Orchestration of the spindle assembly checkpoint by CDK1-cyclin B1

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Abstract

In mitosis, the spindle assembly checkpoint (SAC) monitors the formation of microtubule-kinetochore attachments during capture of chromosomes by the mitotic spindle. Spindle assembly is complete once there are no longer any unattached kinetochores. Here, we will discuss the mechanism and key components of spindle checkpoint signalling. Unattached kinetochores bind the principal spindle checkpoint kinase MPS1. MPS1 triggers the recruitment of other spindle checkpoint proteins and the formation of a soluble inhibitor of anaphase, thus preventing exit from mitosis. On microtubule attachment, kinetochores become checkpoint silent due to the actions of PP2A-B56 and PP1. This SAC responsive period has to be coordinated with mitotic spindle formation to ensure timely mitotic exit and accurate chromosome segregation. We focus on the molecular mechanisms by which the SAC permissive state is created, describing a central role for CDK1-cyclin B1 and its counteracting phosphatase PP2A-B55. Furthermore, we discuss how CDK1-cyclin B1, through its interaction with MAD1, acts as an integral component of the SAC, and actively orchestrates checkpoint signalling and thus contributes to the faithful execution of mitosis.

173 words

The concept of the spindle assembly checkpoint

Effective chromosome segregation requires the duplicated sister chromatids to attach via their kinetochores to microtubules from opposite poles before their separation at anaphase onset. The successful execution of this process is monitored by a kinetochore-localised signal transduction machinery – the spindle assembly checkpoint pathway. Like many key concepts in cell cycle research, the spindle assembly checkpoint was first discovered using genetic screens in yeast. Landmark work on budding yeast mutants defective in arresting their cell cycle after faulty DNA replication had led to the idea that cells have control mechanisms or 'checkpoints' monitoring the completion of one cell cycle event before the initiation of the next. Mutations that allowed commencement of a cell cycle event even when the preceding process was prevented were termed 'relief of inhibition' mutants [1, 2]. This idea was used to investigate the regulatory mechanisms that safeguard correct chromosome segregation. It was known at the time that treatment of yeast cells with microtubule depolymerising drugs such as nocodazole or methyl benzimidazole carbamate resulted in a mitotic cell cycle arrest without chromosome segregation [3]. Since cells lacking functional microtubules cannot divide their chromosomes effectively, unrestrained progress into anaphase in this situation would lead to high numbers of aneuploid offspring and cell death. The cell cycle arrest thus prevents this adverse outcome. Ground-breaking studies by the labs of Andrew Murray and Andrew Hoyt led to the identification of yeast mutants that progressed through cell division despite severe problems with the mitotic spindle apparatus [4, 5]. The mutants that showed the phenotype of budding, indicative of cell cycle progression, in the presence of the microtubule depolymerising drug benzimidazole (BUB; budding uninhibited by benzimidazole, Hoyt lab) instead of arresting in mitosis (MAD;

mitotic arrest deficient, Murray lab) were thus the founding members of the spindle assembly checkpoint - abbreviated to SAC - the signalling network that halts anaphase entry when spindle defects are detected. The initial two screens identified three MAD (MAD1-3) and three BUB (BUB1-3) proteins, one of which, BUB1, is a protein kinase. Of note, the mammalian homologue of MAD3 was found to have a C-terminal extension with a kinase domain similar to BUB1 and is referred to as BUBR1 in mammalian cells [6, 7]. Despite the presence of a kinase domain it is currently unclear whether BUBR1 operates as an active kinase during mitosis [8, 9]. BUB2 was later identified to have a yeast specific function in mitotic exit rather than acting as a core component of the SAC [10-13] and will therefore not be discussed further here. Interestingly, the MAD and BUB proteins are not essential in yeast, thus conforming to the original definition of checkpoint proteins as factors providing “relief of dependence” when mutated. In an unperturbed cell cycle, the loss of such a protein would be without consequence and would not constitute an essential function. While this is true in yeast for SAC proteins, subsequent research revealed that the mammalian homologues are essential proteins required for normal mitotic timing [14-18]. Despite this difference, the overall core wiring of the SAC is largely conserved between yeast and mammalian cells. However, since the original discovery of spindle assembly checkpoint proteins several more metazoan specific molecular players of the spindle assembly checkpoint have been identified. These additional factors are the components of the ROD-Zwilch-ZW10 (RZZ) complex. This large (800 kD) protein complex is named after the Drosophila gene products (ROD, rough deal; Zwilch; ZW10, Zest White 10) and assembles a fibrous corona at the outer kinetochore during mitosis to promote microtubule attachment and SAC signalling by helping to recruit core SAC proteins [19-22]. After microtubule

attachment has been achieved, interaction of the RZZ complex with the dynein adaptor Spindly mediates the dynein-dependent disassembly of the fibrous corona and thus contributes to SAC inactivation [23-27].

Apart from this metazoan specialisation, the mechanisms of the cell cycle arrest that the core SAC gene products jointly control have been largely elucidated and are for the most part conserved from yeast to man [28, 29], although many details still require clarification. Understanding the particulars of the SAC has attracted a lot of attention because of the importance of faithful chromosome segregation for the development and maintenance of tissues and organisms. Aneuploidy is considered both a hallmark and a driving force of cancer, and the SAC is one of the key cellular mechanisms preventing aneuploidy [30]. Elucidating the precise details of how the SAC works may therefore hold useful information for cancer therapy.

Key components of the SAC and their spatio-temporal localisation

We now understand that the purpose of the spindle assembly checkpoint is to ensure that all kinetochores are attached to microtubules emanating from opposite spindle poles before duplicated sister chromatids are separated at the metaphase-to-anaphase onset. Only this geometry will result in the optimal orientation of the sister chromatids for effective segregation. Unattached kinetochores are detected by the spindle assembly checkpoint machinery and catalyse the formation of a diffusible cell cycle inhibitor, the Mitotic Checkpoint Complex (MCC) [31-33] (Figure 1). The key organiser of the SAC signalling cascade is the protein Monopolar Spindle 1 (MPS1) [34-36]. MPS1 was first identified in budding yeast as an essential gene for the duplication of the yeast centrosome, the spindle pole body, and was named accordingly [37]. Remarkably, in contrast to other genes required for spindle pole

body duplication, yeast cells in which MPS1 had been inactivated progressed through the cell cycle despite their inability to form a bipolar mitotic spindle [38, 39] similar to the behaviour of the mitotic checkpoint mutants identified by Hoyt and Murray. It was thus realised that MPS1 had a dual function in controlling spindle pole body duplication as well as the mitotic checkpoint arrest in response to spindle damage. This dual functionality may be restricted to yeast; while the function of MPS1 in the spindle assembly checkpoint is conserved from yeast to human cells, it is less clear whether MPS1 also has a role in centrosome duplication in organisms other than yeast [35, 40-42]. The universal role of MPS1 in the spindle assembly checkpoint, however, is well understood. MPS1 marks unattached, microtubule-free kinetochores, possibly through competition with microtubules for binding sites [43, 44]. Additionally, MPS1 recruitment to kinetochores is promoted by CDK1-Cyclin B1 and the Aurora B kinase [41, 45-48]. Aurora B is the catalytic subunit of the Chromosomal Passenger Complex (CPC), also containing survivin, borealin and INCENP, which is located on centromeric chromatin during early mitosis and plays a pivotal role in resolving erroneous microtubule-kinetochore attachments [49, 50]. This error correction process relies on the fact that centromeric Aurora B can only phosphorylate the outer kinetochore KNL1-MIS12-NDC80 complex (KMN), the main platform for end-on microtubule binding by the kinetochore [51], in situations when microtubule attachment has not created tension [52]. Phosphorylation of the nuclear division cycle (NDC80) complex in particular, lowers the affinity of NDC80 for microtubules so that these are released [53, 54]. Aurora B thus helps to create unattached kinetochores and hence indirectly promotes MPS1 recruitment. In addition, Aurora B has a direct role in facilitating MPS1 localisation to kinetochores made evident by the fact that Aurora B activity is required for MPS1 recruitment even

when microtubules have been depolymerised with nocodazole [45-48, 55]. Although there is some evidence to suggest that NDC80 phosphorylation by Aurora B is required to enable MPS1 binding to the NDC80 complex [44], it is not entirely clear whether the Aurora B phosphorylations that promote MPS1 recruitment are the same as the ones that destabilise microtubule attachment and this issue will require further investigation.

Once at an unattached kinetochore, MPS1 phosphorylates the outer kinetochore protein KNL1 at specific Met-Glu-Leu-Thr (MELT) motifs, generating docking sites for BUB1-BUB3 complexes which in turn recruit BUBR1-BUB3 heterodimers [56-60] (Figure 1). Further MPS1 phosphorylations on BUB1 and MAD1 lead to the recruitment of the MAD1-MAD2 complex and catalyse the formation of the diffusible MCC [61-64]. In mammalian cells, maintenance of MAD1-MAD2 at kinetochores additionally requires the RZZ complex [21, 65] previously alluded to. The inhibitory MCC protein complex consists of the SAC proteins MAD2, BUBR1 and BUB3, and Cell Division Cycle 20 (CDC20), a co-activator of the 1.2 MDa ubiquitin E3 ligase anaphase promoting complex/cyclosome (APC/C) [33, 66]. APC/C is the key enzyme catalysing the metaphase-to-anaphase transition, and inhibition of this activity by the MCC prevents progression into anaphase. An important aspect of MCC formation is that MAD2 can only contribute to MCC generation in one of its possible structural states, the closed or C state (C-MAD2) [67-69]. The catalytic conversion of open (O-MAD2) MAD2 to closed (C-MAD2) happens at MAD1-(C-MAD2) complexes bound to unattached kinetochores, and the vicinity of the other MCC components assembled at unattached kinetochores then allows efficient assembly of the MCC [69, 70]. Interestingly, at least a subset of APC/C molecules is also found at unattached kinetochores, possibly aiding the rapid inhibition of APC/C

by MCC [71-73]. The MCC binds to the APC/C and inhibits its function in at least two ways: MCC acts as a pseudo-substrate preventing substrates from gaining access to the ubiquitin E3 ligase. MCC also affects the conformation of the APC/C and promotes a form of the APC/C that is less able to associate with its E2 enzyme, UBE2C/UBCH10, also preventing substrate ubiquitination. Together, these effects stop the APC/C from ubiquitinating cyclin B (also often referred to by its gene name CCNB1) and securin, both critical substrates for cell cycle progression [74, 75]. Cyclin B1 is part of the cyclin dependent kinase (CDK1-cyclin B1) which drives cells into mitosis and is required for maintaining the mitotic state, while securin is the inhibitor of the protease separase which cleaves sister chromatid cohesion at the metaphase-to-anaphase transition. In addition, in vertebrate cells cyclin B1 has been reported to interact directly with separase. The interaction between cyclin B1 and separase is dependent on prior phosphorylation of separase by CDK1-cyclin B1 and contributes to the inhibition of separase until the onset of anaphase [76, 77]. Stabilising cyclin B1 and securin by preventing APC/C-mediated ubiquitination thus results in the extension of mitosis until inhibition of the APC/C is relieved. Because production of MCC is triggered by the continued presence of unattached kinetochores, successful completion of microtubule attachment and chromosome alignment stops the generation of further MCC molecules and allows the disassembly of existing APC/C-MCC complexes. This is achieved through the concerted action of at least two pathways. The SAC antagonist p31^{comet} (also called MAD2L1BP) and the AAA+ ATPase TRIP13 act together to extract MAD2 from MCC. Binding of p31 ^{comet} to the N-terminal domain of the AAA+ ATPase TRIP13 places MAD2 N-terminus in the correct constellation to interact with TRIP13, which results in a conformational change from C-MAD2 to O-MAD2 and ultimately results

in the restoration of the O-MAD2 pool [78-82]. Despite this important role in the dismantling of the MCC, TRIP13 and p31^{comet} are not essential for cell cycle progression, suggesting that there are alternative pathways for MCC disassembly [83, 84]. One of these is the ubiquitination of CDC20 in the MCC by APC/C-CDC20 itself, promoting the dismantling of MCC. This second pathway requires the presence of the small APC/C subunit APC15 [85-88]. As a consequence of the synergistic action of p31^{comet}/TRIP13 and APC15 mediated MCC disassembly, APC/C becomes active and catalyses the ubiquitination of cyclin B1 and securin [89].

