Recruitment of Mad1 to metaphase kinetochores is sufficient to reactivate the mitotic checkpoint

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The mitotic checkpoint monitors kinetochore–microtubule attachment and prevents anaphase until all kinetochores are stably attached. Checkpoint regulation hinges on the dynamic localization of checkpoint proteins to kinetochores. Unattached, checkpoint-active kinetochores accumulate multiple checkpoint proteins, which are depleted from kinetochores upon stable attachment, allowing checkpoint silencing. Because multiple proteins are recruited simultaneously to unattached kinetochores, it is not known what changes at kinetochores are essential for anaphase promoting complex/cyclosome (APC/C) inhibition. Using chemically induced dimerization to manipulate protein localization with temporal control, we show that recruiting the checkpoint protein Mad1 to metaphase kinetochores is sufficient to reactivate the checkpoint without a concomitant increase in kinetochore levels of Mps1 or BubR1. Furthermore, Mad2 binding is necessary but not sufficient for Mad1 to activate the checkpoint; a conserved C-terminal motif is also required. The results of our checkpoint reactivation assay suggest that Mad1, in addition to converting Mad2 to its active conformation, scaffolds formation of a higher-order mitotic checkpoint complex at kinetochores.

Introduction

The mitotic checkpoint inhibits the anaphase promoting complex/cyclosome (APC/C) in the presence of unattached kinetochores and silences this inhibition once all kinetochores are stably attached to spindle microtubules. Checkpoint activity (i.e., APC/C inhibition) and silencing are correlated with changes in the kinetochore localization of checkpoint proteins, including Mad1, Mad2, Bub1, BubR1, Bub3, Mps1, and Cdc20 (Kops and Shah, 2012). These proteins are enriched at kinetochores until stable MT attachment and are required for checkpoint function. A central challenge in understanding the mitotic checkpoint is to dissect how local changes in checkpoint protein occupancy at kinetochores drive global changes in checkpoint activity.

Preventing the removal of Mad1 or Mps1 from kinetochores via genetic fusion to the stable kinetochore component Mis12 blocks anaphase, which demonstrates that the removal of these proteins is required for checkpoint silencing (Jelluma et al., 2010; Maldonado and Kapoor, 2011). To probe checkpoint activation (i.e., switching the checkpoint from an “off” state to an “on” state), experimental intervention when the checkpoint is silenced, such as at metaphase, is an attractive approach. The checkpoint can be reactivated at metaphase by disrupting kinetochore–microtubule attachments, using either spindle poisons or laser microsurgery (Clute and Pines, 1999; Dick and Gerlich, 2013). It is unknown if the checkpoint can be reactivated after metaphase without compromising kinetochore–microtubule attachment. Metaphase kinetochores are stably attached and depleted of checkpoint proteins, so they provide a context in which to test the effect of increasing the kinetochore concentration of an individual protein in the absence of the full set of signals associated with the unattached state.

We tested whether increasing kinetochore localization of Mad1 at metaphase is sufficient to reactivate the checkpoint. Mad1 and its partner Mad2 are essential checkpoint proteins (Li and Murray, 1991). Mad1 constitutively binds a single copy of Mad2 in the closed conformation, and this bound population of Mad2 serves as the kinetochore receptor for cytosolic, open-conformation Mad2 (Luo et al., 2004; De Antoni et al., 2005; Lara-Gonzalez et al., 2012). Continual recruitment of open Mad2 and its conversion to the closed conformation, concomitant with binding to Cdc20, constitutes the catalytic engine of the spindle assembly checkpoint at kinetochores (Han et al., 2010; Maldonado and Kapoor, 2011). To probe checkpoint activity (i.e., switching the checkpoint from an “off” state to an “on” state), experimental intervention when the checkpoint is silenced, such as at metaphase, is an attractive approach. The checkpoint can be reactivated at metaphase by disrupting kinetochore–microtubule attachments, using either spindle poisons or laser microsurgery (Clute and Pines, 1999; Dick and Gerlich, 2013). It is unknown if the checkpoint can be reactivated after metaphase without compromising kinetochore–microtubule attachment. Metaphase kinetochores are stably attached and depleted of checkpoint proteins, so they provide a context in which to test the effect of increasing the kinetochore concentration of an individual protein in the absence of the full set of signals associated with the unattached state.

