genetic material during mitosis is critical to safeguard genomic integrity. Defects in this process lead to aneuploidy and cell death and are hypothesized to contribute to cancer development (Rieder and Maiato, 2004; Bharadwaj and Yu, 2004; Kops et al., 2005b). Chromosome segregation depends on kinetochores, large mitosis-specific structures that form on centromeres and make stable attachments to spindle microtubules (Santaguida and Musacchio, 2009; Kops et al., 2010). The spindle checkpoint signal is generated by kinetochores and inhibits mitotic progression until all kinetochores have attachments to the spindle (Musacchio and Salmon, 2007; Burke and Stukenberg, 2008). A single unattached kinetochore is sufficient to generate a mitotic arrest, but the mechanisms that initiate the signal at unattached kinetochores and ensure that it is strong enough to arrest cell cycle progression are incompletely understood.

Spindle checkpoint signaling involves the recruitment of mitotic arrest-deficient (Mad) and budding uninhibited by benzimidazoles (Bub) protein family members to kinetochores (Hoyt et al., 1991; Li and Murray, 1991; Gorbsky et al., 1998; Howell et al., 2004). The key effector of the spindle checkpoint is a complex of Mad1 and Mad2. Elegant structural and biophysical studies have demonstrated that Mad2 can exist in an active closed form (Mad2-c) and an inactive open form (Mad2-o; De Antoni et al., 2005). A dimer of Mad1 is recruited to kinetochores bound to Mad2-c. Once at kinetochores the Mad1–Mad2-c can catalyze the formation of soluble Mad2-o to Mad2-c, which generates a signal that inhibits the anaphase promoting complex, stabilizing important cell cycle substrates including cyclin B and securin (Murray and Kirschner, 1989; Li and Murray, 1991; Yamamoto et al., 1996a,b; Zou et al., 1999).

How kinetochores recruit the Mad1–Mad2-c proteins remains an area of active research. The direct binding site of the Mad1 protein is not known but a complex series of dependencies have been identified. Mad1 recruitment requires Bub1, Bub3, and BubR1 (Chen, 2002). These Bub proteins directly bind the kinetochore protein Knl1 on MELT repeats after they are phosphorylated by Mps1 (Krenn et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). Knl1 also recruits the Zwint protein,
which is required to recruit the RZZ complex (Kiyomitsu et al., 2007). The RZZ complex is composed of Rod, ZW10, and Zwilch and these three proteins have a second role in recruiting the minus end–directed motor cytoplasmic dynein (Basto et al., 2000; Chan et al., 2000). The Ndc80 complex is also required for Mad1 recruitment (Martin-Lluesma et al., 2002; McCleland et al., 2004). Once chromosomes begin to align to the metaphase plate, Mad1 is stripped from kinetochores by dynein (Howell et al., 2001).

The mitotic serine/threonine kinase Aurora B has been proposed to be at the top of a signaling cascade that regulates Mad1 recruitment. Aurora B acts as part of the chromosome passenger complex and directly phosphorylates proteins within the kinetochore (Vader et al., 2006; Santaguida et al., 2011). Aurora B is required for a spindle checkpoint arrest generated by Taxol, and Aurora B inhibitors prevent Mad1 recruitment to kinetochores of prometaphase cells (Ditchfield et al., 2003; Hauf et al., 2003). Aurora B activity is required to localize Bub1 and BubR1 to kinetochores, and this is at least in part through recruitment of the Mps1 kinase (van der Waal et al., 2012). Aurora B also phosphorylates the protein Zwint to generate a binding site for RZZ to recruit Mad1 and dynein (Wang et al., 2004; Kasuboski et al., 2011).

Surprisingly, cells in microtubule-depolymerizing drugs generate a functional spindle checkpoint in the presence of Aurora kinase inhibitors, though not after injection of function-blocking antibodies (Kallio et al., 2002; Ditchfield et al., 2003; Hauf et al., 2003). Because the inhibitors reduce but do not eliminate kinase activity the current model is that a small amount of Aurora kinase activity generates the spindle checkpoint signal in nocodazole (Santaguida et al., 2011). We previously performed a genetic screen to understand how cells arrest in mitosis with compromised Aurora B activity (Matson et al., 2012). We demonstrated that a complex containing the centromere protein CENP-I is required to signal the spindle checkpoint if Aurora B activity is compromised and that this role is conserved from yeast to humans. CENP-I can also regulate the dynamics of microtubules in the kinetochore after they generate mature attachments requiring Ndc80 (Amaro et al., 2010). However, it is unclear how CENP-I generates the spindle checkpoint signal after inhibition of Aurora B activity.