Integration with cell cycle progression: CDK1-cyclin B1 licenses the SAC

The kinetochore is a highly conserved, multi-layered proteinaceous structure composed of more than 100 proteins in vertebrate cells, which assembles on the centromeric chromatin and forms the physical connection between chromosomes and microtubules. Based on their spatial localization and functions these proteins can be grouped into three categories: (1) outer kinetochore proteins that interact with microtubules, (2) inner kinetochore proteins that interact with centromeric DNA, and (3) regulatory proteins that control the activities of the kinetochore [90, 91]. The binding of microtubules is mediated by the ten proteins of the KNL1-MIS12-NDC80 (KMN) complex at the outer kinetochore and is critical for the coupling of chromosome movement to microtubule dynamics [51, 53, 92]. The outer kinetochore also forms the platform for the assembly of the molecules that monitor the capture of chromosomes by microtubules, the spindle assembly checkpoint machinery, introduced above. Since the activation of the SAC is critical for safeguarding correct chromosome segregation but at the same time has drastic consequences for cell cycle progression, it is of utmost importance that the SAC remains responsive until

the point of chromosome segregation but does not re-activate and impede chromosome segregation during anaphase [93]. This requirement raises the question of which molecular control mechanisms license SAC responsiveness to the correct time window during mitosis. One possible regulatory mechanism is the availability of kinetochores for the SAC proteins to assemble on. Whereas the inner part of the kinetochore is present on centromeric chromatin throughout the cell cycle, the outer kinetochore only assembles upon mitotic entry in a CDK1-cyclin B1 dependent manner [94] and is disassembled at the end of mitosis when cyclin B1 levels drop. Interestingly, a number of observations indicated that the presence and activity of CDK1-cyclin B1 is important for spindle assembly checkpoint function beyond simply promoting the assembly of the platform for SAC factor recruitment, making CDK1-cyclin B1 a plausible candidate for a SAC licensing factor. Using Xenopus egg extract made spindle checkpoint competent by the addition of demembranated sperm nuclei and nocodazole as an experimental system, Grieco and co-workers demonstrated in 2003 that sustained CDK activity was required to keep the extract in a mitotic state [95]. Addition of the CDK inhibitor roscovitine resulted in disassembly of the MCC and cyclin B1 degradation. These data suggested that CDK activity in some way was required to promote the production of MCC or the maintenance of its levels, independently of its role in kinetochore assembly. One possible CDK target at the level of MCC maintenance identified by this study was the APC/C co-activator CDC20. The CDK phosphorylated form of CDC20 has been suggested to bind more tightly to MCC than to APC/C and may thus bias the system to MCC production. Inhibition of CDK activity would thus lead to dephosphorylation of CDC20 and promote MCC disassembly, as observed in this study [95]. Other reports indicated the presence of further CDK dependent SAC

activating phosphorylations at the level of MCC production [96, 97]. Specifically, it was demonstrated that the prevention of cyclin B1 degradation enabled the re-accumulation of SAC proteins including MPS1 at anaphase kinetochores in both somatic and meiotic cells [96, 97]. These observations suggested that CDK activity was required for an early step in SAC activation, which had to be reversed to allow anaphase entry. While these insights presented an important advance in our understanding of SAC regulation, neither study pinpointed the critical CDK targets which had to be phosphorylated and dephosphorylated for SAC signalling to take place and anaphase to be initiated, respectively. Recent studies, though, have started to change this picture and have begun to identify molecular targets within the spindle assembly checkpoint that are phosphorylated by CDK1. Table 1 lists the known CDK1-cyclin B1 targets and CDK1-counteracting phosphatases at the kinetochore and in the SAC.

Molecular targets of CDK1-cyclin B1

The presence or absence of CDK1 activity affects the kinetochore recruitment of all core SAC proteins implying that one or more upstream components of the SAC are regulated by CDK1 [97]. In line with this idea, it has been suggested that both localisation and activation of MPS1 are controlled by CDK1-cyclin B1 [45, 98, 99]. It has recently been demonstrated that phosphorylation of MPS1 by CDK1-cyclin B1 controls recruitment of MPS1 to the kinetochore in human cells [45]. MPS1 has an N-terminal kinetochore binding domain which associates with the NDC80/NUF2 complex at the kinetochore [40, 43, 44]. It has now been shown that the N-terminal Ser281 within the kinetochore binding domain of MPS1 has to be phosphorylated to allow efficient kinetochore recruitment of MPS1 [45]. These data are consistent with

biochemical data showing an improved binding of Ser281-phosphorylated MPS1 to purified NDC80 complex [44]. Dephosphorylation of pSer281 therefore abrogates MPS1 accumulation and hence checkpoint signalling at unattached kinetochores. The SAC permissive period is thus defined by the phosphorylation status of MPS1-Ser281 [45] (Figure 2). Interestingly, in amphibians the homologous residue Ser283 seems to control MPS1 activity, rather than localisation [98], pointing to subtle differences in the regulation of checkpoint signalling by CDK1 between different model organisms. In mammals, CDK1-cyclin B1 has also been suggested to regulate MPS1 activity in addition to controlling MPS1 localisation by phosphorylation of residue Ser821 in the C-terminal MPS1 kinase domain [99]. MPS1 localisation and activation are thus both controlled by CDK1-cyclin B1. Of note, CDK1-cyclin B1 also regulates the centromeric localisation of the chromosomal passenger complex including Aurora B, essential for MPS1 recruitment [45, 100, 101], so that MPS1 kinetochore recruitment is regulated both directly and indirectly by CDK1. This aspect will be discussed later in more detail. Apart from regulating MPS1 and Aurora B, CDK1-cyclin B1 has recently also been shown to control at least one specific step in the recruitment of other SAC proteins downstream of MPS1. CDK1-cyclin B1 phosphorylation of BUB1 is required to promote the initial recruitment of MAD1 to the kinetochore [61, 62, 64]. The mechanism of this step is interesting; the CDK1 phosphorylation of Ser459 in the sequence Pro-Ser-Pro-Thr in BUB1 creates a “pseudo-MELT”-motif which then allows MPS1 to phosphorylate Thr461, creating a MAD1 binding site [62, 64]. The cooperative phosphorylation of BUB1 by CDK1-cyclin B1 and MPS1 is reminiscent of the relationship of CDK1-cyclin B1 and Polo like kinase 1 (PLK1), in which CDK1-cyclin B1 creates priming docking phosphorylations for PLK1. These allow PLK1 to bind and subsequently phosphorylate its targets [102, 103]. In the case of BUB1, though,

CDK1-cyclin B1 seems to create a substrate target sequence for MPS1 rather than a binding motif. It is possible that there are other recruitment steps within the SAC signalling cascade that are governed by a co-operative CDK1/MPS1 two-step phosphorylation.

CDK1's aforementioned partnership with PLK1 is also used in the SAC through the phosphorylation of BUBR1. CDK1 phosphorylates BUBR1 at Thr620 which creates a PLK1 docking site. Plk1 then in turn phosphorylates Ser676 and Thr680 of BUBR1, and both sites in conjunction with a further CDK1 phosphorylation at Ser670 contribute to the recruitment of the PP2A-family phosphatase PP2A-B56 to BUBR1 [104, 105]. BUBR1-bound PP2A-B56 is an important phosphatase for modulating Aurora B-mediated error correction as well as for the silencing of the SAC [104-108]. By binding to BUBR1 PP2A-B56 is pre-loaded to the correct location to trigger SAC silencing when MPS1 activity locally drops due to incoming microtubules. The fact that CDK1 promotes the SAC by phosphorylating MPS1 and simultaneously prepares the scenario for the silencing of the SAC through the recruitment of PP2A-B56 underpins the overarching role of CDK1 in orchestrating cell cycle progression.

A similar double act is achieved through CDK1 phosphorylation of the APC/C activator and MCC component CDC20. As an integral part of the MCC, CDC20 is a pivotal player in the SAC. At the same time, CDC20 acts an essential APC/C co-activator, promoting mitotic exit [29, 81]. Recent, ground-breaking work revealed that in fact CDC20 carries out both functions simultaneously, and APC/C is bound both to an activatory molecule of CDC20 as well as the inhibitory MCC containing CDC20 [74, 109] (Figure 3). This arrangement enables MCC to inhibit an 'activated' (i.e. CDC20-bound) APC/C, and explains how it is possible at all to prevent any APC/C activity towards cyclin B1 and securin before anaphase onset. CDK1-dependent

phosphorylation of the N-terminus of CDC20 influences the fate of CDC20 by promoting incorporation of phosphorylated CDC20 into MCC, whereas unphosphorylated CDC20 preferentially binds to APC/C [95, 110-112]. CDK1-cyclin B1 also phosphorylates core APC/C itself, and these phosphorylations are essential to relieve an autoinhibitory interaction within the APC/C and enable CDC20 binding [113-115]. The phosphorylation of APC/C is facilitated by the binding of CDK-cyclin complexes to the APC/C, and this is mediated by the CDK regulatory protein Cks [116] (Figure 3). Differential dephosphorylation of CDC20 and APC/C has been suggested to create a time window in which CDC20 and APC/C can interact [111], and this will be discussed later.

Cyclin B1 is a spindle checkpoint protein

The multiple CDK1-cyclin B1 targets within the SAC discussed above highlight that CDK1-cyclin B1 is more intimately involved in the regulation of the SAC than a superficial analysis of SAC wiring may suggest. A further demonstration of the close relationship of CDK1-cyclin B1 with its substrates in the mitotic checkpoint signalling cascade is the finding that cyclin B1 itself is localised to unattached kinetochores and displaced from these upon microtubule binding, just like a bona fide SAC protein [117-119] (Figure 4). Complementary proteomic and cell biological analysis demonstrated that MAD1 is one of the major components of cyclin B1 complexes in mitosis. Indeed, cyclin B1 failed to localize to unattached kinetochores in MAD1 depleted cells, suggesting that MAD1 is the main MPS1 regulated kinetochore receptor for cyclin B1 [117, 120]. Further analysis showed that cyclin B1 is bound to the kinetochore via the first 100 amino acids of the N-terminus of MAD1. When endogenous MAD1 was replaced with a version of MAD1 lacking the cyclin-binding domain, the kinetochore

recruitment of cyclin B1 was abrogated. As a consequence, the length of the mitotic arrest was impaired. In keeping with these findings, the spindle checkpoint was strongly compromised in cyclin B1 depleted cells [117-119]. These observations support the idea that the cyclin B1-MAD1 interaction creates a positive feedback loop because the local concentration of MAD1-bound CDK1-cyclin B1 at an unattached kinetochore allows for more efficient phosphorylation and kinetochore localisation of MPS1, in turn recruiting more MAD1. This mechanism is particularly important at the onset of mitosis when it is critical to rapidly establish the SAC to prevent APC/C activation [117]. On the other hand, accumulation and concentration of cyclin B1 through MAD1 will enable local re-establishment of MPS1 and downstream SAC signalling even in an environment of globally falling cyclin B1 levels in metaphase, should isolated microtubule-kinetochore connections be disrupted. This mechanism ensures that the SAC remains responsive to potential failures of microtubule-kinetochore attachments for as long as possible before the onset of anaphase. Replacement of endogenous MAD1 with a version lacking the cyclin-binding domain impairs the length of the mitotic arrest that cells can maintain in nocodazole, further in line with the idea that the local concentration of cyclin B1 at kinetochores may be a mechanism to impart robustness to the checkpoint signalling network [117].

Mitotic phosphorylations are counteracted by phosphoprotein phosphatases

So far, we have considered the generation of phosphorylations by CDK1-cyclin B1 and the functional consequences thereof. We will now review the removal or dynamic modulation of these sites by phosphatases. During mitosis, enzymes of the phosphoprotein phosphatase (PPP) family of serine/threonine phosphatases containing PP1 and PP2A are of particular importance for counteracting mitotic

kinases of the CDK, as well as Aurora, Plk1 and MPS1 families [121-123]. PPPs are generally multimeric enzyme complexes containing either a catalytic and a regulatory subunit in the case of PP1, or these two subunits as well as a scaffolding subunit in the case of PP2A [121, 122]. Since the catalytic subunits of the PPP enzyme complexes are all closely related, substrate specificity is achieved by the actions of the specific regulatory subunits, rather than the catalytic subunits alone. In the case of PP1 more than 150 regulatory subunits associate with the three mammalian PP1 catalytic subunits PP1α, PP1β, PP1γ (the last of which has two splice variants PP1γ1 and PP1γ2), whereas for PP2A holoenzyme complexes the regulatory subunits fall into four different families, denoted as B55, B or PP2R2A-D; B56, B' or PP2R5A-E; B'' or PPP2R3A-C and B''' or striatins, and associate with either one of the two catalytic and two scaffolding subunits expressed in human cells [122-126]. The regulatory subunits of the B55 and B56 families, comprising four (B55α-δ or PP2R2A-D) and five members (B56α-ε or PP2R5A-E), respectively, are the most important for the control of mitotic progression by PP2A. They impart very different properties on the resulting PP2A holoenzyme complexes which affect localisation, activity and substrate selection, and will be discussed in detail below.

CDK1 phosphorylations have been suggested to be counteracted by phosphatases of both the PP1 and the PP2A-B55 families, and recently significant advances have been made in particular in the identification of specific CDK1-phosphorylated PP2A-B55 substrates [127-132]. At this point it should be noted that in budding yeast, in contrast to mammalian cells, the turn-over of CDK1 substrates important for the metaphase-to-anaphase transition is predominantly governed by the dual specificity phosphatase CDC14 [133]. Although CDC14 is conserved in mammalian cells, it does not have a role at the metaphase-to-anaphase transition or mitotic exit in

mammals [134]. The use of CDC14 instead of a PP phosphatase therefore seems to be a budding yeast specific adaptation. Here we will focus on the phosphatases opposing CDK1 in mitosis in mammalian cells. Table 1 lists the known CDK1-counteracting phosphatases at the kinetochore and in the SAC.

