Cyclin B1 scaffolds MAD1 at the corona to activate the spindle assembly checkpoint

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ABSTRACT

The Cyclin B:CDK1 kinase complex is the master regulator of mitosis that phosphorylates hundreds of proteins to coordinate mitotic progression. We show here that, in addition to these kinase functions, Cyclin B also scaffolds a localised signalling pathway to help preserve genome stability. Cyclin B1 localises to an expanded region of the outer kinetochore, known as the corona, where it scaffolds the spindle assembly checkpoint (SAC) machinery by binding directly to MAD1. In vitro reconstitutions map the key binding interface to a few acidic residues in the N-terminus of MAD1, and point mutations in this region remove corona MAD1 and weaken the SAC. Therefore, Cyclin B1 is the long-sought-after scaffold that links MAD1 to the corona and this specific pool of MAD1 is needed to generate a robust SAC response. Robustness, in this context, arises because Cyclin B1-MAD1 localisation becomes MPS1-independent after the corona has been established. We demonstrate that this allows corona-MAD1 to persist at kinetochores when MPS1 activity fails, ensuring that it can still be phosphorylated on a key C-terminal catalytic site by MPS1. Therefore, this study explains how corona MAD1 generates a robust SAC signal and why stripping of this pool by dynein is essential for SAC silencing. It also reveals that the key mitotic kinase, Cyclin B1-Cdk1, scaffolds the pathway that inhibits its own degradation.

INTRODUCTION

During mitosis all duplicated chromosomes must attach correctly to microtubules so they can segregate properly when the cell divides. This attachment is mediated via the kinetochore, which is a giant molecular complex assembled on chromosomes at the centromere [1]. As well as attaching to microtubules, the kinetochore must also regulate this process to ensure it occurs correctly. One aspect of this regulation involves the activation of the spindle assembly checkpoint (SAC), which blocks mitotic exit until all kinetochores have attached to microtubules. The principle of the SAC is that each unattached kinetochore acts as a factory to produce an inhibitor of mitotic exit, known as the mitotic checkpoint complex or MCC (for further molecular details see [2]). The generation of MCC is so efficient that every single kinetochore signalling centre must eventually be extinguished by microtubule attachment to allow the cell to exit mitosis [3, 4].

This complicated inactivation process, known as SAC silencing, requires the removal of catalysts that are needed at unattached kinetochores to generate the MCC [5]. Two key catalysts in this regard are MAD1, which drives the first step in MCC assembly, and MPS1, the kinase responsible for recruiting and phosphorylating MAD1 as well as other components needed for MCC assembly. Kinetochore-microtubule attachment extinguishes these activities because microtubules displace MPS1 from its binding site on NDC80 [6, 7] and at the same time they provide a highway onto which dynein motors can travel to strip MAD1 away from kinetochores [8-11]. Removal of both MPS1 and MAD1 is essential for SAC silencing because if either one is artificially tethered to kinetochores then the SAC fails to switch off and mitotic exit is blocked [12, 13].

One key unexplained aspect of the SAC concerns the kinetochore binding sites for MAD1. MAD1 is recruited to kinetochores via an established KL1-BUB1 pathway and, in human cells, by an additional pathway involving the ROD/ZW10/Zwilch (RZZ) complex at the kinetochore’s corona (a fibrous crescent that forms around kinetochores to aid the capture of microtubules) [14]. How exactly MAD1 is recruited to the corona and whether this pool of MAD1 can signal to the SAC is unknown. It is crucial to resolve these issues because it is ultimately the RZZ complex that is stripped by dynein to shut down the SAC, implying that this pool of MAD1 is important for MCC generation [8-11]. However, the corona is positioned some distance away from MPS1 and the proposed catalytic centre for MCC generation at the KNL1/MIS12/NDC80 (KMN) network. Therefore, it remains unclear how MAD1 could signal to the SAC from the corona and it is difficult to resolve this issue without knowledge of how MAD1 binds to this region.

We show here that the key mitotic regulator—Cyclin B1/CDK1—acts as the physical adaptor that links MAD1 to the corona. This unanticipated scaffolding function of Cyclin B1 is crucial for a robust SAC response, which we demonstrate is due the ability of corona-tethered MAD1 to respond to low level of kinetochore MPS1 activity. This study therefore reveals how the corona pool of MAD1 signals to the SAC and it explains why MPS1 inhibition and dynein-mediated stripping of the corona are both essential for SAC silencing.

RESULTS

Cyclin B1-MAD1 interaction facilitates Cyclin B1 and MAD1 localisation to unattached kinetochores.

The Cyclin B:CDK1 kinase complex is a master regulator of mitosis that is activated during G2 phase of the cell cycle to initiate mitotic entry and degraded after chromosome alignment to induce mitotic exit. Analysis of endogenously-tagged Cyclin B1-EYFP localisation in RPE1 cells suggested that its localisation was specifically regulated during mitosis. In particular, Cyclin B1 positive foci appeared after nuclear envelope breakdown and disappeared as mitosis progressed (Figure 1A and Movie S1). Immunofluorescence analysis demonstrated that this localisation pattern reflects specific binding to unattached kinetochores, which is reminiscent of the checkpoint protein MAD1 (Figure 1B-C). In particular, Cyclin B depends on MPS1 activity to be established at this location, but thereafter it became largely insensitive to MPS1 inhibition (Figure 1D-E), as also shown previously for MAD1 [15]. To probe for MAD1 and Cyclin B1 association in cells, we recruited LacI-MAD1 to a LacO array on chromosome 1 in U2OS cells. This was sufficient to co-recruit Cyclin B1 in a manner that was dependent on a region between amino acids 41-92 of MAD1 (Figure 1F-G). Therefore, these data are consistent with earlier reports that Cyclin B1 localises to unattached kinetochores [16, 17] in a manner that is dependent on the N-terminus of MAD1 [18, 19]).

To determine the function of Cyclin B1 at kinetochores, we attempted to remove it from this location by knocking down endogenous MAD1 and replacing it with a Cyclin B1-binding defective mutant. However, all of the siRNAs tested only mildly reduced MAD1 protein (results not shown). This may be due to the fact that MAD1 is a very stable protein in cells because it takes over
Figure 1. Cyclin B1-MAD1 interaction helps both proteins to localise to unattached kinetochores. A. Endogenous Cyclin B-YFP localisation during mitosis live in RPE 1cells. B,C. Immunofluorescence images (B) and quantifications (C) of Cyclin B and MAD1 levels at unattached and attached kinetochores in cells arrested in STLC. Each dot represents a kinetochore, data is from 40 kinetochores. Vertical bars show the 95% confidence interval. D,E. Quantification of kinetochore intensities of Cyclin B1 and MAD1 in nocodazole-arrest cells (noco) treated with the MPS1 inhibitors, AZ-3146 (5 μM) or Reversine (500nM), either before (D) or after (E) mitotic entry. Each dot represents a cell and the errors bars display the variation between the experimental repeats (displayed as ±SD of the experimental means). 30 cells from 3 experiments. F. Immunofluorescence images of LacI-MAD1 and Cyclin B1 in U2OS cells containing a LacO arrays on chromosome 1. G. Live imaging of Cyclin B1-mCherry (CyclB1-mCh) in LacO-U2OS cells transfected with LacI-MAD1-FL (full length: aa1-718) or various LacI-Mad1 truncations (amino acid numbers indicated). H,I. Immunofluorescence images (H) and quantifications (I) of Cyclin B and MAD1 kinetochore levels in control (Mad1-WT) or Mad1β HeLa cells (two independent clones: C13 or C24) treated with nocodazole. J,K. Immunofluorescence images (J) and quantification (K) of Cyclin B and MAD1 kinetochore localisation in doxycycline-inducible MAD1α and β knockouts treated with or without dox for 10 days and then arrested in nocodazole. Cells were selected that had full MAD1 knockout in the doxycycline treatment (this constituted approximately 30% of cells). For kinetochore intensity graphs in panel D, E, I and K each dot represents a cell, horizontal lines indicate the median and vertical bars show the 95% confidence interval. D, E and I show 30 cells from 3 experiments and K shows 40 cells from 4 experiments.
a week to fully deplete MAD1 following genetic deletion; see [20]). Therefore, to attempt to fully remove Cyclin B1 from kinetochores, we generated a MAD1 Δ exon 4 knockout cell line that retains only a MAD1β splice variant lacking exon 4 which encodes the Cyclin B1 binding region (hereafter referred to as MAD1β cells; Figure S2) [21]. Surprisingly, Cyclin B1 was reduced but still present at unattached kinetochores in MAD1β cells (Figure 1H-I). This was not due to residual interaction with MAD1β because doxycycline-inducible knockout of both MAD1α and MAD1β [22] completely removed MAD1 from unattached kinetochores but did not further reduce kinetochore Cyclin B1 (Figure J-K; note, the data shown is from 10 days doxycycline treatment which is the minimum time it takes to fully deplete endogenous MAD1 in this system). Therefore, in contrast to a recent report [18], these data demonstrate that MAD1 contributes to Cyclin B1 kinetochore localisation, but it is not the only binding partner for Cyclin B1 at kinetochores. At least one other receptor exists that is sufficient to maintain substantial levels of Cyclin B1 on kinetochores in the absence of MAD1.