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Weiwad et al., 2006; Hoeffer et al., 2008; De Angelis et al., 2009; Gerard et al., 2010), should remove this limitation. We added an artificial miRNA targeting the 3′ UTR of endogenous FKBP to the constitutively expressed Mis12-GFP-FKBP transcript. This miRNA effectively depleted endogenous FKBP, but not exogenous Mis12-GFP-FKBP (Fig. S1 A), without any overt effect on cellular morphology or growth. Endogenous FKBP depletion dramatically improved the efficiency of rapamycin-induced dimerization (Fig. 1 C and Fig. S1, B and C). We also found that the kinetics of FRB recruitment to kinetochores are sensitive to applied rapamycin concentration, with maximal recruitment in ~10 s using 500 nM rapamycin (Fig. S1 D).

We constructed a cell line that combined constitutive expression of FKBP miRNA and Mis12-GFP-FKBP with doxycycline-inducible expression of FRB-mCherry-Mad1. FRB-mCherry-Mad1 localized robustly to misaligned kinetochores (Fig. S2 A) and was removed as kinetochores aligned at the metaphase plate (Fig. 2 A). In the absence of rapamycin, cells expressing FRB-mCherry-Mad1 proceeded through mitosis normally and rarely entered anaphase with misaligned chromosomes. After addition of rapamycin, FRB-mCherry-Mad1 was removed as kinetochores aligned at the metaphase plate (Fig. 2 B), which did not compromise metaphase plate organization or chromosome alignment. This is consistent with the results of constitutive tethering (Maldonado and Kapoor, 2011). These results indicate that FRB-mCherry-Mad1 is functional and does not exert dominant-negative effects at the expression levels in our cells.

Figure 1. Endogenous FKBP depletion improves efficiency of rapamycin-mediated recruitment. (A) Diagram of a DNA cassette used to constitutively express Mis12-GFP-FKBP and miRNA, and inducibly express mCherry-FRB. The cassette is integrated between Lox acceptor sites downstream of the EF1a promoter (Khandelia et al., 2011, for details see Materials and methods). (B) Schematic representation of rapamycin-mediated recruitment of mCherry-FRB to kinetochore-localized Mis12-GFP-FKBP. (C) HeLa cells expressing Mis12-GFP-FKBP, mCherry-FRB, and either an empty miRNA backbone or miRNA against the 3′ UTR of endogenous FKBP were imaged before and ~1 min after the addition of 500 nM rapamycin (rap). Images are representative of three independent experiments (quantified in Fig. S1 C). Bar, 5 µm.
Figure 2. **Mad1 recruitment to metaphase kinetochores activates the mitotic checkpoint.** Cells expressing FRB-mCherry-Mad1, FKBP miRNA, and either Mis12-GFP-FKBP [A, C, and D] or Mis12-FKBP [B] are shown. [A] Time lapse shows FRB-mCherry-Mad1 removal from the last mCherry-positive kinetochore pair before anaphase (at 22 min). [B] Cells were treated with rapamycin (rap) or vehicle (DMSO) control for 10 min, fixed, and stained for Mad2. Control cells, which have not recruited mCherry, were also stained for Hec1. Images shown are representative of three independent replicates. (C and D) Cells were treated with either rapamycin or vehicle at metaphase (t = 0), then monitored for anaphase onset for 30 min. [C] Images show Mad1 recruitment and metaphase arrest in rapamycin-treated but not control cells. Bars, 5 µm. [D] The graph shows the cumulative percentage of cells entering anaphase over time (n ≥ 24 cells for each condition, pooled from six independent replicates).
increase in two other key checkpoint proteins, Mps1 and BubR1, that are enriched at unattached kinetochores. Mps1 kinase activity is required upstream of Mad1, to recruit Mad1 to unattached kinetochores, and BubR1 levels at kinetochores were quantified. Levels of other checkpoint proteins, including Bub1, CENP-E, P150, ROD, and ZW-10, at metaphase kinetochores are not affected by constitutive tethering of Mad1 to kinetochores (Maldonado and Kapoor, 2011).