Unaligned kinetochores nucleate and bundle microtubules into a distinct class of short spindle microtubules known as preformed kinetochore fibers (PreK-fibers; Khodjakov et al., 2003; Tulu et al., 2006; Mishra et al., 2010). These bundles remain closely associated around kinetochores and are distinct from the ordered kinetochore fiber (K-fiber) microtubules that form mature attachments with the Ndc80 complex and facilitate chromosome movements. PreK-fibers exist before K-fibers and have important roles in ensuring the rapid attachment of kinetochores to spindle poles. Recent work out of our laboratory showed they can also recruit additional Aurora B to inner centromeres (Banerjee et al., 2014). Here we demonstrate that CENP-I regulates the dissociation of RZZ and Mad1 from kinetochores, whereas Aurora B dynamically regulates their association rates. CENP-I stabilizes Mad1 at kinetochores by extending its half-life and by inhibiting dynein-mediated stripping of Mad1. This stabilizing activity is required to maintain RZZ and Mad1 at kinetochores with low Aurora B activity and it ensures that a signaling kinetochore recruits a saturating amount of Mad1.

We recently demonstrated that microtubules can regulate Aurora B localization and activity in prometaphase (Banerjee et al., 2014). Here we extend this observation by showing that microtubule stimulation of Aurora B dynamically regulates the association of Mad1 at kinetochores. In addition, we show that the signal generated by microtubule stimulation of Aurora B is contained to a single kinetochore and adjacent kinetochores in the same cell can have distinct signaling events (chromosome autonomy). Our results lead to a model in which Aurora B activity is responsible for the recruitment of RZZ and Mad1 to kinetochores (Fig. 1 A). CENP-I then stabilizes these proteins at kinetochores by greatly enhancing their half-lives and by inhibiting dynein-mediated stripping until mature kinetochore microtubule attachments are formed. Together the local activities of CENP-I and the microtubule stimulation of Aurora B generate a molecular switch that underlies the chromosome autonomous nature of spindle checkpoint signaling.

Results

Kinetochore structure is not dramatically altered 48 h after CENP-I depletion

We examined the levels of a large set of proteins after depleting CENP-I from HeLa cells for 48 h to estimate the overall effect on kinetochore structure. CENP-I was depleted to <5% of control levels by siRNA (Fig. S1, A and B). We did not note any effect on gross chromatin morphology or chromosome structure after CENP-I depletion, although there was an increased number of prometaphase cells after CENP-I depletion as has been shown previously (Fig. S1 C; Liu et al., 2003; Amaro et al., 2010). CENP-I–depleted kinetochores retained CENP-A, Mis12, Knl1, CENP-C, Zwint, Rod, Mps1, Aurora B, P150, dynein, and CENP-F (Fig. S1 D; Matson et al., 2012). However, CENP-I depletion reduced CENP-H, -K, -O, and -P and ∼50% of Hec1 from kinetochores (Matson et al., 2012). Similar results were reported previously after depletion of CENP-H or CENP-K, which are binding partners of CENP-I (Cheeseman and Desai, 2008; Amaro et al., 2010). It is important to not deplete CENP-I for longer than 48 h to avoid the additional depletion of CENP-A, so all experiments are performed at a 48-h time point (Liu et al., 2003, 2006; Okada et al., 2006).

Aurora B activity and CENP-I cooperate to recruit and maintain RZZ and Mad1 at unattached kinetochores

CENP-I is required to send a checkpoint signal in the presence of low Aurora B activity (Matson et al., 2012). We tested whether HeLa cells could recruit the spindle checkpoint proteins RZZ, Mad1, Mad2, and BubR1 to kinetochores depleted of microtubules after Aurora B inhibition, CENP-I depletion, or both. Control or CENP-I–depleted cells were synchronized in S-phase by double thymidine block (Fig. 1 B). The cells were then released and, while still in G2, they were treated with nocodazole to depolymerize microtubules and the proteasome inhibitor MG132 to
inhibit precocious mitotic exit. In addition the cells were treated with either the Aurora B inhibitor ZM447439 (ZM) or DMSO as a control. After the cells entered mitosis they were fixed and processed for immunofluorescence to visualize the localization of checkpoint proteins. Control, ZM-treated, and CENP-I–depleted cells all recruited nearly identical levels of Mad1 to unattached kinetochores (Fig. 1, B and D). However, cells depleted of both Aurora B activity and CENP-I had greatly reduced levels of kinetochore-bound Mad1. The RZZ complex protein ZW10 also required either Aurora B activity or CENP-I to localize to kinetochores in nocodazole (Fig. 1, C and D).

We note that the retention of Mad1 at kinetochores treated with Aurora inhibitors in nocodazole is in apparent contradiction to a previous study (Santaguida et al., 2011). However, the author’s overall conclusion that Aurora B is at the top of a signaling cascade that recruits the checkpoint proteins is supported by our findings.