The spindle checkpoint permissive period is terminated by PP2A-B55

Microtubule attachment to kinetochores results in the silencing of the SAC. Should a specific microtubule attachment be lost after initial silencing, the SAC can be re-instated as long as CDK1 activity is still high enough, but not if CDK1 is inhibited [45, 97]. As described earlier, one reason for this is the requirement of MPS1 to be phosphorylated by CDK1 for kinetochore localisation and full activation [45, 99]. Recently, PP2A-B55 has been identified as the key phosphatase opposing CDK1 acting on MPS1 [45]. PP2A-B55 is thus the phosphatase that terminates the SAC permissive time window. Once MPS1-Ser281 is dephosphorylated by PP2A-B55, MPS1 cannot be recruited to the kinetochore anymore, and consequently the SAC cannot be reactivated anymore (Figure 2). Interestingly, PP2A-B55 itself is indirectly inhibited by CDK1-cyclin B1 via the MASTL-ENSA system outlined below, creating a situation where PP2A-B55 can only become active once cyclin B1 levels are falling. The ability of a cell to be capable of raising a spindle checkpoint signal when challenged by nocodazole exposure can thus be deduced from the level of cyclin B1 present in the cell at a given time [45].

Regulation of PP2A-B55 activity by the MASTL-ENSA system

PP2A-B55 activity is tightly regulated by the binding of the small, unstructured phospho-proteins Arpp19 and ENSA [135, 136]. The ability of these proteins to inhibit

PP2A-B55 is entirely dependent on phosphorylation of the conserved FDSGDY motif in ENSA and Arpp19 by the Greatwall (Gwl) kinase, also known as MASTL in mammalian cells [137]. In their phosphorylated forms Arpp19 and ENSA bind with high affinity to the PP2A-B55 catalytic site and act as extremely effective competitive inhibitors [135, 136, 138]. Their specific mode of inhibition, characterised by high binding affinity to PP2A-B55 and very slow dephosphorylation kinetics, has been termed “unfair inhibition” [138]. The MASTL kinase is activated by CDK1 phosphorylation of its activation loop, and PP2A-B55 activity is hence indirectly determined by the level of cyclin B1 and active CDK1-cyclin B1 complex [45, 139].

Regulation of CDC20 by CDK1 and opposing phosphatases

Apart from dephosphorylating MPS1, PP2A-B55 has also been proposed to be a key phosphatase for CDC20 at the metaphase to anaphase transition. CDC20 is highly phosphorylated in mitosis by CDK1-cyclin B1, MAPK1, BUB1 and Plk1. The CDK1/MAPK1 phosphorylations in particular have been described to modulate the interaction between CDC20 and MCC or APC/C, respectively [110, 140-142]. For efficient interaction of CDC20 with the APC/C, CDC20 has to be in its dephosphorylated form whereas APC/C has to remain phosphorylated [112, 115]. It has been suggested that the requirement for differential dephosphorylation of these two molecules could be explained because the crucial phosphorylation sites are mainly threonines in CDC20, whereas they are serines in APC/C. Due to the known preference of PP2A-B55 for phospho-threonine over phospho-serine [131, 143], CDC20 would be dephosphorylated first and APC/C later, resulting in a time window allowing the productive interaction of the two molecules. While this appears an appealing explanation for the observed kinetics of dephosphorylation, depletion of

PP2A-B55 did not result in delays at the metaphase-to-anaphase transition in an unperturbed mitosis, suggesting that there may be other phosphatases contributing to CDC20 dephosphorylation at this specific point in the cell cycle. Potential candidates are PP1, as suggested by work in *C. elegans*, or the RNA polymerase II-carboxy-terminal domain phosphatase Fcp1 [45, 144-146].

Comparison of PP2A-B55 to other phosphatases modulating the SAC

While CDK1 and PP2A-B55 are important for demarcating the spindle checkpoint permissive period, within the SAC licensed period a large number of phosphorylations are deposited by MPS1 and have to be dynamically controlled and removed before the metaphase-to-anaphase transition to enable SAC silencing and mitotic exit. Because of the specific regulation of PP2A-B55, outlined above, which means that PP2A-B55 will only become active once cyclin B1 levels drop, it is unlikely that PP2A-B55 is also one of the phosphatases dynamically opposing MPS1. Indeed, increasing evidence indicates that the major MPS1 opposing phosphatase is another PP2A family phosphatase, PP2A-B56. Specifically, PP2A-B56 has been shown to dephosphorylate MPS1 phosphorylated MELT motifs in KNL1, the MAD1 docking motif in BUB1, the Ska complex mediating microtubule-kinetochore attachments, autophosphorylation sites on MPS1 itself as well as the MPS1 activation loop important for MPS1 activity [61, 108, 147, 148]. PP2A-B56 exists in different pools in mitotic mammalian cells, one of which is located to the kinetochore via an association with the SAC protein BUBR1 [104-106], as mentioned before. PP2A-B56 binds to a conserved linear LxxIxE motif in BUBR1 [149-151]. It is this specific pool of PP2A-B56 that has been shown to counteract the kinase activities of MPS1 [61, 108, 147]. Interestingly, the same sub-group of PP2A-B56 also opposes Aurora B [104-106].

Loss of this sub-group of PP2A-B56 consequently interferes with both error correction and SAC silencing and hence leads to a penetrant mitotic arrest [107, 108]. Another pool of PP2A-B56 is localised to the centromere via association with the shugoshin proteins that protect centromeric cohesion and is important for the maintenance of centromeric sister chromatid cohesion until the onset of anaphase but is not relevant for the regulation of the SAC [152, 153]. Of note, these mitotic activities of PP2A-B56 are only possible because PP2A-B56 is active throughout mitosis, whereas PP2A-B55 activity is largely suppressed through the Greatwall-ENSA pathway (Figure 2B). This distinct property of PP2A-B56 enables it to modulate both error correction and the SAC dynamically throughout mitosis.

PP1, the third phosphatase important for cell division, is also mostly shut down in mitosis through inhibitory CDK1 phosphorylation on a conserved residue in the C-terminus of the catalytic subunit [154, 155]. However, clustering of PP1 at specific cellular localisations could result in autoactivation of PP1, explaining how some PP1 activity can be observed even in mitosis. In human cells, PP1 has been shown to localise to the mitotic spindle, centrosomes and chromatin. PP1 also accumulates at attached kinetochores in metaphase [156, 157]. In general, PP1 binds to docking proteins via short linear motifs, such as the well-characterised RVxF motif [124, 125]. The outer kinetochore proteins KNL1 and SKA3, as well as the kinetochore localised motor proteins CENP-E and KIF18A have all been described as interaction partners for PP1 at the kinetochore, and in KNL1, CENP-E and KIF18A RVxF motifs have been identified that mediate the interaction [157-160]. RVxF motifs often contain serine or threonine residues in the “x” position, turning them into potential target sequences for basophilic Aurora kinases [161]. As phosphorylation of an RV[S/T]F motif prevents PP1 binding, PP1 recruitment to certain targets is limited as long as Aurora A or B are

active [157, 162]. This mechanism has been shown to regulate PP1 binding to KNL1 and CENP-E, and could explain why PP1 preferentially localises to attached kinetochores where tension has been established and the outer kinetochore has been moved out of the reach of centromeric Aurora B [157, 159, 163]. At attached kinetochores, PP1 has been proposed to promote the metaphase-to-anaphase transition by contributing to SAC silencing by either directly dephosphorylating MELT motifs on KNL1 or dampening MPS1 activity by dephosphorylating the MPS1 activation loop [60, 163, 164].

Relationship between cyclin B1 levels and PP2A-B55 activation status

As the discussion above outlined, the spindle checkpoint permissive period is initiated by CDK1-cyclin B1 and terminated by PP2A-B55, and both Aurora B and the key SAC kinase MPS1 are affected by this form of regulation. Since PP2A-B55 is indirectly inhibited by CDK1-cyclin B1, the SAC permissive time window is determined by the level of cyclin B1 at a given time point and can only be effectively shut down by PP2A-B55 once cyclin B1 levels begin to fall. Experimental measurements of the minimal cellular level of cyclin B1 required to support a SAC response indicated that the threshold for effective SAC signalling is located at approximately 75 nM cyclin B1 (Figure 2) [45]. This relationship allows the SAC to be responsive until the point of chromosome segregation. Live cell imaging of cells under conditions of experimental down- or upregulation of the activity of PP2A-B55 by depletion of PP2A-B55 or depletion of the inhibitory kinase MASTL, respectively, revealed the effect of PP2A-B55 activity on the spindle checkpoint response. When cells have higher than physiological levels of PP2A-B55 activity, the SAC, once silenced, can never be re-activated if microtubule-kinetochore connections are disrupted after the original

silencing event. On the other hand, decreased PP2A-B55 activity results in an extended SAC permissive period, and thus makes cells more likely to re-activate the SAC [45]. This becomes particularly relevant under conditions that slightly impair the efficiency of microtubule-kinetochore attachment formation, such as low level nocodazole or low temperature. An increased ability to re-activate the SAC thus impairs the robustness of the metaphase-to-anaphase transition. In summary, the CDK1-PP2A-B55 interplay seems to have evolved to hit the “Goldilocks” zone with regards to the kinetics with which the spindle assembly checkpoint moves from re-activatable to un-re-activatable.

CDK1 and PP2A-B55 act on both the chromosomal passenger complex and MPS1

In addition to the requirement for phosphorylation of MPS1 by CDK1, we have already explained that the Aurora B kinase is another critical factor for the recruitment of MPS1 to unattached kinetochores. Interestingly, the centromere localisation of Aurora B is regulated by CDK1-cyclin B1 and PP2A-B55 as well (Figure 2). CDK1 promotes CPC localisation to the centromere by multiple mechanisms acting via the different subunits of the CPC: the survivin subunit of the CPC binds to a mitosis-specific phosphorylation of histone H3 at amino acid threonine 3 [165-167]. This phosphorylation is deposited by the Haspin kinase [168]. Haspin is autoinhibited in interphase, and CDK1-CCNB1 triggers a phosphorylation cascade that results in relief of autoinhibition and activation of Haspin, thus restricting Haspin activity to mitosis and therefore CPC recruitment to mitotic chromatin [169, 170]. The Borealin subunit of the CPC is also phosphorylated by CDK1-CCNB1 enabling Borealin to bind to the centromeric shugoshin proteins [101, 171]. Lastly, phosphorylation of Thr59 in INCENP by CDK1 prevents the

interaction of the CPC with the mitotic motor protein MKLP2 [100]. MKLP2 is critical for the extraction of Aurora B from centromeric chromatin at the metaphase to anaphase transition and its transport to the anaphase central spindle [172]. The Inhibition of contact between INCENP and MKLP2 prevents transport of the CPC away from chromatin until anaphase onset, hence contributing to the centromeric accumulation of CPC during mitosis [100]. PP2A-B55 has been shown to be a key phosphatase opposing the influence of CDK1 in localising the CPC [45, 111] and thus contributes to curtailing error correction and SAC signalling by removing the SAC promoting activity of Aurora B.

MPS1 kinetochore association is therefore controlled by CDK1-cyclin B1 in a two-fold fashion. First, direct phosphorylation of the MPS1 kinetochore binding domain by CDK1-cyclin B1 promotes its docking to kinetochore partner proteins. Second, CDK1-cyclin B1 regulating the targeting of Aurora B to the centromere indirectly controls MPS1 kinetochore recruitment. Understanding the relative importance of these two activities is important to accurately appreciate how the SAC is triggered and revoked. Experimentally, the actions of CDK1 and Aurora B on SAC signalling can be separated by the depletion of MKLP2 in mitotic cells. Loss of MKLP2 results in retention of Aurora B on centromeric chromatin, even in the presence of CDK1 inhibitor, and makes it possible to assess the influence of Aurora B activity on MPS1 in isolation of CDK1. Importantly, this experimental situation showed that Aurora B alone, in the absence of CDK1 activity, is not sufficient to promote efficient MPS1 recruitment and SAC signalling [45, 173]. Aurora B and CDK1 activities are thus required to act synergistically to control MPS1 kinetochore localisation. This dual control presumably evolved to ensure that MPS1 is only recruited when all conditions for spindle checkpoint signalling are met.

Dynamic regulation of the SAC by kinase-phosphatase modules

As highlighted here, the regulation of the SAC by distinct kinase-phosphatase modules is both critical for the integration of the SAC into the cell cycle as well as the dynamic response of the SAC to changing microtubule attachment conditions within the SAC permissive period. It is important to realise that the different kinds of kinase-phosphatase modules exert fundamentally distinct roles in the regulation of the mitotic checkpoint: CDK1-cyclin B1 and its counteracting phosphatase PP2A-B55 set the scene for SAC signalling as well as error correction by granting a molecular license to Aurora B and MPS1 to act hand-in-hand in a specified time window during mitosis. This time window is terminated by PP2A-B55, itself under the regulation of CDK1-CCNB1. Within this CDK1 licensed period, Aurora B and MPS1 drive error correction and SAC signalling, and both kinases are dynamically opposed by PP2A-B56. A useful analogy for this relationship is the functioning of a car: Aurora B and MPS1 jointly act as the gas pedal for the connected activities of error correction and the spindle assembly checkpoint and are dynamically opposed by the brake PP2A-B56. The key to the ignition switch for the entire system, however, is CDK1-cyclin B1. Once cyclin B1 is destroyed and PP2A-B55 has terminated the SAC permissive time window, neither Aurora B or MPS1 can act anymore.