To test the effect of recruiting Mad1 to kinetochores after metaphase, we identified metaphase cells in an asynchronous population, added either rapamycin or a vehicle (DMSO) control, then monitored for 30 min to determine progress to anaphase (Fig. 2, C and D). We only considered cells with a clearly defined metaphase plate, without misaligned chromosomes identifiable by Mis12-GFP signal, and without mCherry-Mad1–positive kinetochores. 100% of control cells proceeded to anaphase within 15 min of mock treatment. In contrast, after recruiting Mad1 to metaphase kinetochores, only 34% of cells entered anaphase within 30 min (Fig. 2 D). In a related experiment, rapamycin was added at a fixed time (3 min) after removal of Mad1 from the last unattached kinetochore. In this case, very few (1/11) cells entered anaphase, which is consistent with recent results demonstrating that checkpoint reactivation depends on time in metaphase (Dick and Gerlich, 2013). Levels of other checkpoint proteins, including Bub1, CENP-E, P150, ROD, and ZW-10, at metaphase kinetochores are not affected by constitutive tethering of Mad1 to kinetochores (Maldonado and Kapoor, 2011).

We measured levels of endogenous BubR1 and Mps1 at kinetochores using immunofluorescence. As expected, Mps1 and BubR1, that are enriched at unattached kinetochores. Mps1 kinase activity is required upstream of Mad1, to recruit Mad1 to unattached kinetochores, and downstream of Mad1, to recruit cytosolic Mad2 to kinetochore-bound Mad1–Mad2 (Hewitt et al., 2010). BubR1 contains a conserved Mad3 homology domain that is the key inhibitor of Cdc20 and a pseudokinase domain that is an important signaling scaffold (Suijkerbuijk et al., 2012; Han et al., 2013). Levels of other checkpoint proteins, including Bub1, CENP-E, P150, ROD, and ZW-10, at metaphase kinetochores are not affected by constitutive tethering of Mad1 to kinetochores (Maldonado and Kapoor, 2011).

We next asked whether recruiting Mad1 to metaphase kinetochores activated the checkpoint through a concomitant increase in two other key checkpoint proteins, Mps1 and BubR1, that are enriched at unattached kinetochores. Mps1 kinase activity is required upstream of Mad1, to recruit Mad1 to unattached kinetochores, and downstream of Mad1, to recruit cytosolic Mad2 to kinetochore-bound Mad1–Mad2 (Hewitt et al., 2010). BubR1 contains a conserved Mad3 homology domain that is the key inhibitor of Cdc20 and a pseudokinase domain that is an important signaling scaffold (Suijkerbuijk et al., 2012; Han et al., 2013). Levels of other checkpoint proteins, including Bub1, CENP-E, P150, ROD, and ZW-10, at metaphase kinetochores are not affected by constitutive tethering of Mad1 to kinetochores (Maldonado and Kapoor, 2011).
activate the checkpoint without triggering an increase in kinetochore levels of Mps1 or BubR1.

To determine what functional domains of Mad1 are necessary for checkpoint activation, we first tested the role of the N-terminal domain, which is required for kinetochore localization in *Xenopus laevis* (Chung and Chen, 2002) and mammalian cells (Kim et al., 2012). It is not known whether this domain contributes to checkpoint activation beyond its role in localization. We removed the first 484 residues to generate Mad1^{NAD}, which matches a fragment used for in vitro studies of Mad2 conformational templating (De Antoni et al., 2005). FRB-mCherry-Mad1^{NAD} fails to localize to unattached kinetochores in the absence of rapamycin (Fig. S2 A), as expected, but recruits Mad2 to metaphase kinetochores upon treatment with rapamycin (Figs. 4 B and S3 C) and induces a metaphase arrest (Fig. 4, C and D). These results show that the N-terminal domain of Mad1 is not required for checkpoint activation beyond localizing Mad1 to kinetochores.