Although inhibiting MAD1-Cyclin B1 interaction did not abolish Cyclin B1 recruitment to kinetochores, it did cause a dramatic effect on MAD1 localisation itself. As discussed earlier, MAD1 localises to the kinetochores via two separate pathways in human cells: the KNL1-BUB1 pathway at the outer kinetochore and the RZZ pathway at the corona. Figure 1H shows that Cyclin B and MAD1 both bind to the expanded corona in wild type cells, but strikingly, when their interaction is prevented in MAD1β cells, it is MAD1, and not Cyclin B1, that is lost from the corona. Therefore, this suggested that Cyclin B1 may act as a scaffold to recruit MAD1 to this region. Although MAD1 is well-known to bind the corona [14, 23-31], an interaction with a corona component has never been mapped in vitro. In fact, the only established way to remove MAD1 from the corona is to deplete RZZ subunits, which simply abolishes corona formation altogether. Therefore, we next sought to explore whether Cyclin B1 might be the receptor that directly recruits MAD1 to the corona.

**Cyclin B1 directly scaffolds MAD1 at the corona**

We first assayed for direct MAD1 and Cyclin B1 interaction by obtaining homogeneously purified recombinant MAD1-MAD2 and Cyclin B1:CDK1 complexes and testing their interaction by size-exclusion chromatography (SEC), which separates proteins based on size and shape. When combined stoichiometrically with MBP-MAD1-MAD2, GST-CDK1:Cyclin B1 underwent a prominent shift in elution volume and co-eluted with MAD1-MAD2, indicative of a binding interaction (Figure 2A); MBP and GST are affinity tags that stand for maltose binding protein and glutathione S-transferase, respectively. Early elution of MAD1-MAD2 from the SEC column reflects its high hydrodynamic radius, typical of highly elongated structures rich in coiled-coil. As expected, the elution volume of MAD1-MAD2 was not affected by the interaction with GST-CDK1:Cyclin B1.

In the absence of Cyclin B1, GST-CDK1 did not interact directly with MAD1-MAD2 (Figure S3A). Cyclin B1, on the other hand, interacted with MAD1-MAD2, albeit more weakly than was observed with the GST-CDK1:Cyclin B1 complex (Figure S3B). Removal of residues 1-92 from MAD1 (MAD1Δ1-92:MAD2) abolished the interaction with Cyclin B1 (Figure S3C), indicating that residues 1-92 of MAD1 are necessary for the interaction. Importantly, the N-terminal region of MAD1 was also sufficient to bind Cyclin B1:CDK1, as revealed by SEC experiments with MBP-MAD1Δ1-92 and CDK1:Cyclin B1Δ165-433 (Figure 2B; note, the Cyclin B1 N-terminal region was removed in the experiments to improve yield, stability, and solubility). Therefore, MAD1 binds directly to Cyclin B1:CDK1 via a region located in the first 92 residues of MAD1.

To narrow this region down further, we performed additional truncations of MAD1Δ1-92, which led to the identification of a minimal MAD1 Δ cyclin B1 binding site in residues 41-62 of MAD1 (Figure S3D-F; the full list of tested constructs is in Table S1, Part I). Importantly, MAD1Δ1-62 interacted not only with CDK1:Cyclin B1Δ165-433, but also with the isolated Cyclin B1Δ165-433, thus confirming results in Figure S3 that Cyclin B1 contains the MAD1 binding site (Figure 2C and S3F). To identify determinants of the MAD1Δ1-62:Cyclin B1 interaction, we extensively mutagenized residues in the MAD1Δ1-62 segment and on Cyclin B1 (as summarized in Table S1, Parts II and III; selected examples are shown in Figure S3G-H). Charge reversals at three conserved negatively charged residues in MAD1Δ1-62 (E52, E53, E56) abolished binding to Cyclin B1 (Figure 2C-D). To identify potential binding partners on Cyclin B1 for the MAD1 residues E52, E53, and E56, we mutagenized various clusters of positively charged residues on the surface of Cyclin B1, without however identifying a sufficiently penetrant mutant (Table S1, Part III). Collectively, these results indicate that MAD1 and Cyclin B1:CDK1 interact directly, and that the interaction is mediated primarily or exclusively by residues 41-62 of MAD1 and by Cyclin B1. In addition, a conserved acidic patch in this N-terminal region of MAD1 is essential for Cyclin B1 interaction (Figure 2E).

To assess the effect of inhibiting Cyclin B1-MAD1 interaction in cells, we generated doxycycline-inducible vsv-tagged MAD1-WT or MAD1-3EK HeLa cells and used these to create MAD1 knockouts via CRISPR/Cas9 (with a gRNA targeting Exon 3 to knockout MAD1α and MAD1β; Figure 2F). MAD1 localisation was then assessed in nocodazole-arrested cells, which demonstrated that MAD1-WT and MAD1-3EK displayed a similar localisation pattern in early prometaphase, but only the MAD1-WT was able to localise to the corona when it formed in late prometaphase (Figures 2G). At this stage, MAD1-3EK levels decreased at the kinetochore (Figures 2G-H), which is consistent with the fact that the BUB1-dependent pool of MAD1 is reduced by PP2A as mitosis progresses [31]. Therefore, a MAD1-3EK mutant, which is unable to bind directly to Cyclin B1, is also unable to localise to the corona in nocodazole-arrested cells. This confirms that Cyclin B1 is the scaffold that recruits MAD1 to this region of the kinetochore in human cells. When the corona pool is removed in MAD1-3EK cells, MAD1 kinetochore recruitment is reduced soon after nuclear envelope breakdown (mirroring the localisation and phosphorylation of its other kinetochore receptor, BUB1) [31, 32]. Note, that we also generated YFP-tagged MAD1 cells (MAD1Δ1-62 and MAD1-3EK) but these were both absent from the corona, which suggests that a large N-terminal tag affects MAD1 localisation to this region (Figure S4). This may be why removing the N-terminus of GFP or mCherry-MAD1 was not reported to affect MAD1 kinetochore localisation in previous studies [18, 20]. It is also important to note that the N-terminal vsv-tag on MAD1 is not detected at the corona by immunofluorescence (results not shown), suggesting that this region maybe buried in an interaction interface.