Conclusions and Perspectives

How spindle checkpoint signalling is limited to a specific period during mitosis has been a long-standing but elusive question in the cell cycle field. Recent evidence demonstrates that a spindle checkpoint licensed period during mitosis is established by the interplay of CDK1-cyclin B1 and its opposing phosphatase PP2A-B55. These

counteracting forces both converge on the key spindle checkpoint kinase, MPS1, and determine whether MPS1 is able to localise to unattached kinetochores or not. In addition to MPS1, several other important players in the SAC, including the upstream kinase Aurora B, are also regulated by CDK1-cyclin B1. Of particular interest, cyclin B1 not only sets the temporal boundaries of spindle checkpoint signalling but also directly influences the spindle checkpoint by localising to unattached kinetochores itself, like a bona fide spindle checkpoint protein. In this way a positive feedback loop is established that promotes spindle checkpoint signalling. These new findings provide novel insight into how spindle checkpoint signalling is integrated with cell cycle progression and set the scene for further analysis of the role of CDK1-cyclin B1 in orchestrating spindle checkpoint signalling.

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References

1. Hartwell, L. H. & Weinert, T. A. (1989) Checkpoints: controls that ensure the order of cell cycle events, *Science.* **246**, 629-34.

2. Weinert, T. A. & Hartwell, L. H. (1988) The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae, *Science.* **241**, 317-22.

3. Jacobs, C. W., Adams, A. E., Szaniszlo, P. J. & Pringle, J. R. (1988) Functions of microtubules in the Saccharomyces cerevisiae cell cycle, *J Cell Biol.* **107**, 1409-26.

4. Hoyt, M. A., Totis, L. & Roberts, B. T. (1991) S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function, *Cell.* **66**, 507-17.

5. Li, R. & Murray, A. W. (1991) Feedback control of mitosis in budding yeast, *Cell.* **66**, 519-31.

6. Taylor, S. S., Ha, E. & McKeon, F. (1998) The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase, *J Cell Biol.* **142**, 1-11.

7. Cahill, D. P., Lengauer, C., Yu, J., Riggins, G. J., Willson, J. K., Markowitz, S. D., Kinzler, K. W. & Vogelstein, B. (1998) Mutations of mitotic checkpoint genes in human cancers, *Nature.* **392**, 300-3.

8. Suijkerbuijk, S. J., van Dam, T. J., Karagoz, G. E., von Castelmur, E., Hubner, N. C., Duarte, A. M., Vleugel, M., Perrakis, A., Rudiger, S. G., Snel, B. & Kops, G. J. (2012) The vertebrate mitotic checkpoint protein BUBR1 is an unusual pseudokinase, *Dev Cell.* **22**, 1321-9.

9. Huang, Y., Lin, L., Liu, X., Ye, S., Yao, P. Y., Wang, W., Yang, F., Gao, X., Li, J., Zhang, Y., Zhang, J., Yang, Z., Liu, X., Yang, Z., Zang, J., Teng, M., Wang, Z., Ruan, K., Ding, X., Li, L., Cleveland, D. W., Zhang, R. & Yao, X. (2019) BubR1 phosphorylates CENP-E as a switch enabling the transition from lateral association to end-on capture of spindle microtubules, *Cell Res.* **29**, 562-578.

10. Li, R. (1999) Bifurcation of the mitotic checkpoint pathway in budding yeast, *Proc Natl Acad Sci U S A.* **96**, 4989-94.

11. Bloecher, A., Venturi, G. M. & Tatchell, K. (2000) Anaphase spindle position is monitored by the BUB2 checkpoint, *Nat Cell Biol.* **2**, 556-8.

12. Bardin, A. J., Visintin, R. & Amon, A. (2000) A mechanism for coupling exit from mitosis to partitioning of the nucleus, *Cell.* **102**, 21-31.

13. Pereira, G., Hofken, T., Grindlay, J., Manson, C. & Schiebel, E. (2000) The Bub2p spindle checkpoint links nuclear migration with mitotic exit, *Mol Cell.* **6**, 1-10.

14. Kalitsis, P., Earle, E., Fowler, K. J. & Choo, K. H. (2000) Bub3 gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis, *Genes Dev.* **14**, 2277-82.

15. Perera, D., Tilston, V., Hopwood, J. A., Barchi, M., Boot-Handford, R. P. & Taylor, S. S. (2007) Bub1 maintains centromeric cohesion by activation of the spindle checkpoint, *Dev Cell.* **13**, 566-79.

16. Dobles, M., Liberal, V., Scott, M. L., Benezra, R. & Sorger, P. K. (2000) Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2, *Cell.* **101**, 635-45.

17. May, K. M. & Hardwick, K. G. (2006) The spindle checkpoint, *J Cell Sci.* **119**, 4139-42.

18. Meraldi, P., Draviam, V. M. & Sorger, P. K. (2004) Timing and checkpoints in the regulation of mitotic progression, *Dev Cell.* **7**, 45-60.

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

20. Buffin, E., Lefebvre, C., Huang, J., Gagou, M. E. & Karess, R. E. (2005) Recruitment of Mad2 to the kinetochore requires the Rod/Zw10 complex, *Curr Biol.* **15**, 856-61.

21. Rodriguez-Rodriguez, J. A., Lewis, C., McKinley, K. L., Sikirzhytski, V., Corona, J., Maciejowski, J., Khodjakov, A., Cheeseman, I. M. & Jallepalli, P. V. (2018) Distinct Roles of RZZ and Bub1-KNL1 in Mitotic Checkpoint Signaling and Kinetochore Expansion, *Curr Biol.* **28**, 3422-3429 e5.

22. Pereira, C., Reis, R. M., Gama, J. B., Celestino, R., Cheerambathur, D. K., Carvalho, A. X. & 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 e8.

23. Mosalaganti, S., Keller, J., Altenfeld, A., Winzker, M., Rombaut, P., Saur, M., Petrovic, A., Wehenkel, A., Wohlgemuth, S., Muller, F., Maffini, S., Bange, T., Herzog, F., Waldmann, H., Raunser, S. & Musacchio, A. (2017) Structure of the RZZ complex and molecular basis of its interaction with Spindly, *J Cell Biol.* **216**, 961-981.

24. McHugh, T. & Welburn, J. P. (2017) Dynein at kinetochores: Making the connection, *J Cell Biol.* **216**, 855-857.

25. Howell, B. J., McEwen, B. F., Canman, J. C., Hoffman, D. B., Farrar, E. M., Rieder, C. L. & 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**, 1159-72.

26. Gama, J. B., Pereira, C., Simoes, P. A., Celestino, R., Reis, R. M., Barbosa, D. J., Pires, H. R., Carvalho, C., Amorim, J., Carvalho, A. X., Cheerambathur, D. K. & Gassmann, R. (2017) Molecular mechanism of dynein recruitment to kinetochores by the Rod-Zw10-Zwilch complex and Spindly, *J Cell Biol.* **216**, 943-960.

27. Wojcik, E., Basto, R., Serr, M., Scaerou, F., Karess, R. & Hays, T. (2001) Kinetochore dynein: its dynamics and role in the transport of the Rough deal checkpoint protein, *Nat Cell Biol.* **3**, 1001-7.

28. Lara-Gonzalez, P., Westhorpe, F. G. & Taylor, S. S. (2012) The spindle assembly checkpoint, *Curr Biol.* **22**, R966-80.

29. Musacchio, A. (2015) The Molecular Biology of Spindle Assembly Checkpoint Signaling Dynamics, *Curr Biol.* **25**, R1002-18.

30. Kops, G. J., Weaver, B. A. & Cleveland, D. W. (2005) On the road to cancer: aneuploidy and the mitotic checkpoint, *Nat Rev Cancer.* **5**, 773-85.

31. Fraschini, R., Beretta, A., Sironi, L., Musacchio, A., Lucchini, G. & Piatti, S. (2001) Bub3 interaction with Mad2, Mad3 and Cdc20 is mediated by WD40 repeats and does not require intact kinetochores, *EMBO J.* **20**, 6648-59.

32. Hardwick, K. G., Johnston, R. C., Smith, D. L. & Murray, A. W. (2000) MAD3 encodes a novel component of the spindle checkpoint which interacts with Bub3p, Cdc20p, and Mad2p, *J Cell Biol.* **148**, 871-82.

33. Sudakin, V., Chan, G. K. & Yen, T. J. (2001) Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2, *J Cell Biol.* **154**, 925-36.

34. Abrieu, A., Magnaghi-Jaulin, L., Kahana, J. A., Peter, M., Castro, A., Vigneron, S., Lorca, T., Cleveland, D. W. & Labbe, J. C. (2001) Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint, *Cell.* **106**, 83-93.

35. Stucke, V. M., Sillje, H. H., Arnaud, L. & Nigg, E. A. (2002) Human Mps1 kinase is required for the spindle assembly checkpoint but not for centrosome duplication, *EMBO J.* **21**, 1723-32.

36. Liu, X. & Winey, M. (2012) The MPS1 family of protein kinases, *Annu Rev Biochem.* **81**, 561-85.

37. Winey, M., Goetsch, L., Baum, P. & Byers, B. (1991) MPS1 and MPS2: novel yeast genes defining distinct steps of spindle pole body duplication, *J Cell Biol.* **114**, 745-54.

38. Hardwick, K. G., Weiss, E., Luca, F. C., Winey, M. & Murray, A. W. (1996) Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption, *Science.* **273**, 953-6.

39. Weiss, E. & Winey, M. (1996) The Saccharomyces cerevisiae spindle pole body duplication gene MPS1 is part of a mitotic checkpoint, *J Cell Biol.* **132**, 111-23.

40. Stucke, V. M., Baumann, C. & Nigg, E. A. (2004) Kinetochore localization and microtubule interaction of the human spindle checkpoint kinase Mps1, *Chromosoma.* **113**, 1-15.

41. Santaguida, S., Tighe, A., D'Alise, A. M., Taylor, S. S. & Musacchio, A. (2010) Dissecting the role of MPS1 in chromosome biorientation and the spindle checkpoint through the small molecule inhibitor reversine, *J Cell Biol.* **190**, 73-87.

42. Kwiatkowski, N., Jelluma, N., Filippakopoulos, P., Soundararajan, M., Manak, M. S., Kwon, M., Choi, H. G., Sim, T., Deveraux, Q. L., Rottmann, S., Pellman, D., Shah, J. V., Kops, G. J., Knapp, S. & Gray, N. S. (2010) Small-molecule kinase inhibitors provide insight into Mps1 cell cycle function, *Nat Chem Biol.* **6**, 359-68.

43. Hiruma, Y., Sacristan, C., Pachis, S. T., Adamopoulos, A., Kuijt, T., Ubbink, M., von Castelmur, E., Perrakis, A. & Kops, G. J. (2015) CELL DIVISION CYCLE. Competition between MPS1 and microtubules at kinetochores regulates spindle checkpoint signaling, *Science.* **348**, 1264-7.

44. Ji, Z., Gao, H. & Yu, H. (2015) CELL DIVISION CYCLE. Kinetochore attachment sensed by competitive Mps1 and microtubule binding to Ndc80C, *Science.* **348**, 1260-4.

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

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

47. Zhu, T., Dou, Z., Qin, B., Jin, C., Wang, X., Xu, L., Wang, Z., Zhu, L., Liu, F., Gao, X., Ke, Y., Wang, Z., Aikhionbare, F., Fu, C., Ding, X. & Yao, X. (2013) Phosphorylation of microtubule-binding protein Hec1 by mitotic kinase Aurora B specifies spindle checkpoint kinase Mps1 signaling at the kinetochore, *J Biol Chem.* **288**, 36149-59.

48. Nijenhuis, W., von Castelmur, E., Littler, D., De Marco, V., Tromer, E., Vleugel, M., van Osch, M. H., Snel, B., Perrakis, A. & Kops, G. J. (2013) A TPR domain-containing N-terminal module of MPS1 is required for its kinetochore localization by Aurora B, *J Cell Biol.* **201**, 217-31.

49. Carmena, M., Wheelock, M., Funabiki, H. & Earnshaw, W. C. (2012) The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis, *Nat Rev Mol Cell Biol.* **13**, 789-803.

50. Lampson, M. A. & Cheeseman, I. M. (2011) Sensing centromere tension: Aurora B and the regulation of kinetochore function, *Trends Cell Biol.* **21**, 133-40.

51. Pesenti, M. E., Weir, J. R. & Musacchio, A. (2016) Progress in the structural and functional characterization of kinetochores, *Curr Opin Struct Biol.* **37**, 152-63.

52. Liu, D., Vader, G., Vromans, M. J., Lampson, M. A. & Lens, S. M. (2009) Sensing chromosome bi-orientation by spatial separation of aurora B kinase from kinetochore substrates, *Science.* **323**, 1350-3.

53. DeLuca, J. G., Gall, W. E., Ciferri, C., Cimini, D., Musacchio, A. & Salmon, E. D. (2006) Kinetochore microtubule dynamics and attachment stability are regulated by Hec1, *Cell.* **127**, 969-82.