Because Mad2 binding is the best-characterized activity of Mad1, we tested whether Mad2 binding is sufficient for Mad1 to activate the checkpoint. We truncated Mad1^{NAD} at the C terminus to construct Mad1^{NMD}, comprising residues 485–584, including the Mad2 interaction loop flanked by α-helical regions, as shown in the crystal structure of the tetrameric Mad1–Mad2 complex (Sironi et al., 2002). FRB-mCherry-Mad1^{NMD} recruits Mad2 to metaphase kinetochores upon treatment with rapamycin to similar levels as Mad1 and Mad1—rapamycin to similar levels as Mad1 and Mad1 recruits Mad2 to metaphase kinetochores upon treatment with rapamycin (Figs. 4 B and S3 C) and induces a metaphase arrest (Fig. 4, C and D). These results show that the C-terminal domain of Mad1 is also required.

In *Saccharomyces cerevisiae*, mitotic checkpoint activity can be abolished by mutating three conserved amino acids in the C terminus of *S. cerevisiae* Mad1 to alanine: R653, L654, and K655, which correspond to residues 617–619 in human Mad1 (Brady and Hardwick, 2000). Mutating either R617 or K619 in human cells impairs kinetochore targeting, presumably by disrupting interactions with other kinetochore proteins (Kim et al., 2012), but the role of the RLK motif in checkpoint activation independent of targeting has not been tested. To determine whether checkpoint activation depends on the C-terminal RLK motif, we mutated these three residues to alanines in the context of Mad1ΔN. Mutating the RLK motif does not reduce Mad2 recruitment (Figs. 4 B and S3 C), but almost completely abolishes the ability of Mad1 to activate the checkpoint when recruited to metaphase kinetochores (Fig. 4, C and D). This result demonstrates that a conserved protein interaction motif in the C terminus of Mad1 is required for full checkpoint activity.

In conclusion, our results show that the spindle assembly checkpoint can be reactivated after silencing by relocating Mad1 to metaphase kinetochores. Checkpoint activation does not require the increased levels of Mps1 and BubR1 associated with unattached kinetochores: the basal levels of these proteins present at metaphase kinetochores are sufficient to sustain APC/C inhibition when combined with increased Mad1–Mad2. Both Mps1 and BubR1 exhibit rapid turnover at metaphase kinetochores (Howell et al., 2004), so a large fraction of Mps1 and BubR1 may transiently encounter kinetochore-activated Mad1–Mad2 even though steady-state levels of Mps1 and BubR1 at kinetochores remain low. Additionally, Mps1 and BubR1 play other roles at the kinetochore independent of their checkpoint functions, for example regulating microtubule interactions (Lampson and Kapoor, 2005; Maure et al., 2007; Jelluma et al., 2008; Meyer et al., 2013). It is possible that the increased levels of some checkpoint proteins at unattached kinetochores contribute to functions other than APC/C inhibition.

The results of our Mad1 mutation experiments indicate that the role of Mad1 in APC/C inhibition is not limited to localizing Mad2 to kinetochores. The potent effect of mutating the Mad1 RLK motif strongly suggests that the C terminus of Mad1 mediates interactions with other kinetochore proteins that are essential for checkpoint activity in addition to contributing to Mad1 localization. A growing body of evidence argues that APC/C inhibition is a multistep process in which Cdc20 first binds closed Mad2 (cMad2), which catalyzes the subsequent binding of BubR1 to Cdc20, and that BubR1–Cdc20 is the primary inhibitor of the APC/C (Nilsson et al., 2008; Kulukian et al., 2009; Westhorpe et al., 2011; Han et al., 2013). The C terminus of Mad1 may act as a scaffold to promote either the binding of Cdc20 to cMad2 or the transfer of Cdc20 from Mad2 to BubR1. An important future goal is to determine which proteins interact with the C terminus of Mad1, and what role these interactions play in checkpoint activation.

