Cdk1 promotes kinetochore bi-orientation and regulates Cdc20 expression during recovery from spindle checkpoint arrest

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The spindle assembly checkpoint (SAC), an evolutionarily conserved surveillance pathway, prevents chromosome segregation in response to conditions that disrupt the kinetochore-microtubule attachment. Removal of the checkpoint-activating stimulus initiates recovery during which spindle integrity is restored, kinetochores become bi-oriented, and cells initiate anaphase. Whether recovery ensues passively after the removal of checkpoint stimulus, or requires mediation by specific effectors remains uncertain. Here, we report two unrecognized functions of yeast Cdk1 required for efficient recovery from SAC-induced arrest. We show that Cdk1 promotes kinetochore bi-orientation during recovery by restraining premature spindle elongation thereby extinguishing SAC signalling. Moreover, Cdk1 is essential for sustaining the expression of Cdc20, an activator of the anaphase promoting complex/cyclosome (APC/C) required for anaphase progression. We suggest a model in which Cdk1 activity promotes recovery from SAC-induced mitotic arrest by regulating bi-orientation and APC/C activity. Our findings provide fresh insights into the regulation of mitosis and have implications for the therapeutic efficacy of anti-mitotic drugs.

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Introduction

Establishment of kinetochore bi-orientation (also called amphitelic or bi-polar attachment) is critical for chromosome segregation during mitosis, so much so that failure of a single chromosome to attain bi-orientation brings cell-cycle progression to a grinding halt in metaphase (Li and Nicklaus, 1995; Kline-Smith *et al*, 2005). The cell-cycle arrest in response to unattached kinetochores is imposed by the spindle assembly checkpoint (SAC) ensuring that anaphase is not initiated until all chromosomes are appropriately attached to the spindle (Amon, 1999).

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In budding yeast, the SAC signalling network involves highly conserved proteins Mad1, Mad2, Mad3 (orthologues of human Mad1, Mad2 and BubR1, respectively), Bub1, Bub3 (orthologues of human Bub1 and Bub3), Mps1 (orthologue of human Mps1) and Ipl1 (orthologue of human aurora B kinase) (Musacchio and Salmon, 2007). Bi-orientation defects are detected by sensor kinases, Ipl1, Mps1 and Bub1 (Weiss and Winey, 1996; Farr and Hoyt, 1998; Biggins and Murray, 2001). Activation of these kinases results in rapid recruitment and phosphorylation of downstream effectors such as Bub3, Mad1, Mad2 and Mad3. These proteins assemble into complexes at the unattached kinetochore and inhibit anaphase promoting complex/cyclosome (APC/C)^{Cdc20}. Consequently, the targets of APC/C^{Cdc20}, such as securin (Pds1 in yeast) and Cyclin B (Clbs in yeast), are stabilized, sister chromatids remain tethered by cohesins and cells arrest in metaphase with unsegregated chromosomes and high Cdk1 activity (Li and Cai, 1997; D'Angiolella *et al*, 2003). Interestingly, SAC activation also promotes turnover of Cdc20, leading to lower Cdc20 levels (Pan and Chen, 2004; Nilsson *et al*, 2008), ensuring that checkpoint-imposed arrest is not overridden by Cdc20 hyperaccumulation. During the normal cycle, Cdc20 is regulated both at the transcriptional level and by proteolytic degradation. While *CDC20* gene is expressed in a cell cycle-dependent manner and is driven by a hybrid promoter with Yox1-, Mcm1- and Fkh-binding sites (Zhu *et al*, 2000; Darieva *et al*, 2010), Cdc20 protein is subjected to proteolytic degradation throughout the cell cycle (Prinz *et al*, 1998). Thus, both the transcriptional regulation and proteolytic degradation are relevant to the maintenance of Cdc20 levels during SAC-induced mitotic arrest. But how these processes are regulated remains unclear.

Once the checkpoint stimulus is removed, cells must extinguish SAC signalling, establish bi-orientation and initiate anaphase. Studies in mammalian cells suggest that SAC signalling is actively silenced by specific mechanisms rather than by passive dissipation of the upstream signals once bi-orientation has been achieved (Howell *et al*, 2001; Wassmann *et al*, 2003). For instance, p31 (comet) as well as mechanisms involving Cdc20 ubiquitylation have been implicated in the reversal of Cdc20 inhibition by Mad2 (Xia *et al*, 2004; Reddy *et al*, 2007). While inactivation of the SAC is clearly central to the initiation of recovery, maintenance of the cellular context permissive for silencing of the checkpoint is equally critical. Treatment with the microtubule poison nocodazole, for instance, causes disassembly of mitotic spindle, disrupts kinetochore-microtubule attachment and results in SAC activation. During the arrest, cells must maintain cellular conditions that are conducive to rapid spindle assembly and the establishment of bi-orientation to ensure efficient recovery once nocodazole is removed. However, the cellular requirements for a rapid return to normalcy from SAC-induced arrest remain poorly understood.