**Corona-localised MAD1 generates a robust SAC response**

The ability of Cyclin B1 to recruit MAD1 to the corona could allow Cyclin B to generate the signal that inhibits its own degradation. However, it is unclear if corona-localised MAD1 can signal directly to the SAC and, if it can, how this differs from the conventional KNL1-BUB1-MAD1 pathway at the outer kinetochore. One major difference is that Cyclin B1-MAD1 localisation to the corona is insensitive to MPS1 inhibition after mitotic entry (Figure 1E), whereas MPS1 activity is continually required to phosphorylate KNL1 [33-36] and BUB1 [31, 37-41] to recruit MAD1 to the outer kinetochore. To investigate this major difference between the two pathways, we tested the response of MAD1-WT and MAD1-3EK cells to MPS1 inhibition. As expected [15], MAD1 was preserved on
kinetochores following MPS1 inhibition with AZ-3146 after mitotic entry in MAD1-WT cells (Figure 3A-C). However, in stark contrast, a MAD1-3EK mutant that cannot bind the corona was completely lost from kinetochores under identical conditions (Figure 3A-C). This has considerable impact on the SAC, because MAD1-3EK cells are exquisitely sensitive to MPS1 inhibition in nocodazole, as demonstrated by the fact that these cells immediately exit mitosis at a dose of AZ-3146 that can be tolerated for several hours in MAD1-WT cells (Figure 3D). These data demonstrate that Cyclin B1-MAD1 becomes insensitive to MPS1 inhibition once the corona has been established, which subsequently allows the SAC to tolerate substantial reductions in MPS1 activity.
Figure 3. Inhibiting Cyclin B1-MAD1 interaction weakens the SAC. A-C. Immunofluorescence images (A) and quantifications (B,C) of MAD1 and MAD2 kinetochore levels in indicated cells lines arrested in nocodazole and treated with/without AZ-3146 for 30 min to inhibit MPS1. In all kinetochore intensity graphs, each dot represents a cell, horizontal lines indicate the median and vertical bars show the 95% confidence interval. 60 cells (MAD1) and 30 cells (MAD2) from 3 experiments. D. Duration of mitotic arrest in indicated cell lines arrested in nocodazole and then treated with 1.25 µM AZ-3146. Graph shows cumulative mean (±SEM) of 3 experiments, 50 cells per condition per experiment.

Cyclin B scaffolds MAD1 at the corona to allow the SAC to tolerate MPS1 inhibition

As well as regulating MAD1 recruitment to the outer kinetochore, MPS1 activity is also needed to phosphorylate the C-terminal domain (CTD) of MAD1 and catalyse MCC assembly [37, 38, 42]. Therefore, we reasoned that MAD1 may still need to be phosphorylated by MPS1 to catalyse MCC assembly from the corona. Therefore, to probe this further, we raised a phospho-specific antibody to a key Thr716 MPS1-phosphorylation site on MAD1 and confirmed its specificity in cells (Figure S5). This antibody detects a strong signal at unattached kinetochores in RPE1 and HeLa cells, which is rapidly lost upon MPS1 inhibition (Figures 4A and S5C). Importantly, in nocodazole-arrested cells, although MAD1 decorates the whole corona, the MAD1-pT716 signal is restricted to the outer kinetochore around the KMN network (Figure 4A and S5C). This suggests that MPS1 has a limited zone of activity that is relatively confined to its anchor point on NDC80 [6, 7]. How then can the corona MAD1 support the SAC following MPS1 inhibition? We hypothesised that this MAD1, which is tethered to the corona via Cyclin B1 at its N-terminus, may be able to use its predicted coiled-coil to allow the CTD to reach the zone of MPS1 activity at NDC80 [14].

Therefore, we next set out to visualize the stable complex between MAD1:MAD2 and Cyclin B1:CDK1 by electron microscopy after low-
Figure 4. Cyclin B scaffolds MAD1 at the corona to allow the SAC to tolerate MPS1 inhibition. A. Immunofluorescence images of MAD1 and MAD1-pT716 kinetochore levels in nocodazole-arrested RPE1 cells. B. Electron micrographs of rotary-shadowed MAD1C-MAD2 (top row), MBP-MAD1-MAD2 (middle row), and MBP-MAD1:MAD2 in complex with GST-CDK1: Cyclin-B (bottom row). The scale bar indicates 50 nm. C. Quantifications (top) and corresponding immunofluorescence images (underneath) of kinetochore MAD1 and MAD1-pT716 levels in nocodazole-arrested MAD1-WT and MAD1-3EK treated with different doses of AZ-3146 for 30 min. MG132 was included at the time of AZ-3146 addition to prevent mitotic exit. Each dot represents a cell, horizontal lines indicate the median and error bars show the 95% confidence interval. 30 cells from 3 experiments. D,E. Duration of mitotic arrest in indicated cell lines arrested in nocodazole and then treated with 2.5 μM AZ-3146 (D) or 0.3 μM AZ-3146 (E) or. Graph shows cumulative mean (±SEM) of 3 experiments, 50 cells per condition per experiment.
angle metal shadowing. This highlighted the position of MAD2 near the C-terminal end of MAD1 and demonstrated that MAD1 adopted a thin elongated structure with an apparent length of ~66 nm (Figure 4B). Addition of the globular MBP (43 kDa) allowed the N-terminal end of full-length MAD1 to be recognized within MAD1:MAD2 complexes (Figure 4B). We then inspected a SEC fraction containing Cyclin B1:CDK1 bound to MBP-MAD1:MAD2 and identified an additional density near the N-terminal MBP in a number of complexes (Figure 4B). Thus, CDK1:Cyclin B1 binds the very end of an elongated MAD1:MAD2 complex and the opposite end, which is a substrate for MPS1 [37, 38, 42], lies approximately 66 nm away. This observation suggests that corona-localised MAD1 can still be phosphorylated by MPS1 at the KMN network as long as the anchor point for Cyclin B1 is within ~66 nm of NDC80.

We hypothesised that corona-localised MAD1 could therefore help the SAC to tolerate MPS1 inhibition because, despite the fact the KNL1-MELT and BUB1 are dephosphorylated, corona-MAD1 is still preserved at kinetochores to respond to low levels of MPS1 activity. To test this hypothesis, we stained for MAD1 and MAD1-pT716 in nocodazole-arrested MAD1-WT or MAD1-3EK cells treated with a range of doses of the MPS1 inhibitor AZ-3146. Figures 4C and S6A show that total MAD1 protein and MAD1-pT716 are removed together from kinetochores at very low doses of MPS1 inhibitor in MAD1-3EK cells. However, the MAD1 is preserved at kinetochores following MPS1 inhibition in WT cells, which allows MAD1 phosphorylation to persist until much higher doses of AZ-3146. Quantifying mitotic exit over this dose range shows that the duration of mitotic arrest correlates with the amount of MAD1-pT716 at kinetochores (Figure S6). A persistent mitotic arrest is seen whenever MAD1-pT716 is detected on kinetochores, which equates to ≤0.3 μM AZ-3146 in MAD1-3EK cells and ≤2.5 μM AZ-3146 in MAD1-WT cells. In fact, the length of mitotic arrest is very similar in MAD1-3EK and MAD1-WT cells treated with 0.3 μM and 2.5 μM AZ-3146, respectively (Figure 4D-E), and these two conditions give almost identical levels of MAD1-pT716 (Figures 4C and S6A). The actual MAD1-pT716 signal in these two situations is just detectable on kinetochores, which presumably reflects a minimal sufficient amount to generate a SAC response.