To determine if CENP-I and/or Aurora B activity maintain Mad1 and ZW10 at kinetochores after they are loaded, CENP-I–depleted cells were prearrested in nocodazole for 2 h and then treated with Aurora B inhibitors and MG132 (Fig. S2 A). Cells depleted of either Aurora B activity or CENP-I recruited similar levels of Mad1 and ZW10 to kinetochores as controls (Fig. S2, B and C). However, CENP-I–depleted cells lost virtually all of their Mad1 and ZW10 from kinetochores after 1 h of ZM treatment. Interestingly, Mad1 and ZW10 staining was not dispersed in these cells. Instead they were found on large structures that had completely departed from the kinetochore but seemed to remain stable and in the vicinity of the chromatin for the duration of the experiment (Fig. S2, B and C). These structures also contained Mad2, but not BubR1, whose localization was dependent on Aurora B activity regardless of CENP-I status, as previously reported (Fig. S2, D and E; Ditchfield et al., 2003; Hauf et al., 2003). The structures could also be identified when CENP-I–depleted cells were treated with the structurally distinct Aurora B inhibitor Hesperadin (Fig. S3, A and B; Hauf et al., 2003). We also verified that Mad1 was in these structures using an alternative antibody against Mad1 and cells expressing GFP-Mad1, and we identified the structures using both U20S and 293T cells (Fig. S3, C–G). We conclude that when Aurora B activity is inhibited CENP-I is required to establish and maintain RZZ, Mad1, and Mad2 at kinetochores.

**CENP-I is required for the slow dissociation rate of Mad1 at unattached kinetochores**

There are two pools of Mad1 at unattached kinetochores in PTK2 cells. There is a highly dynamic pool (half-life of \( \sim 12 \text{ s} \)) and a stable pool that has a half-life >15 min (Howell et al., 2004; Shah et al., 2004). We used FRAP to measure the half-life of Mad1 at kinetochores of HeLa cells after Aurora B inhibition or after depletion of CENP-I. Control cells transiently expressing or ZW10 are indicated by yellow arrows. Error bars indicate standard deviation. *, \( P < 0.00005 \). A.U., arbitrary units. Bars: (white) 5 \( \mu \text{m} \); (yellow) 1 \( \mu \text{m} \).
CENP-I–depleted cells than it does in controls. We conclude that CENP-I is required to generate a stable population of Mad1 at unattached kinetochores.

CENP-I–depleted cells rapidly lose Mad1 from kinetochores in the presence of microtubules. Up to this point our experiments had been performed in nocodazole, but Mad1 is reported to be absent in CENP-I–depleted cells when microtubules are present, including during early mitosis (Liu et al., 2003; Matson et al., 2012). We measured the rate that Mad1 is lost from CENP-I–depleted kinetochores after exposure to microtubules. Cells were washed out of nocodazole to allow microtubule polymerization and fixed for immunofluorescence, and the amount of Mad1 at kinetochores was quantified at 4-min time points after washout (Fig. 3, A–C).

Control cells retained Mad1 at most kinetochores 16 min after nocodazole washout even though bipolar spindles had formed. Loss of Mad1 from kinetochores of control cells was only obvious after 20 min, when strong microtubule bundles consistent

GFP-Mad1 in nocodazole had a highly stable pool of Mad1 that did not recover over the course of the experiment and a dynamic pool, although the dynamic pool of Mad1 in HeLa cells appears approximately twice as large as in PTK2 cells (Fig. 2, A–C; Shah et al., 2004). Inhibiting Aurora B activity did not significantly affect Mad1 recovery, suggesting that high Aurora B activity is not required to retain the stable pool of Mad1 at unattached kinetochores. CENP-I–depleted cells had a larger pool of dynamic Mad1 at kinetochores compared with controls, although the half-life of the dynamic pool did not significantly change (Fig. 2 D). Moreover, the stable population of Mad1 displayed a steady rate of recovery in CENP-I–depleted cells. Consistent with previous analyses we found that Mad1 recovery followed biphasic kinetics that were best fit using the sum of two exponentials (Fig. 2 D; Howell et al., 2004). In control cells the second phase of recovery was extremely slow with a half-life of 145 min, reflecting the remarkably stable nature of this population. However, the slow phase of recovery had a half-life of only 4 min in CENP-I–depleted cells. Thus, the population of stable Mad1 at kinetochores turns over ∼36 times faster in CENP-I–depleted cells than it does in controls. We conclude that CENP-I is required to generate a stable population of Mad1 at unattached kinetochores.

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Dynein prematurely strips Mad1 from kinetochores in the presence of microtubules. The disappearance of Mad1 from kinetochores of CENP-I–depleted cells and its appearance at spindle poles is consistent with dynein-dependent movements. Dynein does not normally strip RZZ and Mad1 from kinetochores to silence the spindle checkpoint until kinetochores form mature attachments to spindle microtubules (Howell et al., 2001). However, the rapid kinetics of Mad1 loss from unaligned kinetochores in CENP-I–depleted cells suggested that dynein stripping was occurring before proper kinetochore attachments had formed.