Finally, our work demonstrates the potential for inducible protein dimerization as a tool to manipulate protein localization at kinetochores on a rapid timescale. Dynamic localization is a hallmark of many mitotic proteins, and much has been learned from disrupting localization by mutating targeting domains or conferring new localization through constitutive tethering, but these techniques are fundamentally limited by a lack of temporal control. Chemically induced dimerization overcomes this limitation, and allows us to study the effects of altering protein localization in real time.

**Materials and methods**

**Cell culture, creation of stable cell lines, and rapamycin treatment** All experiments were performed with stable HeLa cell lines generated by recombinase-mediated cassette exchange (RMCE) using the HILO RMCE system (obtained from E.V. Makeyev, Nanyang Technological University, Singapore; reported in Khandelia et al., 2011). This system allowed us to reproducibly insert transgene cassette at a single genomic locus. In brief: a monoclonal acceptor cell line with the C terminus of Mad1 may act as a scaffold to promote either the binding of Cdc20 to cMad2 or the transfer of Cdc20 from Mad2 to BubR1. An important future goal is to determine which proteins interact with the C terminus of Mad1, and what role these interactions play in checkpoint activation.

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except as noted in Fig. S1 D. Rapamycin was stored as a 500 mM stock in DMSO, then diluted in medium to a 0.75- or 1-mM intermediate dilution in medium and prewarmed before addition to cells.

within each population of cells, the variation was reproducible and similar between different cell lines produced with this technique. All rapamycin experiments were done at a final working concentration of 500 nM rapamycin, except as noted in Fig. S1 D. Rapamycin was stored as a 500 mM stock in DMSO, then diluted in medium to a 0.75- or 1-mM intermediate dilution in medium and prewarmed before addition to cells.
Plasmids
pEM734 expressing nuclear-localized Cre recombinase and the donor cassette plasmid pEM791 were obtained from E.V. Makeyev (Khandelia et al., 2011). All donor cassettes used for this study were derived from pEM791. pEM791 is designed for inducible expression of miRNA-based shRNA and a reporter gene. pEM791 contains: a Puro resistance gene (Pur) positioned for constitutive transcription from the EF1α promoter upstream of the LoxP site in the acceptor locus, the gene for reverse-tetracycline transactivator 3 (rtTA3) constitutively expressed from a UBC promoter, and a TRE promoter for inducible transcription of an artificial miRNA-based shRNA nested in an intron upstream of a GFP reporter gene (Du et al., 2006; Khandelia et al., 2011).

For this study, we modified pEM791 for constitutive transcription of an additional mRNA and protein sequence. Between the LoxP site and Pur, we added: (1) an miRNA-based shRNA against the 3’ UTR of FKBP12, nested within an intron; (2) Miss12-GFP-FKBP or Miss12-FKBP in both cases; and (3) an internal ribosomal entry sequence (IRES). These modifications allowed constitutive polycistronic coexpression of FKBP miRNA, Miss12-targeted FKBP, and the Puro resistance gene from the EF1α promoter in the acceptor locus. FKBP miRNA was designed using the miRNA function within the Block-iT RNAi Designer (Life Technologies). The following oligos were used for FKBP shRNA: 5’-TGCTGAATGGGTGATG-3’, 5’-TGCTGAATGGGTGATG-3’. mCherry-FRB expression was measured for each cell in the pre-rapamycin condition. Each group was composed of at least eight cells that were averaged for each cell type in three independent replicates. All image acquisition and processing was performed using ImageJ.