**DISCUSSION**

We show here that Cyclin B1 allows the SAC to tolerate substantial MPS1 inhibition by tethering MAD1 at the corona in a manner that is MPS1-independent once the corona has been established. Although this distinguishes corona-localised MAD1 from the canonical pool at the KMN network, it most likely behaves similarly in all other respects, because MPS1 phosphorylation at the C-terminus of MAD1 is most likely still required to catalyse MCC assembly [37, 38, 42]. We speculate that the thin elongated structure of MAD1 facilitates this process by providing the necessary reach to orient MAD2 and the MAD1 CTD towards MPS1 at the KMN network.

This link between corona MAD1 and the kinetochore SAC signal explains why end-on microtubule attachments rapidly silence the SAC in human cells: microtubules compete with MPS1 for NDC80 binding [6, 7], and in addition, they allow dynein motors to remove corona-localised MAD1 away from the zone of MPS1 activity [8-11]. Both components are necessary to fully extinguish the SAC because preventing either MPS1 or MAD1 removal from kinetochores is sufficient to also prevent SAC silencing [12, 13]. The final model is presented in figure 5.

There are two other recent studies that also demonstrate that MAD1-Cyclin B1 interaction is important during mitosis, but for different reasons. Alfonso-Peres et al demonstrated that knockdown of MAD1, or removal of its first 100 amino acids, causes a partial SAC defect in nocodazole and reduces the amount of Cyclin B1 and MPS1 on kinetochores (by approximately 75% and 50%, respectively) [18]. It should be noted, however, that the concentration of nocodazole used in these studies (0.3μM) may be insufficient to fully depolymerise microtubules [43], which complicates interpretations about direct effects on the SAC and on MAD1/Cyclin B1/MPS1 localisation to unattached kinetochores. Furthermore, removal of the N-terminal region of MAD1 has previously been shown to affect MCC assembly from the nuclear pore [20], which may also have contributed to the observed SAC defects. Nevertheless, the authors put forward an important hypothesis by proposing that kinetochore Cyclin B1/CDK1 may drive localised CDK1 activity to support the SAC; for example, by increasing CDK1-mediated phosphorylation of MPS1 on Ser281 to enhance MPS1 localisation (see accompanying paper by the same groups [44]). Although kinetochore localised Cyclin B1 has not so far been demonstrated to drive CDK1 substrate phosphorylation at kinetochores, it will be important to test this hypothesis in future because, if it does, then this has the potential to impact on many processes, including the SAC. As well as MPS1 localisation, CDK1 positively and negatively regulates a number of other key enzymes at the kinetochore (for review see: [45]), and therefore these substrates may change dramatically upon kinetochore-microtubule attachment when Cyclin B1/MAD1 is stripped away down microtubules. Furthermore, the removal of the corona itself may depend on localised CDK1 inactivation because acute inhibition of CDK1 is known to cause premature corona detachment [27, 46]. To address the potential importance of such localised kinetochore CDK1 regulation, however, we believe it will be crucial to first identify the other receptor(s) for Cyclin B1 at the corona so that this pool can then be fully removed from unattached kinetochores.

Very recent data from Jackman et al has also demonstrated the importance of Cyclin B1-MAD1 interaction during mitosis, but this time in the release of MAD1 from the nuclear pore [19]. In this study, preventing the interaction delays MAD1 accumulation at kinetochores until late prophase, as well as weakening the SAC and enhancing the level of chromosomal instability (CIN). The authors use an elegant approach to mutate two key acidic residues in MAD1 (E53K/E56K) at the endogenous locus of RPE cells by CRISPR/Cas9. Although this reduces the amount of Cyclin B1 that co-precipitates with MAD1, it is possible that the additional E52K mutation included in our 3EK mutant may produce a more penetrant phenotype, since all three glutamates lie on the same face of the predicted helix in MAD1 [19]. Nevertheless, the subsequent results on MAD1 dissociation from the nuclear pore are entirely consistent with our data, since these focus on an earlier stage of mitosis and the authors do not examine the effects later in prometaphase at unattached kinetochores. It is likely, therefore, that Cyclin B1-MAD1 functions at the nuclear pore in early mitosis and then again at the corona following nuclear envelope breakdown to safeguard chromosome segregation and prevent CIN. It will be interesting to determine whether this interaction is commonly perturbed in cancer cells that frequently missegregate their chromosomes to become chromosomally unstable.

In summary, this study reveals how the main mitotic kinase, Cyclin B1/CDK1, plays a key role in scaffolding the SAC machinery to the corona. Considering that Cyclin B1 is ultimately degraded by the APC/C once the SAC has been silenced, this important scaffolding function most likely helps to ensure the SAC cannot be re-established following anaphase onset.
Figure 5. Model for how Cyclin B1-MAD1 at the corona helps the SAC to respond to low levels of MPS1 activity. A. On unattached kinetochore MAD1 is recruited via MPS1-phosphorylated BUB1 at the KMN network and Cyclin B1 at the corona. B. If MPS1 activity is lowered without microtubule attachment, then the BUB1-dependent pool of MAD1 is removed at the KMN network but the corona MAD1 pool persists to respond to low level of MPS1 activity and generate a SAC signal. C. When microtubules attach then MPS1 activity is lowered and the corona is stripped to remove all MAD1 and silence the localised SAC signal.

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AUTHOR CONTRIBUTIONS
A.T.S discovered the MAD1-Cyclin B1 connection whilst working in the lab of G.K. (figures 1A-C, 1F-G). L.A. performed most other cell biology experiments, with important contributions from M.R. The in vitro biochemistry was performed by Y.L. and the EM of MAD1 by P.H.V., under the supervision of A.M. A.T.S. wrote the manuscript with comments from all authors.
MATERIALS AND METHODS

Cell culture and reagents. RPE1 were purchased from ATCC and HeLa Flp-in cells were a gift from S Taylor (University of Manchester, UK) [47]. The RPE1 Cyclin B1-EYFP cells have been published previously [48], as have the U2OS with LacO array on chromosome 1 [49]. All cells were authenticated by STR profiling (Eurofins) and screened every 4-8 weeks to ensure they were mycoplasma free. Cells were cultured in DMEM supplemented with 9% FBS and 50 µg/ml penicillin/streptomycin. Except during fluorescence time-lapse analysis, when they were cultured in Leibovitz’s L-15 media (900 mL D+ Galactose, 5mM Sodium Pyruvate, no phenol red). Doxycycline (1µg/ml), STL C (S-Trityl-L-cystiene: 10 µM) and thymidine (2 mM) were purchased from Sigma-Aldrich, nocardazole (3.3 µM) from Millipore, puromycin and hygromycin B from Santa Cruz Biotechnology, MG132 (10 µM) from Selleckbio, AZ -3146 (at indicated concentrations) from Axon, Rapamycin (100nM) from LC Laboratories, and reversionis (at indicated concentrations) from Cayman Chemicals.