To test if CENP-I prevents the premature stripping of Mad1 by dynein we washed cells out of nocodazole to synchronize the stripping of Mad1 from kinetochores and inhibited dynein activity with K-fibers appeared. In contrast, Mad1 was lost from most kinetochores of CENP-I–depleted cells 8 min after nocodazole washout and was virtually unobservable after 12 min. Interestingly, at 12 and 16 min many CENP-I–depleted cells had lost Mad1 from kinetochores and this correlated with the accumulation of Mad1 at microtubule foci that are most likely forming spindle poles. When cells were washed out of nocodazole in the presence of ZM Mad1 was retained at kinetochores for up to 20 min (Fig. 3 C). However, we found that ZM treatment also resulted in a slowed rate of microtubule polymerization after nocodazole washout compared with controls and CENP-I–depleted cells (Fig. S3 H). We conclude that the dissociation rate of Mad1 exceeds the association rate in CENP-I–depleted cells when microtubules are present, resulting in the premature dissociation of Mad1 from kinetochores.
by expressing CC1-GFP. This CC1 fragment of P150/Dynactin inhibits dynein walking activity without affecting dynein localization (Quintyne et al., 1999). Mad1 remained at kinetochores of control cells 10 min after nocodazole washout (Fig. 4 A). In contrast, Mad1 staining was lost from kinetochores and could be visualized at spindle poles in CENP-I–depleted cells. After dynein inhibition both control and CENP-I–depleted cells retained Mad1 at kinetochores after nocodazole washout (Fig. 4, A–C). We conclude that a function of CENP-I is to prevent dynein-mediated stripping of Mad1 at kinetochores that have lateral attachments with microtubules but have not formed mature attachments to the spindle. Furthermore, dynein stripping does not normally carry sufficient Mad1 to poles for it to be localized as discrete foci. Our ability to localize Mad1 to poles in CENP-I–depleted cells is consistent with an increased flow of Mad1 from kinetochores caused by increased dynein loading rates and poor retention of Mad1 at kinetochores.

Development of an assay to visualize the association dynamics of spindle checkpoint proteins

Our data demonstrate that CENP-I regulates the dissociation of Mad1 from kinetochores by increasing its half-life and inhibiting premature stripping by dynein. However, we did not detect a role for Aurora B activity in stabilizing existing Mad1 at unattached kinetochores (Fig. 2). This is somewhat surprising because Aurora B activity and CENP-I can each localize RZZ and Mad1 to unattached kinetochores independently of each other (Fig. 1). One explanation is that there are two pathways that can independently recruit RZZ and Mad1: one pathway that requires Aurora B activity and another pathway that requires CENP-I. An alternative and simpler model is that Aurora B activity regulates the association kinetics of RZZ and Mad1 to kinetochores whereas CENP-I regulates their dissociation rates. Because the dissociation rate of stable Mad1 from kinetochores is essentially zero (Fig. 2), even weak Aurora B activity would eventually saturate the kinetochore binding sites. We strongly favor this model where Aurora B regulates the association of Mad1 and CENP-I inhibits its dissociation for three reasons. First, when cells are injected with function-blocking antibodies against Aurora B they lose checkpoint activity even though CENP-I is present, arguing for a single loading pathway (Kallio et al., 2002). Consistent with the idea that low levels of Aurora activity are sufficient to generate a spindle checkpoint signal there is residual Aurora activity in cells treated with Aurora inhibitors and Ipl1 mutants in budding yeast can be rescued through inhibition of PP1 phosphatase (Francisco and Chan, 1994; Santaguida et al., 2010). Second, Aurora B phosphorylates Zwint to drive loading of RZZ, Mad1, and other outer kinetochore proteins, which provides for a direct role for Aurora B in Mad1 recruitment (Kasuboski et al., 2011). Third, our data show that CENP-I regulates two different dissociation reactions: the half-life of Mad1 is reduced as measured by FRAP and dynein can prematurely strip spindle checkpoint proteins in the absence of CENP-I.

We designed a test to determine if Aurora B activity controls the recruitment of RZZ and Mad1 to kinetochores and if the CENP-I pathway regulates their dissociation. Our assay is based on three recent findings. First, recent work in our laboratory showed that Aurora B localization and activity is stimulated by PreK-fibers (Banerjee et al., 2014). Second, in our nocodazole

Figure 4. CENP-I-depleted kinetochores fail to inhibit dynein-mediated stripping of Mad1. (A) Immunofluorescence images of Mad1 in control and CENP-I–depleted cells 10 min after nocodazole washout, with or without expression of the dynein inhibitor CC1. Control cells retain Mad1 at kinetochores after nocodazole washout, but CENP-I–depleted cells rapidly lose Mad1 from kinetochores and accumulate it at spindle poles in a dynein-dependent manner. (B) Quantification of the total number of Mad1–positive kinetochores in cells from conditions depicted in A. (C) Immunofluorescence images of CENP-I–depleted cells demonstrating that inhibition of dynein does not prevent recruitment of Mad1 to unattached kinetochores, but does prevent loss of Mad1 from kinetochores after nocodazole washout. Centromeres are labeled to demonstrate that Mad1 is at kinetochores. Blue arrows indicate position of spindle poles. Cy5–labeled anti-Mad1 antibody is displayed here in green for ease of viewing. Error bars indicate standard deviation. *, P < 10^{-7}; **, P < 10^{-3}. Bars, 5 µm.
Microtubules at kinetochores recruit Aurora B to centromeres to phosphorylate kinetochores