Immunofluorescence
All cell fixation procedures were done at room temperature (−22°C). For Mad2 staining in Figs. 2, 4, and 3, cells were fixed in PBS + 3.7% formaldehyde and 0.5% Triton X-100 for 10 min. For BubR1 staining in Fig. 3, A3 cells were preextracted in PBS + 0.5% Triton X-100 for 3 min, then fixed in PBS + 3.7% formaldehyde and 0.5% Triton X-100 for 10 min. For Mps1 staining in Fig. 3, B cells were prefixed for 3 s in PBS + 3.7% formaldehyde, extracted in PBS + 0.5% Triton X-100 for 1 min, then fixed in PBS + 3.7% formaldehyde and 0.5% Triton X-100 for 10 min. For Mps1 staining in Fig. 3, B cells were prefixed for 5 s in PBS + 3.7% formaldehyde, extracted in PBS + 0.5% Triton X-100 for 7 min, then fixed in PBS + 3.7% formaldehyde and 0.5% Triton X-100 for 10 min. The following primary antibodies were used: rabbit polyclonal anti-Mad2 (1:500; PRB-652C; Covance), mouse monoclonal anti-Hec1 9G3 (1:1,000; ab3613; Abcam), rabbit anti-BubR1 monoclonal (1:1,000; a gift from W. Dai, New York Medical College, Valhalla, NY), rabbit anti–CENP-C polyclonal (1:1,000; a gift from B. Black, University of Pennsylvania, Philadelphia, PA), and mouse anti-Mps1 monoclonal NT (1:100; 05-682; EMD Millipore).

Immunoblotting
Whole cell lysates were prepared from asynchronous populations of cells. Western blot analysis was performed using the following antibodies: rabbit anti-FKBP12 (1:2,000; ab2918; Abcam), rabbit anti-GFP polyclonal (1:10,000; a gift from B. Black), and mouse anti-tubulin monoclonal DM1α (1:10,000; Sigma-Aldrich).

Image acquisition and processing
For live imaging, cells were plated on 22 × 22-mm glass coverslips (no. 1.5; Thermo Fisher Scientific) coated with poly-L-lysine (Sigma-Aldrich). Coverslips were mounted in magnetic chambers (Chamlide CM221-1, LC) using L:1 5 medium without phenol red (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin. Temperature was maintained at 22°C. For Mad2 staining in Figs. 2, 4, and 3, cells were fixed in PBS + 3.7% formaldehyde and 0.5% Triton X-100 for 10 min. For BubR1 staining in Fig. 3, A3 cells were preextracted in PBS + 0.5% Triton X-100 for 10 min, then fixed in PBS + 3.7% formaldehyde and 0.5% Triton X-100 for 10 min. For Mps1 staining in Fig. 3, B cells were prefixed for 5 s in PBS + 3.7% formaldehyde, extracted in PBS + 0.5% Triton X-100 for 1 min, then fixed in PBS + 3.7% formaldehyde and 0.5% Triton X-100 for 10 min. Temperature was maintained at 22°C. For Mad2 staining in Figs. 2, 4, and 3, cells were fixed in PBS + 3.7% formaldehyde and 0.5% Triton X-100 for 10 min. For Mps1 staining in Fig. 3, B cells were prefixed for 5 s in PBS + 3.7% formaldehyde, extracted in PBS + 0.5% Triton X-100 for 1 min, then fixed in PBS + 3.7% formaldehyde and 0.5% Triton X-100 for 10 min. The following primary antibodies were used: rabbit polyclonal anti-Mad2 (1:500; PRB-652C; Covance), mouse monoclonal anti-Hec1 9G3 (1:1,000; ab3613; Abcam), rabbit anti-BubR1 monoclonal (1:1,000; a gift from W. Dai, New York Medical College, Valhalla, NY), rabbit anti–CENP-C polyclonal (1:1,000; a gift from B. Black, University of Pennsylvania, Philadelphia, PA), and mouse anti-Mps1 monoclonal NT (1:100; 05-682; EMD Millipore).