Generation of knockout cell lines by CRISPR/Cas9 gene editing. To generate MAD1Δα knockout cells (i.e. MAD1β cells), a guide RNA targeting exon 4 of MAD1 (CCGCTCACCTGGATGAGTGGG) was cloned into a lentiviral vector that co-expresses Cas9 and a puromycin resistivis marker (pLentiCRISPRv2; addgene #52961) to create pLentiCas9-9-g4-MAD1. Cyclin B-YFP-FKBP HeLa Flp-in cells (generated by CRISPR/Cas9-mediated homologous recombination) were transfected with pLentiCas9-9-g4 plasmid and selected in puromycin to obtain single cell clones. These were screened for the absence of nuclear MAD1 by immunofluorescence, since only MAD1Δα, and not MAD1Δβ, is localised to the nucleus in interphase [21], and 2 clones were subsequently validated (C13 and C24; Figure S2). Note, the original aim was to target endogenous MAD1 and replace exon 4 with an FRB cassette to lose Cyclin B interaction and regain it upon rapamycin addition (to induce Cyclin B-YFP-FKBP interaction). The knock-in gene-editing was however unsuccessful, therefore MAD1Δα knockouts were used instead. To generate doxycycline-inducible vsv or YFP-MAD1 WT and -3EK cell lines HeLa Flp-in cells were transfected with Mad1 in pCDNAs/FRT/T0 vector (Invitrogen) together with the FLP recombinase, pOG44 (Invitrogen) using Eugene HD (Promega) according to the manufacturer’s protocol. Stable transfecants were selected in media containing 200 µg/mL hygromycin-B. Subsequently, to knock-out endogenous Mad1, these cells were transfected with a guide RNA targeting exon 3 of Mad1 (CTTCATCTCAGCGTTGGG) in pLentiCRISPRv2 for 24h and thereafter selected in puromycin for a further 24h. Cells were then cultured continually in the presence of Dox to maintain viability after knock-out of endogenous Mad1 by inducing expression of vsv- or YFP-Mad1 WT or 3EK. Individual clones were isolated and screened for loss of endogenous Mad1 by western analysis for vsv-tagged Mad1 (Figure S4) and 10 days after removal of Dox for vsv-tagged Mad1 (Figure 2F). 2 clones for each construct were validated and used subsequently (vsv-Mad1 WT: C1 & 13; vsv-Mad1 3EK: C14 & C20; YFP-Mad1 WT: C5 & C19; YFP-Mad1 3EK: C6 & C18).

Antibodies. The following primary antibodies were used for immunofluorescence imaging (at the indicated final concentration diluted in 3% BSA in PBS): chicken α-GFP (ab13997 from Abcam, 1:5000), guinea pig α-Cenp-C (BT20278 from Caltag + Medsystems), human ACA (90C-CS1058 from Fitzgerald, 1:2000), rabbit Cyclin B1 (#122315 from Cell signalling technology, 1:1000), mouse Mad1 (clone BB3-8, MABE867 from Millipore, 1:1000), mouse Tubulin (clone 5-12 from Sigma, 1:5000), rabbit ZW10 (ab21582 from abcam, 1:1000), MAD2 (A300-301A-T from Bethyl, 1:1000). The MAD1-pT716 used in this study (MAD1-pT716-p1) was a custom rabbit polyclonal phospho-specific antibody generated by Biomatik. Secondary antibodies used for immunofluorescence were highly-cross absorbed goat, α-chicken, α-rabbit, α-mouse or a-guinea pig coupled to Alexa Fluor 488, Alexa Fluor 568, or Alexa Fluor 647 (Thermo Fischer). The primary antibodies used for Western blotting were Actin (A2066 from Sigma, 1:5000), FKBPD12 (Clone H-5 from Santa Cruz, 1:250), mouse Mad1 (clone BB3-8, MABE867 from Millipore, 1:5000), tubulin (clone 5-12-L from Sigma, 1:5000), GST (clone B-326, MA4-004 from Thermo Fischer, 1:1000) and Cyclin B (C8831 from Sigma, 1:1000). The secondary antibodies used for Western blotting were goat β-mouse IgG HRP conjugate (170-6516 from Bio-Rad; 1:2000) and goat β-rabbit IgG HRP conjugate (170-6515 from Bio-Rad; 1:5000).

Time-lapse analyses. For fluorescence imaging, cells were imaged in 8-well chamber slides (ibidi), on either a Zeiss Axio Observer 7 with a CMOS Orca flash 4.0 camera or a DELTAvision Elite equipped with Photometrics CascadeII-1024 EMCCD or CoolSNAP HQ (Photometrics) camera. The objectives used for fluorescent imaging where either 20x/0.8 NA or 40x/1.3NA. For brightfield imaging, cells were imaged in a 24-well plate in DMEM on a Zeiss Axiosvert 200M using Hamamatsu ORCA-ER camera and controlled by Micro-manager software (open source: https://mijro-manager.org/), or a Zeiss Axio Observer 7 as detailed above. The air objectives used for brightfield imaging were either 10x/0.5 NA or 20x/0.4 NA. Mitotic exit was defined by cells flattening down in the presence of nocardazole and Mps 1 inhibitor.

Immunofluorescence. Cells, plated on High Precision 1.5H 12-mm coverslips (Marienfeld), were treated and then pre-extracted with 0.1% Triton X-100 in PEM (100 mM Pipes, pH 6.8, 1 mM MgCl2 and 5 mM EGTA) for 1 minute before addition of 4% paraformaldehyde (PFA) in PBS for 10 min. Experiments involving MAD1-pT716 were not pre-extracted and fixed directly in 4% PFA Coverslips were washed with PBS and blocked with 3% BSA in PBS + 0.5% Triton X-100 for at least 30 min. Thereafter coverslips were incubated with primary antibodies overnight at 4°C, washed with PBS and incubated with secondary antibodies plus DAPI (4,6-diamidino2-phenylindole, Thermo Fischer) for an additional 2-4 hours at room temperature in the dark. Coverslips were then washed with PBS and mounted on glass slides using ProLong antifade reagent (Molecular Probes). All images were acquired on a DeltaVision Core or Elite system equipped with a heated 37°C chamber, with a 100x/1.4 NA Plan S Apochromat objective using softWoRx software (Applied precision). Images were acquired at 1x1 binning using a CoolSNAP HQ or HQ2 camera (Photometrics) and processed using softWorx software and ImageJ (National Institutes of Health). All immunofluorescence images displayed maximum intensity projections of deconvoluted stacks and were chosen to most closely represent the mean quantified data.

Image quantification. For quantification of immunostainings, all images to be compared directly were acquired with identical illumination settings. An ImageJ macro was used to threshold and select all kinetochores and all chromosome areas (excluding kinetochores) using the DAPI and anti-kinetochore antibody channels, as previously [50]. This was used to calculate the relative mean kinetochore intensity of various proteins ([kinetochores-chromosome arm intensity (test protein)] / [kinetochores-chromosome arm intensity (CENP-C)]. For the quantification of kinetochore localisation on attached or unattached kinetochore, cells were arrested in STLC and MAD1 intensity used to define attached (MAD1 negative) or unattached (MAD1 positive) kinetochores. The signal in the MAD1 and Cyclin B channel was then expressed as a percentage of CenPC on these individual kinetochores (after normalising for surrounding background intensities). Graphs were generated using either Graphpad or PlotsOfData [51].

DNA cloning, Protein Expression and Purification. Constructs for
the recombinant expression of GST-CDK1-Cyclin B1 and CDK1-Cyclin B1<sup>1413</sup> complexes were gifts of Jane Endicott and Martin Noble, and the complex was expressed and purified as previously described [52]. The MBP-MAD1:MAD2 complex and MAD1<sup>718</sup>-MAD2 complex were expressed and purified as described [37]. DNA sequences encoding MAD1 N-terminal fragments were amplified from a full-length MAD1 synthetic gene encoding the HsMAD1 sequence [37] by the polymerase chain reaction (PCR) and sub-cloned into the previously generated pETDuet-MBP8His vector [53]. All the mutually versions of recombinant proteins were produced by QuickChange Mutagenesis kit to mutate the plasmid DNA (Agilent Technologies). The MAD1 fragments and Cyclin B1 were transformed into BL21(DE3) Rosetta 2 competent cells. Cells were grown in Terrific broth at 37°C to an OD<sub>600</sub> of about 1.2. Protein expression was induced by addition of 1 mM IPTG at 37°C, and cells were further allowed to grow for 5 hours. Cell pellets were resuspended in binding buffer (20 mM Tris/HCl pH 8.0, 500 mM NaCl, 5% (v/v) glycerol, 1 mM EDTA, 1 mM TCEP), lysed by sonication and cleared by centrifugation at 10000g for 45 min. The cleared supernatant was purified through Histrap HP and gel-filtered on a Superdex 200 10/300 columns (GE Healthcare). The Lact-GFP-MAD1 fragments were generated by PCR amplification of MAD1 and insertion into the Lact-GFP vector [54]. To create the guide RNA-resistant MAD1-WT and 3EK plasmids, gene blocks were synthesised that encode for amino acids 1-97 of MAD1, with or without mutations to change E52K, E53K and E56K (denoted as 3EK), and containing additional silent mutations in the gRNA sequences. These gene blocks were inserted into full length MAD1 by Gibson assembly to replace the region encoding for amino acids 1-97.