54. Welburn, J. P., Vleugel, M., Liu, D., Yates, J. R., 3rd, Lampson, M. A., Fukagawa, T. & Cheeseman, I. M. (2010) Aurora B phosphorylates spatially distinct targets to differentially regulate the kinetochore-microtubule interface, *Mol Cell.* **38**, 383-92.

55. Santaguida, S., Vernieri, C., Villa, F., Ciliberto, A. & Musacchio, A. (2011) Evidence that Aurora B is implicated in spindle checkpoint signalling independently of error correction, *EMBO J.* **30**, 1508-19.

56. Overlack, K., Primorac, I., Vleugel, M., Krenn, V., Maffini, S., Hoffmann, I., Kops, G. J. & Musacchio, A. (2015) A molecular basis for the differential roles of Bub1 and BubR1 in the spindle assembly checkpoint, *Elife.* **4**, e05269.

57. Primorac, I., Weir, J. R., Chiroli, E., Gross, F., Hoffmann, I., van Gerwen, S., Ciliberto, A. & Musacchio, A. (2013) Bub3 reads phosphorylated MELT repeats to promote spindle assembly checkpoint signaling, *Elife.* **2**, e01030.

58. Yamagishi, Y., Yang, C. H., Tanno, Y. & Watanabe, Y. (2012) MPS1/Mph1 phosphorylates the kinetochore protein KNL1/Spc7 to recruit SAC components, *Nat Cell Biol.* **14**, 746-52.

59. Shepperd, L. A., Meadows, J. C., Sochaj, A. M., Lancaster, T. C., Zou, J., Buttrick, G. J., Rappsilber, J., Hardwick, K. G. & Millar, J. B. (2012) Phosphodependent recruitment of Bub1 and Bub3 to Spc7/KNL1 by Mph1 kinase maintains the spindle checkpoint, *Curr Biol.* **22**, 891-9.

60. London, N., Ceto, S., Ranish, J. A. & Biggins, S. (2012) Phosphoregulation of Spc105 by Mps1 and PP1 regulates Bub1 localization to kinetochores, *Curr Biol.* **22**, 900-6.

61. Qian, J., Garcia-Gimeno, M. A., Beullens, M., Manzione, M. G., Van der Hoeven, G., Igual, J. C., Heredia, M., Sanz, P., Gelens, L. & Bollen, M. (2017) An Attachment-Independent Biochemical Timer of the Spindle Assembly Checkpoint, *Mol Cell.* **68**, 715-730 e5.

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

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

64. Ji, Z., Gao, H., Jia, L., Li, B. & Yu, H. (2017) A sequential multi-target Mps1 phosphorylation cascade promotes spindle checkpoint signaling, *Elife.* **6**.

65. Silio, V., McAinsh, A. D. & Millar, J. B. (2015) KNL1-Bubs and RZZ Provide Two Separable Pathways for Checkpoint Activation at Human Kinetochores, *Dev Cell.* **35**, 600-613.

66. Visintin, R., Prinz, S. & Amon, A. (1997) CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis, *Science.* **278**, 460-3.

67. Luo, X., Tang, Z., Xia, G., Wassmann, K., Matsumoto, T., Rizo, J. & Yu, H. (2004) The Mad2 spindle checkpoint protein has two distinct natively folded states, *Nat Struct Mol Biol.* **11**, 338-45.

68. Sironi, L., Mapelli, M., Knapp, S., De Antoni, A., Jeang, K. T. & Musacchio, A. (2002) Crystal structure of the tetrameric Mad1-Mad2 core complex: implications of a 'safety belt' binding mechanism for the spindle checkpoint, *EMBO J.* **21**, 2496-506.

69. De Antoni, A., Pearson, C. G., Cimini, D., Canman, J. C., Sala, V., Nezi, L., Mapelli, M., Sironi, L., Faretta, M., Salmon, E. D. & Musacchio, A. (2005) The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint, *Curr Biol.* **15**, 214-25.

70. Kulukian, A., Han, J. S. & Cleveland, D. W. (2009) Unattached kinetochores catalyze production of an anaphase inhibitor that requires a Mad2 template to prime Cdc20 for BubR1 binding, *Dev Cell.* **16**, 105-17.

71. Vigneron, S., Prieto, S., Bernis, C., Labbe, J. C., Castro, A. & Lorca, T. (2004) Kinetochore localization of spindle checkpoint proteins: who controls whom?, *Mol Biol Cell.* **15**, 4584-96.

72. Topper, L. M., Campbell, M. S., Tugendreich, S., Daum, J. R., Burke, D. J., Hieter, P. & Gorbsky, G. J. (2002) The dephosphorylated form of the anaphase-promoting complex protein Cdc27/Apc3 concentrates on kinetochores and chromosome arms in mitosis, *Cell Cycle.* **1**, 282-92.

73. Acquaviva, C., Herzog, F., Kraft, C. & Pines, J. (2004) The anaphase promoting complex/cyclosome is recruited to centromeres by the spindle assembly checkpoint, *Nat Cell Biol.* **6**, 892-8.

74. Alfieri, C., Chang, L., Zhang, Z., Yang, J., Maslen, S., Skehel, M. & Barford, D. (2016) Molecular basis of APC/C regulation by the spindle assembly checkpoint, *Nature.* **536**, 431-436.

75. Yamaguchi, M., VanderLinden, R., Weissmann, F., Qiao, R., Dube, P., Brown, N. G., Haselbach, D., Zhang, W., Sidhu, S. S., Peters, J. M., Stark, H. & Schulman, B. A. (2016) Cryo-EM of Mitotic Checkpoint Complex-Bound APC/C Reveals Reciprocal and Conformational Regulation of Ubiquitin Ligation, *Mol Cell.* **63**, 593-607.

76. Holland, A. J. & Taylor, S. S. (2006) Cyclin-B1-mediated inhibition of excess separase is required for timely chromosome disjunction, *J Cell Sci.* **119**, 3325-36.

77. Stemmann, O., Zou, H., Gerber, S. A., Gygi, S. P. & Kirschner, M. W. (2001) Dual inhibition of sister chromatid separation at metaphase, *Cell.* **107**, 715-26.

78. Eytan, E., Wang, K., Miniowitz-Shemtov, S., Sitry-Shevah, D., Kaisari, S., Yen, T. J., Liu, S. T. & Hershko, A. (2014) Disassembly of mitotic checkpoint complexes by the joint action of the AAA-ATPase TRIP13 and p31(comet), *Proc Natl Acad Sci U S A.* **111**, 12019-24.

79. Ye, Q., Kim, D. H., Dereli, I., Rosenberg, S. C., Hagemann, G., Herzog, F., Toth, A., Cleveland, D. W. & Corbett, K. D. (2017) The AAA+ ATPase TRIP13 remodels HORMA domains through N-terminal engagement and unfolding, *EMBO J.* **36**, 2419-2434.

80. Ma, H. T. & Poon, R. Y. C. (2016) TRIP13 Regulates Both the Activation and Inactivation of the Spindle-Assembly Checkpoint, *Cell Rep.* **14**, 1086-1099.

81. Sivakumar, S. & Gorbsky, G. J. (2015) Spatiotemporal regulation of the anaphase-promoting complex in mitosis, *Nat Rev Mol Cell Biol.* **16**, 82-94.

82. Alfieri, C., Chang, L. & Barford, D. (2018) Mechanism for remodelling of the cell cycle checkpoint protein MAD2 by the ATPase TRIP13, *Nature.* **559**, 274-278.

83. Choi, E., Zhang, X., Xing, C. & Yu, H. (2016) Mitotic Checkpoint Regulators Control Insulin Signaling and Metabolic Homeostasis, *Cell.* **166**, 567-581.

84. Ma, H. T. & Poon, R. Y. C. (2018) TRIP13 Functions in the Establishment of the Spindle Assembly Checkpoint by Replenishing O-MAD2, *Cell Rep.* **22**, 1439-1450.

85. Reddy, S. K., Rape, M., Margansky, W. A. & Kirschner, M. W. (2007) Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation, *Nature.* **446**, 921-5.

86. Foster, S. A. & Morgan, D. O. (2012) The APC/C subunit Mnd2/Apc15 promotes Cdc20 autoubiquitination and spindle assembly checkpoint inactivation, *Mol Cell.* **47**, 921-32.

87. Uzunova, K., Dye, B. T., Schutz, H., Ladurner, R., Petzold, G., Toyoda, Y., Jarvis, M. A., Brown, N. G., Poser, I., Novatchkova, M., Mechtler, K., Hyman, A. A., Stark, H., Schulman, B. A. & Peters, J. M. (2012) APC15 mediates CDC20 autoubiquitylation by APC/C(MCC) and disassembly of the mitotic checkpoint complex, *Nat Struct Mol Biol.* **19**, 1116-23.

88. Mansfeld, J., Collin, P., Collins, M. O., Choudhary, J. S. & Pines, J. (2011) APC15 drives the turnover of MCC-CDC20 to make the spindle assembly checkpoint responsive to kinetochore attachment, *Nat Cell Biol.* **13**, 1234-43.

89. Kim, D. H., Han, J. S., Ly, P., Ye, Q., McMahon, M. A., Myung, K., Corbett, K. D. & Cleveland, D. W. (2018) TRIP13 and APC15 drive mitotic exit by turnover of interphase- and unattached kinetochore-produced MCC, *Nat Commun.* **9**, 4354.

90. Gascoigne, K. E. & Cheeseman, I. M. (2011) Kinetochore assembly: if you build it, they will come, *Curr Opin Cell Biol.* **23**, 102-8.

91. Musacchio, A. & Desai, A. (2017) A Molecular View of Kinetochore Assembly and Function, *Biology (Basel).* **6**.

92. Cheeseman, I. M., Chappie, J. S., Wilson-Kubalek, E. M. & Desai, A. (2006) The conserved KMN network constitutes the core microtubule-binding site of the kinetochore, *Cell.* **127**, 983-97.

93. Kops, G. J. (2014) Cell division: SACing the anaphase problem, *Curr Biol.* **24**, R224-6.

94. Gascoigne, K. E. & Cheeseman, I. M. (2013) CDK-dependent phosphorylation and nuclear exclusion coordinately control kinetochore assembly state, *J Cell Biol.* **201**, 23-32.

95. D'Angiolella, V., Mari, C., Nocera, D., Rametti, L. & Grieco, D. (2003) The spindle checkpoint requires cyclin-dependent kinase activity, *Genes Dev.* **17**, 2520-5.

96. Rattani, A., Vinod, P. K., Godwin, J., Tachibana-Konwalski, K., Wolna, M., Malumbres, M., Novak, B. & Nasmyth, K. (2014) Dependency of the spindle assembly checkpoint on Cdk1 renders the anaphase transition irreversible, *Curr Biol.* **24**, 630-7.

97. Vazquez-Novelle, M. D., Sansregret, L., Dick, A. E., Smith, C. A., McAinsh, A. D., Gerlich, D. W. & Petronczki, M. (2014) Cdk1 inactivation terminates mitotic checkpoint surveillance and stabilizes kinetochore attachments in anaphase, *Curr Biol.* **24**, 638-45.

98. Morin, V., Prieto, S., Melines, S., Hem, S., Rossignol, M., Lorca, T., Espeut, J., Morin, N. & Abrieu, A. (2012) CDK-dependent potentiation of MPS1 kinase activity is essential to the mitotic checkpoint, *Curr Biol.* **22**, 289-95.

99. Diril, M. K., Bisteau, X., Kitagawa, M., Caldez, M. J., Wee, S., Gunaratne, J., Lee, S. H. & Kaldis, P. (2016) Loss of the Greatwall Kinase Weakens the Spindle Assembly Checkpoint, *PLoS Genet.* **12**, e1006310.

100. Hummer, S. & Mayer, T. U. (2009) Cdk1 negatively regulates midzone localization of the mitotic kinesin Mklp2 and the chromosomal passenger complex, *Curr Biol.* **19**, 607-12.

101. Tsukahara, T., Tanno, Y. & Watanabe, Y. (2010) Phosphorylation of the CPC by Cdk1 promotes chromosome bi-orientation, *Nature.* **467**, 719-23.

102. Elia, A. E., Rellos, P., Haire, L. F., Chao, J. W., Ivins, F. J., Hoepker, K., Mohammad, D., Cantley, L. C., Smerdon, S. J. & Yaffe, M. B. (2003) The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain, *Cell.* **115**, 83-95.

103. Elia, A. E., Cantley, L. C. & Yaffe, M. B. (2003) Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates, *Science.* **299**, 1228-31.

104. Kruse, T., Zhang, G., Larsen, M. S., Lischetti, T., Streicher, W., Kragh Nielsen, T., Bjorn, S. P. & Nilsson, J. (2013) Direct binding between BubR1 and B56-PP2A phosphatase complexes regulate mitotic progression, *J Cell Sci.* **126**, 1086-92.

105. Suijkerbuijk, S. J., Vleugel, M., Teixeira, A. & Kops, G. J. (2012) Integration of kinase and phosphatase activities by BUBR1 ensures formation of stable kinetochore-microtubule attachments, *Dev Cell.* **23**, 745-55.