Online supplemental material
Fig. 1 shows the effects of endogenous FKBP knockdown on rapamycin-mediated recruitment to kinetochores. Fig. 2 shows a characterization of Mad1 truncation mutants. Fig. 3 shows that Mad2-binding is required for checkpoint activation by Mad1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201311113/DC1.

The authors declare no competing financial interests.

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References

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Figure S1.  Effects of endogenous FKBP knockdown on rapamycin-mediated recruitment to kinetochores. HeLa cells expressed Mis12-GFP-FKBP, mCherry-FRB, and either empty miRNA backbone or miRNA against the 3′ UTR of endogenous FKBP. (A) Cells were analyzed by immunoblotting for FKBP (top), GFP (middle), and tubulin (bottom) as a loading control. Arrows indicate expected bands for endogenous FKBP and Mis12-GFP-FKBP. Note that our Mis12-FKBP constructs include a tandem trimer of FKBP, resulting in a total molecular weight of 88 kD for Mis12-GFP-FKBP. miRNA expression in this system effectively depletes endogenous FKBP. (B and C) Cells were imaged before and ~1 min after treatment with 500 nM rapamycin (rap). (B) Images show mCherry at different expression levels of mCherry-FRB. In the presence of endogenous FKBP, mCherry-FRB recruitment is only observed at higher expression levels, but not at low expression levels (Fig. 1 C). (C) mCherry-FRB recruitment to kinetochores was quantified by measuring the increase in mCherry fluorescence (indicated by GFP signal, not depicted) after rapamycin treatment. Each data point represents a single cell; n ≥ 138 cells for each condition, pooled from three independent replicates. Knockdown of endogenous FKBP improves recruitment at all expression levels. (D) Cells expressing Mis12-GFP-FKBP, mCherry-FRB, and FKBP miRNA were imaged before and after treatment with 50 nM or 500 nM rapamycin, as indicated. Bars, 5 µm.
Figure S2. Characterization of Mad1 truncation mutants. Cells expressing Mis12-GFP-FKBP, FKBP miRNA, and FRB-mCherry-Mad1 fragments are shown as indicated. (A) In early prometaphase cells, full-length Mad1, but not N-terminal truncation mutants, localizes to unaligned kinetochores in the absence of rapamycin. Note: the Mad1ΔN-AAA cell was imaged separately (as indicated by the broken line), so localization can be compared with the other images, but not overall brightness. (B) Cells were imaged live before and after the addition of DMSO (t = 0) as a vehicle control to the rapamycin experiment in Fig. 4 C. Images are representative of at least three independent replicates.
Figure S3. Mad2 binding is required for checkpoint activation by Mad1. (A) Schematic representation of truncation mutant Mad1ΔN549 lacking the Mad2-binding region, compared with full-length Mad1. (B) Cells expressing Mis12-GFP-FKBP, FKBP miRNA, and FRB-mCherry-Mad1ΔN549 were treated with rapamycin or vehicle for 10 min, then fixed and stained for DNA, Mad2, and Hec1. Mad1ΔN549 does not recruit Mad2 to kinetochores after rapamycin treatment. (C) Parental HeLa cells and cells expressing Mis12-GFP-FKBP, FKBP miRNA, and the indicated FRB-mCherry-Mad1 constructs were treated with rapamycin for 10 min, then fixed and stained for DNA, Mad2, and Hec1. Mad2 levels at metaphase kinetochores were quantified in more than eight cells per condition in each of three independent replicates. Error bars represent SEM. (D and E) Cells expressing Mis12-GFP-FKBP, FKBP miRNA, and FRB-mCherry-Mad1ΔN549 are shown as indicated. (D) Cells were treated with rapamycin or vehicle at metaphase (t = 0), then monitored for anaphase onset for 38 min. (E) The graph shows the cumulative percentage of cells entering anaphase over time (n ≥ 31 cells, pooled from three independent replicates). Bars, 5 µm.