**Analytical Size-Exclusion Chromatography.** All samples were eluted under isocratic conditions at 4°C in SEC buffer (20 mM Tris, 150 mM NaCl, 1 mM TCEP) at a flow rate of 0.1 ml/min. The elution profiles of proteins were monitored at 280 nm. To form the complex, proteins were mixed at 1:1 molar ration and a typical concentration of 10 μM) and incubated for 1 hour on ice. The loading volume for each injection was 25 μl and the final concentration of each protein is 5 μM. SDS-PAGE, followed by Coomassie staining, was used to detect proteins.

**Low-angle metal shadowing and electron microscopy.** Fractions from size-exclusion chromatography containing protein complexes of interest were diluted 1:1 with spraying buffer (200 mM ammonium acetate and 60% glycerol) and air-sprayed onto freshly cleaved mica pieces (V1 quality, Plano GmbH). Specimens were mounted and dried in a MEDD020 high-vacuum metal coater (Bal-tec). A Platinum layer of approximately 1 nm and a 7 nm Carbon support layer were evaporated subsequently onto the rotating specimen at angles of 6-7° and 45° respectively. Pt/C replicas were released from the mica on water, captured by freshly glow-discharged 400-mesh Pd/Cu grids (Plano GmbH), and visualized using a LaB<sub>6</sub> equipped JEM-1400 transmission electron microscope (JEOL) operated at 120 kV. Images were recorded at a nominal magnification of 60000x on a 4k x 4k CCD camera F416 (TVIPS), resulting in 0.18 nm per pixel. Representative particles were manually selected using EMAN2 [55].

**REFERENCES**

1. Musacchio, A., and Desai, A. (2017). A Molecular View of Kinetochore Assembly and Function. Biology (Basel) 6, 1-35.
2. Corbett, K.D. (2017). Molecular Mechanisms of Spindle Assembly Checkpoint Activation and Silencing. Prog Mol Subcell Biol 56, 429-455.
3. Dick, A.E., and Gerlich, D.W. (2013). Kinetic framework of spindle assembly checkpoint signalling. Nat Cell Biol 15, 1370-1377.
4. Rieder, C.L., Cole, R.W., Khodjakov, A., and Sluder, G. (1995). The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. J Cell Biol 130, 941-948.
5. Etemad, B., and Kops, G.J. (2016). Attachment issues: kinetochore transformations and spindle checkpoint silencing. Curr Opin Cell Biol 39, 101-108.
6. Hiruma, Y., Sacristán, C., Pachis, S.T., Adamopoulos, A., Kuijt, T., Ubbink, M., von Castelmuir, E., Perrakis, A., and Kops, G.J. (2015). CELL DIVISION CYCLE. Competition between MPS1 and microtubules at kinetochores regulates spindle checkpoint signaling. Science 348, 1264-1267.
7. Ji, Z., Gao, H., and Yu, H. (2015). CELL DIVISION CYCLE. Kinetochore attachment sensed by competitive Mps1 and microtubule binding to Ndc80C. Science 348, 1260-1264.
8. Howells, B.J., McEwen, B.F., Canman, J.C., Hoffman, D.B., Farrar, E.M., Rieder, C.L., and Salmon, E.D. (2001). Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. J Cell Biol 155, 1159-1172.
9. Mische, S., He, Y., Ma, L., Li, M., Serr, M., and Hays, T.S. (2008). Dynemin light intermediate chain: an essential subunit that contributes to spindle checkpoint inactivation. Mol Biol Cell 19, 4918-4929.
10. Sivaram, M.V., Wadzinski, T.L., Redick, S.D., Manna, T., and Doxsey, S.J. (2009). Dynemin light intermediate chain 1 is required for progress through the spindle assembly checkpoint. EMBO J 28, 902-914.
11. Wojcik, E., Basto, R., Serr, M., Scaerou, F., Karess, R., and Hays, T. (2001). Kinetochore dynein: its dynamics and role in the transport of the Rough deal checkpoint protein. Nat Cell Biol 3, 1001-1007.
12. Jelluna, N., Dansen, T.B., Sliedrecht, T., Kwiatkowski, N.P., and Kops, G.J. (2010). Release of Mps1 from kinetochores is crucial for timely anaphase onset. J Cell Biol 191, 281-290.
13. Maldonado, M., and Kapoor, T.M. (2011). Constitutive Mad1 targeting to kinetochores uncouples checkpoint signalling from chromosome biorientation. Nat Cell Biol 13, 475-482.
14. Luo, Y., Ahmad, E., and Liu, S.T. (2018). MAD1: Kinetochore Receptors and Catalytic Mechanisms. Front Cell Dev Biol 6, 51.
15. Hewitt, L., Tighe, A., Santaguida, S., White, A.M., Jones, C.D., Musacchio, A., Green, S., and Taylor, S.S. (2010). Sustained Mps1 activity is required in mitosis to recruit O-Mad2 to the Mad1-C-Mad2 core complex. J Cell Biol 190, 25-34.
16. Bentley, A.M., Normand, G., Hoyt, J., and King, R.W. (2007). Distinct sequence elements of cyclin B1 promote localization to chromatin, centrosomes, and kinetochores during mitosis. Mol Biol Cell 18, 4847-4858.
17. Chen, Q., Zhang, X., Jiang, Q., Clarke, P.R., and Zhang, C. (2008). Cyclin B1 is localized and regulates spindle checkpoint signaling. J Cell Biol 176, 268-280.
18. Alfonso-Perez, T., Hayward, D., Holder, J., Gruneberg, U., and Barr, F.A. (2019). MAD1-dependent recruitment of CDK1-CCNB1 to kinetochores promotes spindle checkpoint signaling. J Cell Biol.
32. Rodríguez-Bravo, V., Maciejowski, J., Corona, J., Buch, H.K., Collin, P., Kanemaki, M.T., Shah, J.V., and Jallepalli, P.V. (2014). Nuclear pores protect genome integrity by assembling a premitotic and Mad1-dependent anaphase inhibitor. Cell 156, 1017-1031.

33. Sze, K.M., Ching, Y.P., Jin, D.Y., and Ng, I.O. (2008). Role of a novel splice variant of mitotic arrest deficient 1 (MAD1), MAD1beta, in mitotic checkpoint control in liver cancer. Cancer Res 68, 9194-9201.

34. McKinley, K.L., and Cheeseman, I.M. (2017). Large-Scale Analysis of CRISPR/Cas9 Cell-Cycle Knockouts Reveals the Diversity of p53-Dependent Responses to Cell-Cycle Defects. Developmental cell 40, 405-420 e402.

35. Caldás, G.V., Lynch, T.R., Anderson, R., Afreen, S., Varma, D., and Deluca, J.G. (2015). The RZZ complex requires the N-terminus of KNL1 to mediate optimal Mad1 kinetochore localization in human cells. Open Biol 5.