We first asked whether Aurora B was specifically enriched at kinetochores with associated microtubules during spindle formation after nocodazole washout in unperturbed cells. We found that kinetochores with microtubules (either as small foci or connected to forming poles) had levels of centromere Aurora B that were almost three times higher than kinetochores without detectable microtubules (Fig. 5, A and B; and Fig. S4 B). In addition, the mean Aurora B activity across all centromeres was significantly enhanced (Fig. 5, A and C). Aurora B activity was similarly enriched at kinetochores with microtubules and low at kinetochores without microtubules after nocodazole washout. Yellow arrows indicate select examples of kinetochores without detectable microtubules. Blue arrows indicate select examples of kinetochores with associated microtubules. Each image represents multiple Z-slices. Error bars indicate standard deviation. *, P < 0.05; **, P < 5 × 10⁻⁷. Noc, nocodazole; A.U., arbitrary units. Bars: (white) 5 µm; (yellow) 1 µm.
that CENP-I has no role in Aurora B localization or activation (Fig. S4, D and E). We conclude that the presence of microtubules at kinetochores correlates with chromosome-autonomous recruitment of Aurora B.

Chromosome-autonomous recruitment of RZZ and Mad1 to kinetochores is revealed in CENP-I-depleted cells

Having demonstrated that we could generate and visualize chromosome-autonomous localization of Aurora B in a nocodazole washout assay, we used the system to monitor the role of local Aurora B activity in spindle checkpoint signaling. We performed nocodazole washout experiments in both control and CENP-I-depleted cells and stained for Mad1, Mad2, or ZW10. In control cells, all kinetochores retained Mad1, Mad2, and ZW10 (Fig. 6, A and B; and Fig. S4, G–P). This is expected if CENP-I prevents the dissociation of spindle checkpoint proteins and keeps Mad1 levels at kinetochores saturated when there is low Aurora B activity. We reasoned that we could uncover the chromosome-autonomous nature of checkpoint protein recruitment by depleting CENP-I. In fact, kinetochores associated with PreK-fibers or spindle microtubules recruited Mad1, Mad2, and ZW10 in CENP-I-depleted cells, whereas the kinetochores that were not associated with microtubules had fivefold lower amounts of Mad1 at kinetochores with or without microtubules, whereas CENP-I-depleted cells have fivefold more Mad1 at kinetochores with microtubules. Yellow arrows indicate select examples of kinetochores without microtubules. Blue arrows indicate select examples of kinetochores with associated microtubules. Insets contain multiple Z-sections for clarity. Error bars indicate standard deviation. *, P < 0.00005; **, P < 0.005. Noc, nocodazole; A.U., arbitrary units. Bars: (white) 5 µm; (yellow) 1 µm.
they also demonstrate that the enrichment of Aurora B by PreK-fibers and spindle microtubules can dynamically recruit spindle checkpoint proteins.

CENP-I-depleted kinetochores fail to retain Mad1 at anti-poleward kinetochores in Monastrol

We sought a nocodazole-independent method to test the hypothesis that CENP-I inhibits the dissociation of Mad1 from kinetochores with premature kinetochore attachments. Cells treated with the Eg5 inhibitor Monastrol generate an ideal situation to test our hypothesis. In these cells, poleward-facing kinetochores are attached to the central pole and lack Mad1, whereas anti-poleward kinetochores have immature attachments to PreK-fibers and recruit Mad1 (shown schematically in Fig. 7 A; Kapoor et al., 2000; Maliga et al., 2002; Cochran et al., 2005). If CENP-I is required for kinetochores with immature microtubule attachments to retain Mad1 then we predict that CENP-I-depleted cells would lack Mad1 at anti-poleward kinetochores in Monastrol.

As expected, Mad1 and Mad2 were only observed at anti-poleward kinetochores, whereas BubR1 was found at all kinetochores. CENP-I-depleted cells can still recruit BubR1 to kinetochores but fail to retain Mad1 and Mad2 at anti-poleward kinetochores. Together our data demonstrate that the CENP-I pathway prevents the loss of Mad1 from kinetochores that have not generated mature microtubule attachments.

Mad1 mislocalization in CENP-I-depleted cells is not a result of depletion of Hec1

The kinetochore binding protein Hec1 (also known as Ndc80) is another protein hypothesized to prevent the premature stripping of Mad1 (DeLuca et al., 2003). CENP-I depletion for >72 h
Discussion

A single unattached kinetochore is sufficient to generate a spindle checkpoint signal robust enough to arrest the metaphase to anaphase transition. Our data provides a mechanistic framework to understand the on/off nature of this signal. The key event is the localization of RZZ and Mad1 to the kinetochore, which is both necessary and sufficient to generate a spindle checkpoint signal (Maldonado and Kapoor, 2011). The proper localization of proteins to a subcellular structure is a function of the number of available binding sites, the association kinetics, and the dissociation kinetics. We have demonstrated that a pathway requiring CENP-I regulates the dissociation of RZZ and Mad1 from individual kinetochores. We also demonstrated that Aurora B activity regulates the association of RZZ and Mad1 onto each kinetochore.