106. Xu, P., Raetz, E. A., Kitagawa, M., Virshup, D. M. & Lee, S. H. (2013) BUBR1 recruits PP2A via the B56 family of targeting subunits to promote chromosome congression, *Biol Open.* **2**, 479-86.

107. Foley, E. A., Maldonado, M. & Kapoor, T. M. (2011) Formation of stable attachments between kinetochores and microtubules depends on the B56-PP2A phosphatase, *Nat Cell Biol.* **13**, 1265-71.

108. Espert, A., Uluocak, P., Bastos, R. N., Mangat, D., Graab, P. & Gruneberg, U. (2014) PP2A-B56 opposes Mps1 phosphorylation of Knl1 and thereby promotes spindle assembly checkpoint silencing, *J Cell Biol.* **206**, 833-42.

109. Izawa, D. & Pines, J. (2015) The mitotic checkpoint complex binds a second CDC20 to inhibit active APC/C, *Nature.* **517**, 631-4.

110. Yudkovsky, Y., Shteinberg, M., Listovsky, T., Brandeis, M. & Hershko, A. (2000) Phosphorylation of Cdc20/fizzy negatively regulates the mammalian cyclosome/APC in the mitotic checkpoint, *Biochem Biophys Res Commun.* **271**, 299-304.

111. Hein, J. B., Hertz, E. P. T., Garvanska, D. H., Kruse, T. & Nilsson, J. (2017) Distinct kinetics of serine and threonine dephosphorylation are essential for mitosis, *Nat Cell Biol.* **19**, 1433-1440.

112. Labit, H., Fujimitsu, K., Bayin, N. S., Takaki, T., Gannon, J. & Yamano, H. (2012) Dephosphorylation of Cdc20 is required for its C-box-dependent activation of the APC/C, *EMBO J.* **31**, 3351-62.

113. Qiao, R., Weissmann, F., Yamaguchi, M., Brown, N. G., VanderLinden, R., Imre, R., Jarvis, M. A., Brunner, M. R., Davidson, I. F., Litos, G., Haselbach, D., Mechtler, K., Stark, H., Schulman, B. A. & Peters, J. M. (2016) Mechanism of APC/CCDC20 activation by mitotic phosphorylation, *Proc Natl Acad Sci U S A.* **113**, E2570-8.

114. Fujimitsu, K., Grimaldi, M. & Yamano, H. (2016) Cyclin-dependent kinase 1-dependent activation of APC/C ubiquitin ligase, *Science.* **352**, 1121-4.

115. Zhang, S., Chang, L., Alfieri, C., Zhang, Z., Yang, J., Maslen, S., Skehel, M. & Barford, D. (2016) Molecular mechanism of APC/C activation by mitotic phosphorylation, *Nature.* **533**, 260-264.

116. Patra, D. & Dunphy, W. G. (1998) Xe-p9, a Xenopus Suc1/Cks protein, is essential for the Cdc2-dependent phosphorylation of the anaphase- promoting complex at mitosis, *Genes Dev.* **12**, 2549-59.

117. Alfonso-Perez, T., Hayward, D., Holder, J., Gruneberg, U. & Barr, F. A. (2019) MAD1-dependent recruitment of CDK1-CCNB1 to kinetochores promotes spindle checkpoint signaling, *J Cell Biol.* **218**, 1108-1117.

118. Chen, Q., Zhang, X., Jiang, Q., Clarke, P. R. & Zhang, C. (2008) Cyclin B1 is localized to unattached kinetochores and contributes to efficient microtubule attachment and proper chromosome alignment during mitosis, *Cell Res.* **18**, 268-80.

119. Bentley, A. M., Normand, G., Hoyt, J. & King, R. W. (2007) Distinct sequence elements of cyclin B1 promote localization to chromatin, centrosomes, and kinetochores during mitosis, *Mol Biol Cell.* **18**, 4847-58.

120. Pagliuca, F. W., Collins, M. O., Lichawska, A., Zegerman, P., Choudhary, J. S. & Pines, J. (2011) Quantitative proteomics reveals the basis for the biochemical specificity of the cell-cycle machinery, *Mol Cell.* **43**, 406-17.

121. Barr, F. A., Elliott, P. R. & Gruneberg, U. (2011) Protein phosphatases and the regulation of mitosis, *J Cell Sci.* **124**, 2323-34.

122. Nilsson, J. (2019) Protein phosphatases in the regulation of mitosis, *J Cell Biol.* **218**, 395-409.

123. Shi, Y. (2009) Serine/threonine phosphatases: mechanism through structure, *Cell.* **139**, 468-84.

124. Heroes, E., Lesage, B., Gornemann, J., Beullens, M., Van Meervelt, L. & Bollen, M. (2013) The PP1 binding code: a molecular-lego strategy that governs specificity, *FEBS J.* **280**, 584-95.

125. Bollen, M., Peti, W., Ragusa, M. J. & Beullens, M. (2010) The extended PP1 toolkit: designed to create specificity, *Trends Biochem Sci.* **35**, 450-8.

126. Janssens, V. & Goris, J. (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling, *Biochem J.* **353**, 417-39.

127. Godfrey, M., Touati, S. A., Kataria, M., Jones, A., Snijders, A. P. & Uhlmann, F. (2017) PP2A(Cdc55) Phosphatase Imposes Ordered Cell-Cycle Phosphorylation by Opposing Threonine Phosphorylation, *Mol Cell.* **65**, 393-402 e3.

128. Mochida, S., Ikeo, S., Gannon, J. & Hunt, T. (2009) Regulated activity of PP2A-B55 delta is crucial for controlling entry into and exit from mitosis in Xenopus egg extracts, *EMBO J.* **28**, 2777-85.

129. Schmitz, M. H., Held, M., Janssens, V., Hutchins, J. R., Hudecz, O., Ivanova, E., Goris, J., Trinkle-Mulcahy, L., Lamond, A. I., Poser, I., Hyman, A. A., Mechtler, K., Peters, J. M. & Gerlich, D. W. (2010) Live-cell imaging RNAi screen identifies PP2A-B55alpha and importin-beta1 as key mitotic exit regulators in human cells, *Nat Cell Biol.* **12**, 886-93.

130. McCloy, R. A., Parker, B. L., Rogers, S., Chaudhuri, R., Gayevskiy, V., Hoffman, N. J., Ali, N., Watkins, D. N., Daly, R. J., James, D. E., Lorca, T., Castro, A. & Burgess, A. (2015) Global Phosphoproteomic Mapping of Early Mitotic Exit in Human Cells Identifies Novel Substrate Dephosphorylation Motifs, *Mol Cell Proteomics.* **14**, 2194-212.

131. Cundell, M. J., Hutter, L. H., Nunes Bastos, R., Poser, E., Holder, J., Mohammed, S., Novak, B. & Barr, F. A. (2016) A PP2A-B55 recognition signal controls substrate dephosphorylation kinetics during mitotic exit, *J Cell Biol.* **214**, 539-54.

132. Wu, J. Q., Guo, J. Y., Tang, W., Yang, C. S., Freel, C. D., Chen, C., Nairn, A. C. & Kornbluth, S. (2009) PP1-mediated dephosphorylation of phosphoproteins at mitotic exit is controlled by inhibitor-1 and PP1 phosphorylation, *Nat Cell Biol.* **11**, 644-51.

133. Bouchoux, C. & Uhlmann, F. (2011) A quantitative model for ordered Cdk substrate dephosphorylation during mitotic exit, *Cell.* **147**, 803-14.

134. Mocciaro, A. & Schiebel, E. (2010) Cdc14: a highly conserved family of phosphatases with non-conserved functions?, *J Cell Sci.* **123**, 2867-76.

135. Gharbi-Ayachi, A., Labbe, J. C., Burgess, A., Vigneron, S., Strub, J. M., Brioudes, E., Van-Dorsselaer, A., Castro, A. & Lorca, T. (2010) The substrate of Greatwall kinase, Arpp19, controls mitosis by inhibiting protein phosphatase 2A, *Science.* **330**, 1673-7.

136. Mochida, S., Maslen, S. L., Skehel, M. & Hunt, T. (2010) Greatwall phosphorylates an inhibitor of protein phosphatase 2A that is essential for mitosis, *Science.* **330**, 1670-3.

137. Castro, A. & Lorca, T. (2018) Greatwall kinase at a glance, *J Cell Sci.* **131**.

138. Williams, B. C., Filter, J. J., Blake-Hodek, K. A., Wadzinski, B. E., Fuda, N. J., Shalloway, D. & Goldberg, M. L. (2014) Greatwall-phosphorylated Endosulfine is both an inhibitor and a substrate of PP2A-B55 heterotrimers, *Elife.* **3**, e01695.

139. Blake-Hodek, K. A., Williams, B. C., Zhao, Y., Castilho, P. V., Chen, W., Mao, Y., Yamamoto, T. M. & Goldberg, M. L. (2012) Determinants for activation of the atypical AGC kinase Greatwall during M phase entry, *Mol Cell Biol.* **32**, 1337-53.

140. Chung, E. & Chen, R. H. (2003) Phosphorylation of Cdc20 is required for its inhibition by the spindle checkpoint, *Nat Cell Biol.* **5**, 748-53.

141. Tang, Z., Shu, H., Oncel, D., Chen, S. & Yu, H. (2004) Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint, *Mol Cell.* **16**, 387-97.

142. Jia, L., Li, B. & Yu, H. (2016) The Bub1-Plk1 kinase complex promotes spindle checkpoint signalling through Cdc20 phosphorylation, *Nat Commun.* **7**, 10818.

143. Pinna, L. A., Donella, A., Clari, G. & Moret, V. (1976) Preferential dephosphorylation of protein bound phosphorylthreonine and phosphorylserine residues by cytosol and mitochondrial "casein phosphatases", *Biochem Biophys Res Commun.* **70**, 1308-15.

144. Visconti, R., Palazzo, L., Della Monica, R. & Grieco, D. (2012) Fcp1-dependent dephosphorylation is required for M-phase-promoting factor inactivation at mitosis exit, *Nat Commun.* **3**, 894.

145. Kim, T., Lara-Gonzalez, P., Prevo, B., Meitinger, F., Cheerambathur, D. K., Oegema, K. & Desai, A. (2017) Kinetochores accelerate or delay APC/C activation by directing Cdc20 to opposing fates, *Genes Dev.* **31**, 1089-1094.

146. Cundell, M. J., Bastos, R. N., Zhang, T., Holder, J., Gruneberg, U., Novak, B. & Barr, F. A. (2013) The BEG (PP2A-B55/ENSA/Greatwall) pathway ensures cytokinesis follows chromosome separation, *Mol Cell.* **52**, 393-405.

147. Maciejowski, J., Drechsler, H., Grundner-Culemann, K., Ballister, E. R., Rodriguez-Rodriguez, J. A., Rodriguez-Bravo, V., Jones, M. J. K., Foley, E., Lampson, M. A., Daub, H., McAinsh, A. D. & Jallepalli, P. V. (2017) Mps1 Regulates Kinetochore-Microtubule Attachment Stability via the Ska Complex to Ensure Error-Free Chromosome Segregation, *Dev Cell.* **41**, 143-156 e6.

148. Hayward, D., Bancroft, J., Mangat, D., Alfonso-Perez, T., Dugdale, S., McCarthy, J., Barr, F. A. & Gruneberg, U. (2019) Checkpoint signaling and error correction require regulation of the MPS1 T-loop by PP2A-B56, *bioRxiv*.

149. Wang, X., Bajaj, R., Bollen, M., Peti, W. & Page, R. (2016) Expanding the PP2A Interactome by Defining a B56-Specific SLiM, *Structure.* **24**, 2174-2181.

150. Hertz, E. P. T., Kruse, T., Davey, N. E., Lopez-Mendez, B., Sigurethsson, J. O., Montoya, G., Olsen, J. V. & Nilsson, J. (2016) A Conserved Motif Provides Binding Specificity to the PP2A-B56 Phosphatase, *Mol Cell.* **63**, 686-695.

151. Wang, J., Wang, Z., Yu, T., Yang, H., Virshup, D. M., Kops, G. J., Lee, S. H., Zhou, W., Li, X., Xu, W. & Rao, Z. (2016) Crystal structure of a PP2A B56-BubR1 complex and its implications for PP2A substrate recruitment and localization, *Protein Cell.* **7**, 516-26.

152. Tanno, Y., Kitajima, T. S., Honda, T., Ando, Y., Ishiguro, K. & Watanabe, Y. (2010) Phosphorylation of mammalian Sgo2 by Aurora B recruits PP2A and MCAK to centromeres, *Genes Dev.* **24**, 2169-79.

153. Kitajima, T. S., Sakuno, T., Ishiguro, K., Iemura, S., Natsume, T., Kawashima, S. A. & Watanabe, Y. (2006) Shugoshin collaborates with protein phosphatase 2A to protect cohesin, *Nature.* **441**, 46-52.

154. Kwon, Y. G., Lee, S. Y., Choi, Y., Greengard, P. & Nairn, A. C. (1997) Cell cycle-dependent phosphorylation of mammalian protein phosphatase 1 by cdc2 kinase, *Proc Natl Acad Sci U S A.* **94**, 2168-73.