36. Kops, G.J., Kim, Y., Weaver, B.A., Mao, Y., McLeod, I., Yates, J.R., 3rd, Tagaya, M., and Cleveland, D.W. (2005). ZW10 links mitotic checkpoint signaling to the structural kinetochore. J Cell Biol 169, 49-60.

37. Buffin, E., Lefebvre, C., Huang, J., Gagou, M.E., and Karess, R.E. (2005). Recruitment of Mad2 to the kinetochore requires the Rod/Zw10 complex. Curr Biol 15, 856-861.

38. Rodríguez-Rodriguez, J.A., Lewis, C., McKinley, K.L., Sikirzhytski, V., Corona, J., Maciejowski, J., Khodjakov, A., Cheeseman, I.M., and Jallepalli, P.V. (2018). Distinct Roles of Rzz and Bub1-KNl1 in Mitotic Checkpoint Signaling and Kinetochore Expansion. Curr Biol 28, 3422-3429 e3425.

39. Pereira, C., Reis, R.M., Gama, J.B., Celestino, R., Cheerambathur, D.K., Carvalho, A.X., and Gassmann, R. (2018). Self-Assembly of the RZZ Complex into Filaments Drives Kinetochore Expansion in the Absence of Microtubule Attachment. Curr Biol 28, 3408-3421 e3408.

40. Sacristan, C., Ahmad, M.U.D., Keller, J., Fermie, J., Groenewold, V., Tromer, E., Fish, A., Melero, R., Carazo, J.M., Klumperman, J., et al. (2018). Dynamic kinetochore size regulation promotes microtubule capture and chromosome biorientation in mitosis. Nat Cell Biol 20, 800-810.

41. Sillo, V., McAinsh, A.D., and Millar, J.B. (2015). KNL1-Bubs and RZZ Provide Two Separable Pathways for Checkpoint Activation at Human Kinetochores. Developmental cell 35, 600-613.

42. Wynne, D.J., and Funabiki, H. (2015). Kinetochore function is controlled by a phospho-dependent coexpression of inner and outer components. J Cell Biol 210, 899-916.

43. Qian, J., Garcia-Gimeno, M.A., Beullens, M., Manzoni, M.G., Van der Hoeven, G., Igual, J.C., Heredia, M., Sanz, P., Gelen, L., and Bollen, M. (2017). An Attachment-Independent Biochemical Timer of the Spindle Assembly Checkpoint. Molecular cell 68, 715-730 e715.

44. Nijenhuis, W., Vallardi, G., Teixeira, A., Kops, G.J., and Saurin, A.T. (2014). Negative feedback at kinetochores underlies a responsive spindle checkpoint signal. Nat Cell Biol 16, 1257-1264.

45. London, N., Ceto, S., Ranish, J.A., and Biggins, S. (2012). Phosphoregulation of Spc105 by Mps1 and PP1 regulates Bub1 localization to kinetochores. Curr Biol 22, 900-906.

46. Shepperd, L.A., Meadows, J.C., Sochaj, A.M., Lancaster, T.C., Zou, J., Buttrick, G.J., Rappaport, J., Hardwick, K.G., and Millar, J.B. (2012). Phosphodependent recruitment of Bub1 and Bub3 to Spc7/KNl1 by Mph1 kinase maintains the spindle checkpoint. Curr Biol 22, 891-899.

47. Yamagishi, Y., Yang, C.H., Tanno, Y., and Watanabe, Y. (2012). MPS1/Mph1 phosphorylates the kinetochore protein KNL1/Spc7 to recruit SAC components. Nat Cell Biol 14, 746-752.

48. Vleugel, M., Omerzu, M., Groenewold, V., Hadders, M.A., Lens, S.M., and Kops, G.J. (2015). Sequential multisite phospho-regulation of KNL1-BUB3 interfaces at mitotic kinetochores. Molecular cell 57, 824-835.

49. Faessen, A.C., Thanasoula, M., Maffini, S., Breit, C., Muller, F., van Gerwen, S., Bange, T., and Musacchio, A. (2017). Basis of catalytic assembly of the mitotic checkpoint complex. Nature 542, 498-502.

50. Ji, Z., Gao, H., Jia, L., Li, B., and Yu, H. (2017). A sequential multi-target Mps1 phosphorylation cascade promotes spindle checkpoint signaling. eLife 6.

51. Zhang, G., Kruse, T., Lopez-Mendez, B., Sylvestersen, K.B., Garvanska, D.H., Schopper, S., Nielsen, M.L., and Nilsson, J. (2017). Bub1 positions Mad1 close to KNL1 MELT repeats to promote checkpoint signalling. Nature Communications 8, 15822.

52. London, N., and Biggins, S. (2014). Mad1 kinetochore recruitment by Mps1-mediated phosphorylation of Bub1 signals the spindle checkpoint. Genes Dev 28, 140-152.

53. Mora-Santos, M.D., Hervas-Aguilar, A., Sewart, K., Lancaster, T.C., Meadows, J.C., and Millar, J.B. (2016). Bub3-Bub1 Binding to Spc7/KNl1 Toggles the Spindle Checkpoint Switch by Licensing the Interaction of Bub1 with Mad1-Mad2. Curr Biol 26, 2642-2650.

54. Ji, W., Luo, Y., Ahmad, E., and Liu, S.T. (2018). Direct interactions of mitotic arrest deficient 1 (MAD1) domains with each other and MAD2 conformers are required for mitotic checkpoint signaling. J Biol Chem 293, 484-496.

55. Yang, Z., Kenny, A.E., Brito, D.A., and Rieder, C.L. (2009). Cells satisfy the mitotic checkpoint in Taxol, and do so faster in concentrations that stabilize syntelic attachments. J Cell Biol 186, 675-684.

56. Hayward, D., Alfonso-Perez, T., Cundell, M.J., Hopkins, M., Holder, J., Bancroft, J., Hutter, L.H., Novak, B., Barr, F.A., and Gruneberg, U. (2019). CDK1-CCNB1 creates a spindle checkpoint-permissive state by enabling MPS1 kinetochore localization. J Cell Biol 218, 1182-1199.

57. Saurin, A.T. (2018). Kinase and Phosphatase Cross-Talk at the Kinetochore. Front Cell Dev Biol 6, 62.

58. Sacristan, C., Ahmad, M.K., Keller, J., Fermie, J., Groenewold, V., Tromer, E., Fish, A., Melero, R., Carazo, J.M., Klumperman, J., et al. (2018). Dynamic kinetochore size regulation promotes microtubule capture and chromosome biorientation in mitosis. Nat Cell Biol 20, 800-810.

59. Tirand, C., O'Neill, K., and Millar, J.B. (2018). Phosphorylation of Mps1 promotes structural and functional changes of the kinetochore. J Cell Biol 217, 1717-1728.

60. Shaltiel, I.A., Apriela, M., Saurin, A.T., Chowdhury, D., Kops, G.J., Voest, E.E., and Medema, R.H. (2014). Distinct phosphatases antagonize the p53 response in different phases of the cell cycle. Proceedings of the National Academy of Sciences of the United States of America 111, 7313-7318.

61. Janicki, S.M., Tsukamoto, T., Salghetti, S.E., Tansey, W.P., Sachidanandam, R., Prasanth, K.V., Ried, T., Shav-Tal, Y., and McKinley, K.J., Voest, E.E., and Medema, R.H. (2014). Distinct phosphatases antagonize the p53 response in different phases of the cell cycle. Proceedings of the National Academy of Sciences of the United States of America 111, 7313-7318.
Bertrand, E., Singer, R.H., et al. (2004). From silencing to gene expression: real-time analysis in single cells. Cell 116, 683-698.