We suggest that the independent regulation of both association and dissociation reactions is an essential feature of building this tightly controlled molecular switch (Fig. 8 A). Individual kinetochores can exist in three states during prometaphase and metaphase (Fig. 8 B): unattached without PreK-fibers, with lateral (immature) attachments to spindle microtubules or PreK-fibers mediated by dynein, or with properly attached "end-on" to spindle microtubules through the Ndc80 complex. In the first case weak Aurora B activity is sufficient to generate a robust spindle checkpoint signal because CENP-I ensures that the dissociation rate of RZZ and Mad1 is essentially zero. The event that is regulated by CENP-I is not known and could either be the recruitment of a protein receptor or a posttranslational modification to a protein that recruits RZZ. A reasonable candidate is the phosphorylation of Zwint by Aurora B, which is required for kinetochore localization of RZZ (Kasuboski et al., 2011). In this case, CENP-I could inhibit the opposing phosphatase. The spindle checkpoint signal remains robust at kinetochores with lateral attachments because CENP-I continues to inhibit the dissociation of Mad1 from kinetochores. There are two nonexclusive models. First CENP-I may make the attachment of RZZ to kinetochores so tight that it cannot be displaced by dynein. Second, CENP-I could alter the cargo loading of dynein so that it generates PreK-fibers but cannot strip Mad1. In addition, Aurora B signaling is stronger in the presence of immature microtubule attachments, which leads to robust loading of Mad1 (Salimian et al., 2011). After attachments mature, RZZ and Mad1 are quickly removed because these attachments...
down-regulate Aurora B activity and inhibit CENP-I, which activates dynein stripping (Fig. 8, A and B).

The CENP-H/I/K complex has an established role at the kinetochores in regulating the microtubule dynamics of “end-on” attached microtubules (Amaro et al., 2010). We have previously shown that CENP-I plays no role in the spindle checkpoint signal generated by Taxol, suggesting that CENP-I’s spindle checkpoint function is turned off by the presence of end-on attached microtubules (Matson et al., 2012). Thus a reasonable hypothesis is that the CENP-H/I/K complex locks Mad1 in a stable kinetochore complex until this activity is turned off through the engagement of CENP-H/I/K with end-on attached microtubules (Fig. 8 B).

It is well-established that spindle checkpoint proteins dissociate from kinetochores that form mature microtubule attachments and align to the metaphase plate (Gorbsky et al., 1998; Waters et al., 1998). These experiments have entrenched the concept of chromosome-autonomous dissociation of checkpoint proteins from kinetochores. Our data suggest that the pathway involving CENP-I is the key regulator of RZZ and Mad1 release after microtubule attachment. Moreover, whether the association of RZZ and Mad1 was regulated or constitutive could not previously be measured because the dissociation reaction is so tightly regulated by the CENP-I pathway. However, by depleting CENP-I we enhanced RZZ and Mad1 dissociation and could visualize their association dynamics. Thus our data also demonstrate that the enrichment of Aurora B activity to kinetochores by immature microtubule attachments can dynamically drive recruitment of RZZ and Mad1 to kinetochores.

Our model can explain a confusing observation. Aurora B activity must be high to maintain a spindle checkpoint arrest in Taxol but not nocodazole (Hauf et al., 2003; Ditchfield et al., 2003; Matson et al., 2012). Cells in nocodazole can tolerate the reduced loading of Mad1 by Aurora B inhibition because Mad1 dissociation is inhibited by CENP-I. However, the CENP-I pathway is turned off by the stable kinetochore–microtubule attachments in Taxol-arrested cells and are therefore dependent on continuous Mad1 loading by Aurora B (Matson et al., 2012). Our model cannot fully explain why Aurora B is required during prometaphase to localize RZZ, because it predicts that CENP-I should prevent the removal of RZZ until end-on attachments are generated, like it does in nocodazole (Kasuboski et al., 2011; Kops et al., 2005a). It is possible that there are transient end-on attachments that inhibit CENP-I, because of high phosphorylation of the Ndc80 complex. The recent identification of a direct binding event between Bub1 and Mad1 in Caenorhabditis elegans is an exciting finding (Moyle et al., 2014). However, it is also a potential source of confusion because the levels of Bub1/BubR1 are reduced after inhibition of Aurora B in both prometaphase and nocodazole (Ditchfield et al., 2003; Hauf et al., 2003; Fig. S2). We suggest that there are more molecules of Knl1/Bub1 in the kinetochores than there are RZZ and that RZZ is the limiting component for Mad1 binding. Consistent with this idea, the levels of RZZ, not Bub1, are more closely correlated with Mad1 binding and Xenopus laevis kinetochores have approximately three times more KMN components than RZZ components in nocodazole (Emanuele et al., 2005). There remains much to be learned about the dynamic conversion from lateral to end-on kinetochore–microtubule attachments and how this is coordinated with spindle checkpoint signaling during prometaphase.

An important future direction is to identify how CENP-I controls the dissociation of RZZ and Mad1 from kinetochores. The simplest model is that CENP-I produces a tight binding event between RZZ and kinetochores, which generates an extended half-life and prevents dynein from stripping it off. Alternatively, it is possible that CENP-I has two independent functions: one that increases the stability of the checkpoint complexes at kinetochores and a second that inhibits dynein stripping. CENP-H depletion was shown to increase the stability of K-fiber microtubules in metaphase (Amaro et al., 2010). Thus it is also possible that CENP-I functions to prevent the untimely maturation of lateral attachments. In any case, some event must occur after proper microtubule attachment to allow for stripping of RZZ and Mad1.