155. Dohadwala, M., da Cruz e Silva, E. F., Hall, F. L., Williams, R. T., Carbonaro-Hall, D. A., Nairn, A. C., Greengard, P. & Berndt, N. (1994) Phosphorylation and inactivation of protein phosphatase 1 by cyclin-dependent kinases, *Proc Natl Acad Sci U S A.* **91**, 6408-12.

156. Trinkle-Mulcahy, L., Andrews, P. D., Wickramasinghe, S., Sleeman, J., Prescott, A., Lam, Y. W., Lyon, C., Swedlow, J. R. & Lamond, A. I. (2003) Time-lapse imaging reveals dynamic relocalization of PP1gamma throughout the mammalian cell cycle, *Mol Biol Cell.* **14**, 107-17.

157. Liu, D., Vleugel, M., Backer, C. B., Hori, T., Fukagawa, T., Cheeseman, I. M. & Lampson, M. A. (2010) Regulated targeting of protein phosphatase 1 to the outer kinetochore by KNL1 opposes Aurora B kinase, *J Cell Biol.* **188**, 809-20.

158. Sivakumar, S., Janczyk, P. L., Qu, Q., Brautigam, C. A., Stukenberg, P. T., Yu, H. & Gorbsky, G. J. (2016) The human SKA complex drives the metaphase-anaphase cell cycle transition by recruiting protein phosphatase 1 to kinetochores, *Elife.* **5**.

159. Kim, Y., Holland, A. J., Lan, W. & Cleveland, D. W. (2010) Aurora kinases and protein phosphatase 1 mediate chromosome congression through regulation of CENP-E, *Cell.* **142**, 444-55.

160. De Wever, V., Nasa, I., Chamousset, D., Lloyd, D., Nimick, M., Xu, H., Trinkle-Mulcahy, L. & Moorhead, G. B. (2014) The human mitotic kinesin KIF18A binds protein phosphatase 1 (PP1) through a highly conserved docking motif, *Biochem Biophys Res Commun.* **453**, 432-7.

161. Kettenbach, A. N., Schweppe, D. K., Faherty, B. K., Pechenick, D., Pletnev, A. A. & Gerber, S. A. (2011) Quantitative phosphoproteomics identifies substrates and functional modules of Aurora and Polo-like kinase activities in mitotic cells, *Sci Signal.* **4**, rs5.

162. Nasa, I., Rusin, S. F., Kettenbach, A. N. & Moorhead, G. B. (2018) Aurora B opposes PP1 function in mitosis by phosphorylating the conserved PP1-binding RVxF motif in PP1 regulatory proteins, *Sci Signal.* **11**.

163. Nijenhuis, W., Vallardi, G., Teixeira, A., Kops, G. J. & Saurin, A. T. (2014) Negative feedback at kinetochores underlies a responsive spindle checkpoint signal, *Nat Cell Biol.* **16**, 1257-64.

164. Moura, M., Osswald, M., Leca, N., Barbosa, J., Pereira, A. J., Maiato, H., Sunkel, C. E. & Conde, C. (2017) Protein Phosphatase 1 inactivates Mps1 to ensure efficient Spindle Assembly Checkpoint silencing, *Elife.* **6**.

165. Yamagishi, Y., Honda, T., Tanno, Y. & Watanabe, Y. (2010) Two histone marks establish the inner centromere and chromosome bi-orientation, *Science.* **330**, 239-43.

166. Wang, F., Dai, J., Daum, J. R., Niedzialkowska, E., Banerjee, B., Stukenberg, P. T., Gorbsky, G. J. & Higgins, J. M. (2010) Histone H3 Thr-3 phosphorylation by Haspin positions Aurora B at centromeres in mitosis, *Science.* **330**, 231-5.

167. Kelly, A. E., Ghenoiu, C., Xue, J. Z., Zierhut, C., Kimura, H. & Funabiki, H. (2010) Survivin reads phosphorylated histone H3 threonine 3 to activate the mitotic kinase Aurora B, *Science.* **330**, 235-9.

168. Higgins, J. M. (2010) Haspin: a newly discovered regulator of mitotic chromosome behavior, *Chromosoma.* **119**, 137-47.

169. Ghenoiu, C., Wheelock, M. S. & Funabiki, H. (2013) Autoinhibition and Polo-dependent multisite phosphorylation restrict activity of the histone H3 kinase Haspin to mitosis, *Mol Cell.* **52**, 734-45.

170. Zhou, L., Tian, X., Zhu, C., Wang, F. & Higgins, J. M. (2014) Polo-like kinase-1 triggers histone phosphorylation by Haspin in mitosis, *EMBO Rep.* **15**, 273-81.

171. Watanabe, Y. (2005) Shugoshin: guardian spirit at the centromere, *Curr Opin Cell Biol.* **17**, 590-5.

172. Gruneberg, U., Neef, R., Honda, R., Nigg, E. A. & Barr, F. A. (2004) Relocation of Aurora B from centromeres to the central spindle at the metaphase to anaphase transition requires MKlp2, *J Cell Biol.* **166**, 167-72.

173. Vazquez-Novelle, M. D. & Petronczki, M. (2010) Relocation of the chromosomal passenger complex prevents mitotic checkpoint engagement at anaphase, *Curr Biol.* **20**, 1402-7.

174. Elowe, S., Hummer, S., Uldschmid, A., Li, X. & Nigg, E. A. (2007) Tension-sensitive Plk1 phosphorylation on BubR1 regulates the stability of kinetochore microtubule interactions, *Genes Dev.* **21**, 2205-19.

175. Huang, H., Hittle, J., Zappacosta, F., Annan, R. S., Hershko, A. & Yen, T. J. (2008) Phosphorylation sites in BubR1 that regulate kinetochore attachment, tension, and mitotic exit, *J Cell Biol.* **183**, 667-80.

176. Kramer, E. R., Scheuringer, N., Podtelejnikov, A. V., Mann, M. & Peters, J. M. (2000) Mitotic regulation of the APC activator proteins CDC20 and CDH1, *Mol Biol Cell.* **11**, 1555-69.

Table 1

CDK1 target	Phospho-site	Function	Phosphatase	Reference
MPS1	S281 (H. sapiens)	Kinetochore localisation	PP2A-B55	[45]
MPS1	S283 (X. laevis)	Activity	N.D.	[98]
	S821	Activity	PP2A-B55	[99]
BUB1	S459	MAD1 recruitment	N.D.	[61, 62, 64]
BUBR1	T620	PLK1 recruitment	N.D	[174]
	T670	PP2A-B56 recruitment	N.D.	[175]
CDC20	S41, S51, T55, T59, T70, T106, T157, S487	Regulation of CDC20-MAD2 and CDC20-APC/C interaction	PP2A-B55; PP1; Fcp1	[110-112, 130, 144, 145, 176]
APC1	S362, S364, S372, S373, S377 and other sites	Regulation of APC/C-CDC20 interaction	N.D.	[113-115]
APC3	~50 sites on APC3	Regulation of APC/C-CDC20 interaction	PP2A-B55	[111, 113-115]
INCENP (CPC)	T59	Regulation of CPC interaction with MKLP2	PP2A-B55	[100, 111]
Borealin (CPC)	T106, T172, T185, T189, T199, T204 S219	Recruitment of the chromosomal passenger complex to centromeres	N.D.	[101]
Haspin	T128 (H. sapiens), T206 (X. laevis)	Initiation of Haspin activation by creation of PLK1 docking site	N.D.	[169, 170]

N.D.; not determined

Figure Legends

Figure 1. Overview of the Spindle Assembly Checkpoint

A. Key of phosphorylations by CDK1, MPS1 and Aurora B, and dephosphorylation by PP2A-B56. **B.** Diagram of the Spindle Assembly Checkpoint at an unattached kinetochore. **i.** MPS1 is recruited to the NDC80 complex as a result of Aurora B phosphorylations of the NDC80 N-terminus and CDK1 phosphorylation of MPS1 at S281. **ii.** MPS1 phosphorylates multiple MELT motifs on KNL1 leading to recruitment of BUB3, BUB1 and BUBR1 to the outer kinetochore. **iii.** Phosphorylation of BUB1 by CDK1 allows MPS1 to phosphorylate BUB1-T461, leading to MAD1 recruitment, which in complex with MAD2 acts to catalyse the conversion of open MAD2 (O-MAD2) to closed MAD2 (C-MAD2). MAD1 also localises CDK1-cyclin B1 to the kinetochore. **iv.** C-MAD2, BUBR1 and BUB3 form the Mitotic Checkpoint Complex (MCC) alongside the Anaphase Promoting Complex (APC/C) co-activator CDC20, blocking APC/C activation. Phosphorylation of the CDC20 N-terminus by CDK1 increases its affinity to the MCC, whilst CDK1 phosphorylations of the APC/C are critical to enable CDC20 binding. **v.** PP2A-B56, which dephosphorylates the KNL1 MELT motifs, is recruited to the kinetochore in a CDK1 and MPS1 dependent manner, whilst PP1 recruitment is promoted by PP2A-B56 mediated dephosphorylation of the KNL1 N-terminal RVSF motif **C.** Microtubule attachments at kinetochores lead to the loss of SAC proteins from the kinetochore. Once all kinetochores are attached to microtubules, the MCC no longer inhibits the APC/C, which now, active, targets cyclin B1 and Securin for degradation leading to chromosome segregation.

Figure 2. Opposing activities of CDK1-Cyclin B1 and PP2A-B55 limit the spindle checkpoint licensed period

A. i. At cyclin B1 levels above 75 nM, PP2A-B55 is inhibited, and cytoplasmic MPS1 is phosphorylated at S281 and can localise to unattached kinetochores [45]. Should a metaphase microtubule-kinetochore detachment occur in this situation, MPS1 is still phosphorylated at S281 and re-localises to kinetochores. MPS1 generates a SAC response at that kinetochore, inhibiting the APC/C and halting cyclin B1 degradation. **ii.** At cyclin B1 concentrations below 75 nM, PP2A-B55 activity surpasses that of CDK1, leading to MPS1 S281 dephosphorylation. The CPC is also dephosphorylated, triggering Aurora B translocation away from centromeres. The combination of these changes prevents MPS1 from localising to kinetochores and initiating an APC/C inhibiting SAC signal. **B.** Graphical illustration of relative activity levels of CDK1-cyclin B1 and PP2A-B55 and levels of APC/C inhibition and MPS1-S281 phosphorylation throughout mitosis.

Figure 3. Regulation of the APC/C by phosphorylation and two distinct pools of CDC20

i. APC/C is phosphorylated by CDK-cyclin. **ii.** This reaction is enhanced by CDK-cyclin binding to CDK-phosphorylated APC/C, mediated by Cks proteins (not shown). **iii.** The phosphorylation of APC/C allows CDC20 binding, and thus activation of APC/C. **iv.** At the same time, CDC20 is phosphorylated by CDK-cyclin. This reaction may favour CDC20 incorporation into MCC. **v.** MCC, containing phosphorylated CDC20, binds and inhibits APC/C with a bound molecule of CDC20. In mitosis, APC/C is thus associated with two functionally distinct molecules of CDC20.

Figure 4. Cyclin B1 is a spindle assembly checkpoint protein

A-B. Cyclin B1 localizes to unattached SAC positive kinetochores and is excluded from attached kinetochores. Images of HeLa cells expressing cyclin B1 endogenously tagged with GFP at the C-terminus (in green) are shown. Single kinetochore pairs are enlarged. SAC protein MAD1 labels unattached kinetochores (**A**) and kinetochore protein Astrin marks attached kinetochores (**B**) in red.