50. Saurin, A.T., van der Waal, M.S., Medema, R.H., Lens, S.M., and Kops, G.J. (2011). Aurora B potentiates Mps1 activation to ensure rapid checkpoint establishment at the onset of mitosis. Nature communications 2, 316.

51. Postma, M., and Goedhart, J. (2019). PlotsOfData-A web app for visualizing data together with their summaries. PLoS Biol 17, e3000202.

52. Brown, N.R., Korolchuk, S., Martin, M.P., Stanley, W.A., Moukhametzianov, R., Noble, M.E.M., and Endicott, J.A. (2015). CDK1 structures reveal conserved and unique features of the essential cell cycle CDK. Nature communications 6, 6769.

53. Liu, Y., Petrovic, A., Rombaut, P., Mosalaganti, S., Keller, J., Raunser, S., Herzog, F., and Musacchio, A. (2016). Insights from the reconstitution of the divergent outer kinetochore of Drosophila melanogaster. Open Biol 6, 150236.

54. Vleugel, M., Tromer, E., Omerzu, M., Groenewold, V., Nijenhuis, W., Snel, B., and Kops, G.J. (2013). Arrayed BUB recruitment modules in the kinetochore scaffold KNL1 promote accurate chromosome segregation. J Cell Biol 203, 943-955.

55. Tang, G., Peng, L., Baldwin, P.R., Mann, D.S., Jiang, W., Rees, I., and Ludtke, S.J. (2007). EMAN2: an extensible image processing suite for electron microscopy. J Struct Biol 157, 38-46.
Table S1 MAD1 and Cyclin B constructs used for *in vitro* studies

**Part I Identification of a minimal Cyclin B1-binding region in MAD1**

| Deletion tests on MAD1<sup>41-92</sup> | Binding to Cyclin B<sup>165-433</sup> |
|-----------------------------------|---------------------------------------|
| MAD1<sup>41-62</sup>              | Yes                                   |
| MAD1<sup>41-65</sup>              | Yes                                   |
| MAD1<sup>41-73</sup>              | Yes                                   |
| MAD1<sup>41-77</sup>              | Yes                                   |
| MAD1<sup>41-80</sup>              | Yes                                   |
| MAD1<sup>41-92</sup>              | Yes                                   |
| MAD1<sup>58-92</sup>              | No                                    |
| MAD1<sup>60-92</sup>              | No                                    |
| MAD1<sup>65-92</sup>              | No                                    |
| MAD1<sup>60-74</sup>              | No                                    |
| MAD1<sup>60-77</sup>              | No                                    |

**Part II Mutagenesis of MAD1<sup>41-62</sup> to identify Cyclin B1-binding determinants**

| MAD1<sup>41-62</sup> Mutation Group | Mutations              | Binding to Cyclin B<sup>165-433</sup> |
|-------------------------------------|------------------------|---------------------------------------|
| M1                                 | Q42A, Q44A, Q46A, Q47A, Q50A, Q57A | Yes                                   |
| M2                                 | Q57A, S60A, S62A        | Yes                                   |
| M3                                 | E52K, E53K, E56K       | No                                    |
| M4                                 | Y45A, M49A, L51A, 158A | Yes                                   |

**Part III Mutagenesis of Cyclin B1 to identify MAD1-binding determinants**

| Cyclin B<sup>165-433</sup> Mutation Group | Mutations              | Binding to MBP-Mad1<sup>41-62</sup> |
|--------------------------------------------|------------------------|-------------------------------------|
| M1                                         | K171E                  | Yes                                 |
| M2                                         | K214E, R216E           | Yes                                 |
| M3                                         | R306E, R307E, K310E    | Yes                                 |
| M4                                         | K395E, K407E, K410E    | Yes                                 |
| M1 + M2 + M4                               | K171E, K214E, R216E, K395E, K407E, K410E | Yes |
| M5                                         | R187E, K189E, R194E    | Yes                                 |
| M6                                         | K240E, K241E           | Yes                                 |
| M7                                         | K287E, R290E           | Yes                                 |
| M6 + M7                                    | K240E, K241E, K287E, R290E | Yes |
| M5 + M7                                    | R187E, K189E, R194E, K287E, R290E | Yes |
Figure S1. **MPS1 is needed to establish Cyclin B at unattached kinetochores.** Stills from movie of Cyclin B1-EYFP RPE1 cells enter mitosis and in the presence of nocodazole with or without 500nM Reversine.
Figure S2. Validation of Mad1α knockout cell clones. A,B. Western blot (A) and immunofluorescence images (B) of MAD1 in control on MAD1α knockout HeLa cells (2 clones: MAD1α-C13 and C24).
Figure S3. Cyclin B1, not CDK1, interacts with the MAD1:MAD2 complex via an acidic patch in the MAD1 N-terminus. A–C. Elution profiles and SDS-PAGE for SEC runs on a Superose 6 elution profile and SDS-PAGE as in Figure 2D are shown as controls. D. Elution profiles and SDS-PAGE for SEC runs on a Superose 6 Increase 5/150 GL column of (A) GST-CDK1 complex (black), MBP-MAD1FL (green), and their combination (red), (B) Cyclin B1 (black), MBP-MAD1:MAD2 (green), and their combination (red); (C) Cyclin B1 (black), MBP-MAD160-92 (green), and their combination (red). Western blots were obtained with duplicates of the shown SDS-PAGE gels.
Figure S4. YFP-MAD1-WT and YFP-MAD1-3EK do not localise to the corona. A. Western blot analysis of indicated YFP-MAD1-WT and YFP-MAD1-3EK clones treated with doxycycline. B. Immunofluorescence images showing MAD1 and ZW10 kinetochore levels in nocodazole-arrested YFP-MAD1-WT-C19 AND 3EK-C6 cells just after nuclear envelope breakdown (early prometaphase - EPM) or later in mitosis when the chromatin is condensed (late prometaphase - LPM). C. Quantification of MAD1 kinetochore localisation from cells treated as in (B). In all kinetochore intensity graphs, each dot represents a cell, horizontal lines indicate the median and vertical bars show the 95% confidence interval. 60 cells from 3 experiments.
Figure S5. Validating the MAD1-pT716 antibody in cells. A,B. Immunofluorescence images (A) and quantification (B) of MAD1 and MAD1-pT716 kinetochore levels in dox-inducible vsv-MAD1-WT cells with doxycycline removed (- Dox) and MAD1-WT or MAD1-T716A re-expressed. C. Immunofluorescence images of MAD1 and MAD1-pT716 kinetochore levels in nocodazole-arrested HeLa FRT cells.
Figure S6. Corona MAD1 allows the SAC to tolerate MPS1 inhibition by preserving MAD1 at kinetochores and enhancing MAD1-pT716 levels. A. Quantifications (top) and corresponding immunofluorescence images (underneath) of kinetochore MAD1 and MAD1-pT716 levels in nocodazole-arrested MAD1-WT-C1 and 3EK-C20 treated with different doses of AZ-3146 for 30 min. MG132 was included at the time of AZ-3146 addition to prevent mitotic exit. Each dot represents a cell, horizontal lines indicate the median and error bars show 95% confidence interval. 30 cells from 3 experiments. B,C. Duration of mitotic arrest in MAD1-WT C1 (B) or MAD1-3EK C20 (C) cells arrested in nocodazole and then treated with indicated concentrations of AZ-3146. Graph shows cumulative mean (±SEM) of 3 experiments, 50 cells per condition per experiment. Some results are replicated in Figures 3D, 4D-E to allow a complete comparison of the full dose range.