Finally, it is established that PreK-fibers can increase the rate of spindle–kinetochore attachment by extending the spindle capture surface (Khodjakov et al., 2003). However, the presence of PreK-fibers has not been considered in terms of spindle checkpoint signaling. Our data suggest that spindle checkpoint mechanisms are exquisitely tuned to work with this class of microtubules. We demonstrate that PreK-fibers can enrich Aurora B kinase at inner centromeres, increase the phosphorylation of adjacent kinetochores, and recruit spindle checkpoint proteins. We also show that CENP-I is required to prevent dynein stripping along PreK-fibers. Yet it has been standard practice for 30 years to trigger mitotic arrest with microtubule-destabilizing drugs. Clearly, when CENP-I is active the basal amount of Aurora B activity in nocodazole is sufficient to generate a checkpoint signal and microtubule-dependent stimulation is not essential. However, in the future the concentration of spindle poisons will need to be carefully noted. There are significant amounts of kinetochore-associated microtubules at 0.33 µM nocodazole and in a recent paper we demonstrate that these can recruit additional Aurora B (Jordan et al., 1992; Banerjee et al., 2014). However, at 3.3 µM nocodazole there are no microtubules around kinetochores or additional Aurora B at centromeres. Thus, depending on the concentration of drug used one can induce or repress additional inner centromere Aurora B recruitment and activity.

Materials and methods

Cell culture, transfections, and immunoblotting

Hela 1Trex (Invitrogen), U2OS, and 293T cells were maintained in DMEM supplemented with 10% FBS. Cells were plated at 30% confluency onto laminin-coated coverslips in 12-well dishes (Corning) overnight. siRNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen) and plasmid transfections were performed using Lipofectamine2000 (Invitrogen) according to the manufacturer’s protocol. A smart pool of siRNA oligos against CENP-I were purchased from Thermo Fisher Scientific (M02961-701; 5’-GGUACAAGGGUGAAUAUAUUU-3’; 5’-CAGCAAAGACUUUAUGAAGAA-3’; 5’-GCGUGGUAUAUGACUAAA-3’; and 5’-GUAGAGCUACUUCCGUUAUU-3’) and cells were treated with a final concentration of 20 nM siRNA. Experiments were performed 48 h later. HeLa knockdowns were performed using a custom oligo (5’-GAGGAGACUAGGAAUGUUU-3’; Qiagen). Cells were thymidine arrested for 24 h before treatment with 75 nM siRNA and released into fresh media. 12 h later the cells were treated with an additional 75 nM siRNA and thymidine arrested. After 12 h, the cells were released from thymidine and assayed 8 h later when the majority of the population was in mitosis.
For dynein inhibition experiments, the eGFP-CC1 vector expressing CC1-eGFP was provided by K. Pfister (University of Virginia, Charlottesville, VA) and contains the CC1 gene fragment initially reported by T. Schroer (Johns Hopkins University, Baltimore, MD). This vector contains amino acids 217–548 (a region known as CC1) of P150Glued with a C-terminal eGFP fusion expressed under the control of a CMV promoter. Cells in a 12-well dish were transfected with 200 ng of eGFP-CC1 or control plasmid and assayed after 24 h. Monoclonal anti–Centrin-2 antibody was used at 100 µM for 2 h. ZM447439 (Enzo Life Sciences) was used at 2 µM final concentration and Hesperadin (Tocris Bioscience) was used at 100 nM unless otherwise noted. Cytoskeletons for Western blotting were generated by scraping cells from the culture plates and pelleting them at 1,000 rpm in a tabletop centrifuge. Pellets were washed once in PBS, resuspended in 2x SDS sample buffer, sonicated, and loaded onto gels.

Nocodazole treatments and nocodazole washout assays
Nocodazole (Sigma-Aldrich) was used at 3.3 µM throughout the study, a concentration sufficient to depolymerize all microtubules, and cells were arrested in nocodazole for 2 h and the media were aspirated. The nocodazole-containing media was replaced with fresh media, washed once in PBS, and then incubated in fresh media for 10 min unless noted otherwise.