Figure 1

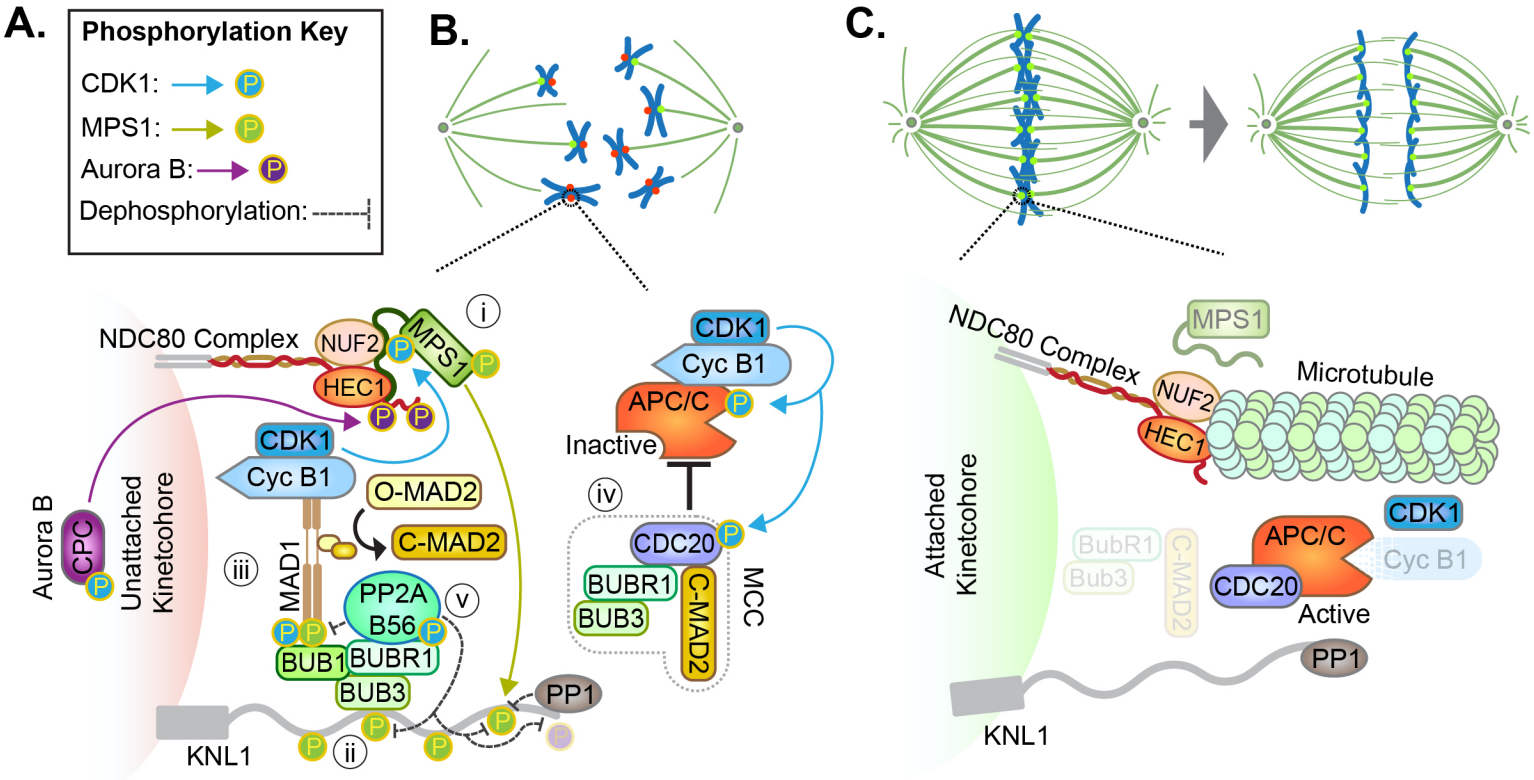
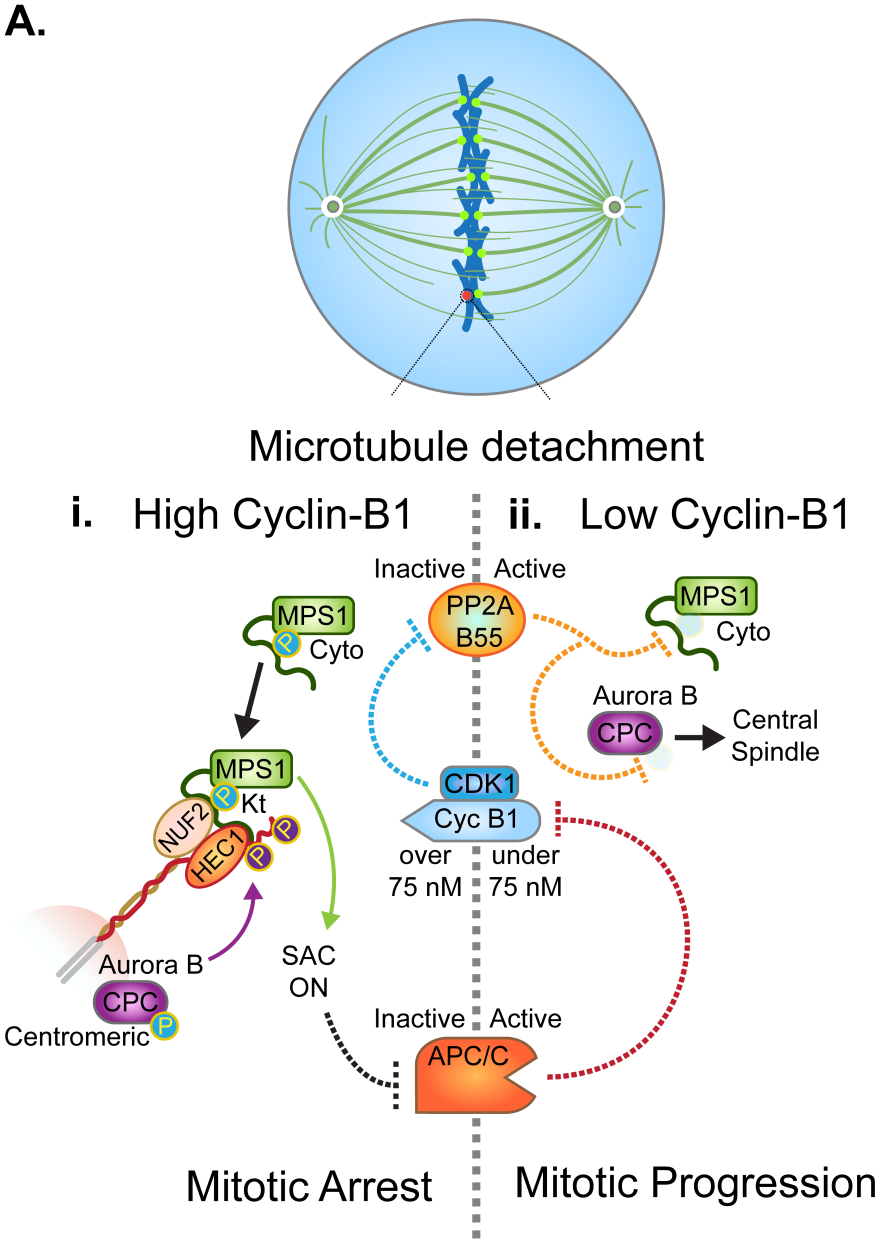

A.
Phosphorylation Key
CDK1:
MPS1:
Aurora B:
Dephosphorylation:
B.
C.
NDC80 Complex
NUF2
HEC1
MPS1
CDK1
Cyc B1
O-MAD2
C-MAD2
MAD1
PP2A
B56
BUB1
BUBR1
BUB3
PP1
KNL1
Aurora B
CPC
Unattached Kinetochore
APC/C
Inactive
CDC20
MCC
Microtubule
Attached Kinetochore
BubR1
Bub3
Active

Figure 2

A.


Microtubule detachment
i. High Cyclin-B1
ii. Low Cyclin-B1
Inactive
Active
MPS1
Cyto
PP2A
B55
MPS1
Cyto
Aurora B
CPC
Central
Spindle
MPS1
Kt
NUF2
HEC1
CDK1
Cyc B1
over
75 nM
under
75 nM
Aurora B
CPC
Centromeric
SAC
ON
Inactive
Active
APC/C
Mitotic Arrest
Mitotic Progression

B.

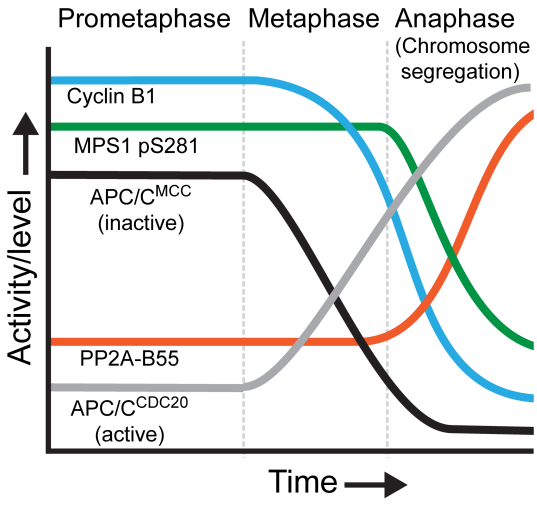

Prometaphase
Metaphase
Anaphase
(Chromosome
segregation)
Cyclin B1
MPS1 pS281
APC/C^MCC
(inactive)
PP2A-B55
APC/C^CDC20
(active)
Activity/level
Time

Figure 3

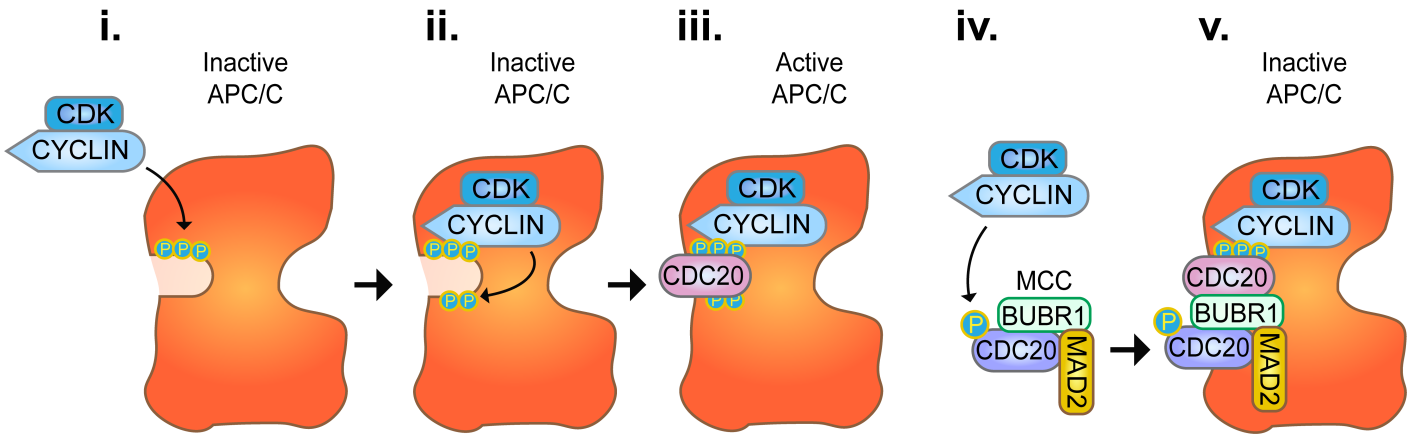
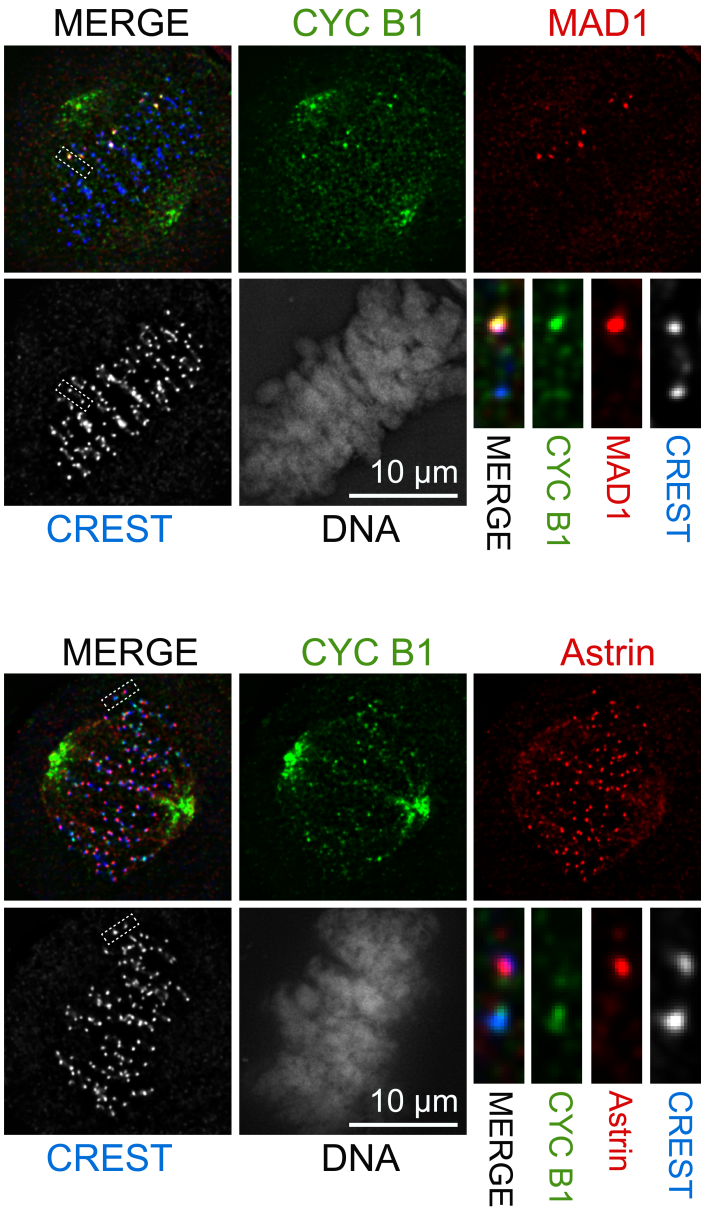

i.
Inactive APC/C
CDK
CYCLIN
ii.
Inactive APC/C
CDK
CYCLIN
iii.
Active APC/C
CDK
CYCLIN
CDC20
iv.
CDK
CYCLIN
MCC
BUBR1
CDC20
MAD2
v.
Inactive APC/C
CDK
CYCLIN
CDC20
BUBR1
CDC20
MAD2

Figure 4


MERGE
CYC B1
MAD1
CREST
DNA
10 µm
MERGE
CYC B1
MAD1
CREST
MERGE
CYC B1
Astrin
CREST
DNA
10 µm
MERGE
CYC B1
Astrin
CREST