Immunofluorescence and quantitative immunofluorescence
Unless otherwise stated, cells on poly-lysine–coated coverslips were fixed in 4% paraformaldehyde and 0.5% Triton X-100 for 20 min and stained in 4% paraformaldehyde and 0.5% Triton X-100 for 20 min and then washed in PBST and stained in PBST plus 5% BSA. For staining with anti-dynein and anti–Centrin-2 antibodies, cells were fixed in –20°C methanol for 10 min and then washed in PBST and stained in PBST plus 5% BSA. For staining with the anti-ZW10 antibody, cells were first fixed in PBS plus 3.5% paraformaldehyde for 7 min and then extracted in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% BSA, and 0.2% Triton X-100 for 2 min. Staining was then performed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% BSA. Staining was performed using the following primary antibodies: anti–CENP-I (rabbit polyclonal antibody against full-length human CENPI), anti-Mad1 (rabbit polyclonal antibody against full-length human Mad1), and anti-UBiquitin (rabbit polyclonal antibody against X. laevis Ubiquitin) obtained from P.T. Stukenberg, University of Virginia, Charlottesville, VA; anti-ZW10 (rabbit, rod, and rabbit antisera; anti–CENP-F (rabbit), anti–CENP-C (rabbit), and anti-Mis12 (rabbit; all gifts from J.T. Yan, Fox Chase Cancer Center, Philadelphia, PA); anti-Zwint (rabbit) and an alternate anti-ZW10 (rabbit) antibody (a gift from G.K. Chan, University of Alberta, Edmonton, Alberta, Canada); anti-Mad1 (rabbit; a gift from P. Meraldi, Swiss Federal Institute of Technology Zurich, Zurich, Switzerland); anti-Hec1 (mouse; Genetex); anti–centromere antibodies (ACA; human; Antibodies Inc.); anti-Mad2 (rabbit; a gift from G. Cortegana, Oklahoma Medical Research Foundation, Oklahoma City, OK); anti-Tubulin DM1a (mouse; Sigma-Aldrich); anti–Aurora B (mouse; BD); and anti-p57CENPI-A (rabbit; Cell Signaling Technology). Quantitative immunofluorescence was performed using ImageJ software (National Institutes of Health) and calculations were performed in Microsoft Excel. In brief, a circular region encompassing one kinetochore was measured and mean gray level intensity was measured for both the experimental antibody signal and ACA, as well as background in both channels. Final intensity was calculated by taking the intensity of the experimental antibody minus background and dividing it by the intensity of the corresponding ACA signal minus background. Approximately 10 kinetochores were measured in 10 cells corresponding to 100 kinetochores per reported intensity value. To measure whether kinetochores had microtubules or not, ACA signals were identified within a Z-series composite image with the fluorescence intensity value. To measure whether kinetochores had microtubules or not, ACA signals were identified within a Z-series composite image with the fluorescence intensity value. To measure whether kinetochores had microtubules or not, ACA signals were identified within a Z-series composite image with the fluorescence intensity value. To measure whether kinetochores had microtubules or not, ACA signals were identified within a Z-series composite image with the fluorescence intensity value. To measure whether kinetochores had microtubules or not, ACA signals were identified within a Z-series composite image with the fluorescence intensity value. To measure whether kinetochores had microtubules or not, ACA signals were identified within a Z-series composite image with the fluorescence intensity value. 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of Aurora B and spindle checkpoint proteins. Fig. S5 comprises immuno-fluorescence data on the effects of Hec1 depletion on the localization of spindle checkpoint proteins. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201307137/DC1.

We thank P. Meraldi for the Mad1 antibody, G.K. Chan for the Zwint and ZW10 antibodies, and T.J. Yen for the ZW10, CENP-F, CENP-C, Rod, and Mis12 antibodies. We additionally would like to thank E.D. Salmon for the GFP-Mad1 expression construct, D.J. Burke for extensive help with statistical methods, and D.J. Burke, J.V. Shah, and G.J. Gorbsky for critical review of the manuscript. We thank the University of Virginia Advanced Microscopy Facility for assistance with FRAP experiments. This work was funded by grants GM063045 to P.T. Stukenberg. D.R. Matson is supported by National Institutes of Health training grants T32GM007267 and T32GM008136. D.R. Matson and P.T. Stukenberg designed and executed the experiments and wrote the manuscript. The authors declare no competing financial interests.

Submitted: 23 July 2013
Accepted: 17 April 2014

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Published May 26, 2014
Downloaded from http://www.jcb.org/ on April 14, 2017

Matson and Stukenberg
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Figure S4. **Aurora B and Aurora B activity localize in a microtubule-dependent manner and dynamically recruit ZW10, Mad1, and Mad2 to kinetochores.**

(A) Immunofluorescence images showing the origins of polymerizing microtubules after nocodazole washout. Microtubules form from the centrosomes as indicated by centrin-2 staining, but also at a population of kinetochores as indicated by ACA. (B and C) Immunofluorescence images of Aurora B and p(S7)CENP-A after nocodazole washout demonstrating microtubule-dependent localization. Two channels are shown individually and then overlaid for clarity and comparison purposes. Select Z-slices shown for clarity. (D and E) Immunofluorescence images of CENP-I–depleted cells depicting Aurora B localization and Aurora B activity indicated by p(S7)CENP-A. Aurora B and Aurora B activity are enhanced at kinetochores with microtubules after nocodazole washout similarly to controls. Select Z-slices shown for clarity. (F) Additional immunofluorescence images of nocodazole washout cells depleted of CENP-I demonstrating the microtubule-associated localization of Mad1 to kinetochores. (G and H) Immunofluorescence images demonstrating that control cells re-

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Note: The image contains several panels labeled A to H, each showing immunofluorescence images and corresponding merge images, illustrating the localization and activity of various proteins in the context of microtubule dynamics and kinetochore function.
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