The involvement of Cdk1 kinase activity in the maintenance of SAC-induced arrest has been previously documented (Li and Cai, 1997; D'Angiolella *et al*, 2003). However, it remains unknown whether there is any specific requirement for high Cdk1 activity in establishing the cellular context permissive for recovery from SAC arrest. In this study, we have uncovered two important functions for Cdk1 activity that facilitate recovery from SAC-induced arrest: (i) coordination of bi-orientation and spindle extension when the mitotic spindle is prone to premature extension due to high levels of elongation-conductive proteins Ase1 and Cin8 (ii) maintenance of the cellular levels of APC/C activator Cdc20 through transcription regulation. Failure to fulfill these requirements in the absence of Cdk1 activity makes it virtually impossible for cells to recover from SAC-induced arrest and resume cell-cycle progression.

Results

Cells deficient in Cdk1/Clb activity fail to recover from SAC arrest

To understand the role of Cdk1 activity in recovery from spindle checkpoint-induced arrest, we utilized *cdc28-as1* (*cdk1-as1*) strain in which the mitotic activity of Cdk1 can be abolished by treatment with the ATP analogue 1NM-PP1 (Bishop *et al*, 2000). Treatment with 500 nM 1NM-PP1 is effective both *in vitro* and *in vivo*, as it abolishes the mitotic kinase activity of Cdk1 *in vitro* (Supplementary Figure S1A), prevents cells from assembling a bi-polar spindle and inhibits entry into mitosis after release from G1 arrest (Supplementary Figure S1B). Since Cdk1 kinase inhibition in mitotic cells causes premature spindle breakdown and cytokinesis (Supplementary Figure S1C, left panel), precocious activation of these events may mask Cdk1's role during recovery from SAC. To prevent the activation of mitotic exit-related events initiated by a reduction in Cdk1 activity, we introduced a telophase trap consisting of the temperature-sensitive mutation *cdc15-2* and a deletion in *SLK19* gene to inactivate both MEN (Mitotic Exit Network) and FEAR (Fourteen Early Anaphase Release) pathways required for efficient mitotic exit (Mah *et al*, 2001; Stegmeier *et al*, 2002). *slk19Δ cdc15-2* cells do not show any observable spindle abnormality and can efficiently recover from nocodazole-induced SAC arrest, albeit with a minor delay (Supplementary Figure S1D). The checkpoint arrest was induced by nocodazole treatment and recovery was initiated by nocodazole removal. Although the use of nocodazole treatment may be relatively harsh, we deemed it helpful in exposing the underlying regulatory design. The SAC-induced arrest was monitored by Mad1 phosphorylation and the stabilization of securin Pds1 (Figure 1A), while the checkpoint inactivation and cells' recovery were ascertained by the loss of Mad1 phosphorylation, degradation of Pds1 and mitotic spindle reassembly and extension, followed by chromosome segregation.

cdc28-as1 slk19Δ cdc15-2 cells were synchronized in G1 by α factor treatment and then released into nocodazole-containing medium at restrictive temperature (30°C). After the arrest in mitosis (Figure 1A), nocodazole was removed and cells were allowed to recover in the absence or presence of 1NM-PP1. Without 1NM-PP1, cells recovered and assembled metaphase spindles ~20 min after the release, with 75% of cells

exhibiting long spindles and divided nuclei at 120 min (Figure 1B, left panel). Correspondingly, Pds1 was degraded and Mad1 phosphorylation declined (Figure 1B and C). In the presence of 1NM-PP1, however, while the cells assembled a bipolar spindle after nocodazole removal, >80% remained arrested with short spindles and undivided nuclei at 120 min (Figure 1B and C, right panel). Pds1 levels and Mad1 phosphorylation remained largely unchanged, suggesting that SAC is not extinguished. Stabilization of Clb2 (Figure 1B) and FACS data confirmed that cells had not exited mitosis. These observations suggest that without Cdk1 activity, SAC signalling remains active despite the removal of nocodazole; hence, cells are unable to recover from nocodazole-induced arrest and fail to progress into anaphase.

It is important to add here a brief cautionary note concerning other cellular changes elicited by the inhibition of Cdk1 activity during mitosis that are relevant to the execution of SAC. As described in greater depth later, the inhibition of Cdk1 activity in SAC-arrested cells leads to a dramatic reduction in the levels of Cdc20 (a major target of SAC). Hence, cells' inability to initiate anaphase during recovery from SAC-induced arrest in the absence (or very low levels) of Cdk1 activity could, in principle, be explained entirely in terms of the absence of Cdc20 protein. However, as shown in Supplementary Figure S2A and B, cells are unable to undergo anaphase during recovery from SAC in the absence of Cdk1 activity even when supplied with constant levels of Cdc20, suggesting that Cdk1 serves some other role(s) during the process of recovery, in addition to the maintenance of Cdc20 levels. We explore in-depth this notion in the following sections.

Cells are unable to re-establish bi-orientation during recovery in the absence of Cdk1 activity

One possible reason why SAC signalling persists in *cdc28-as1 slk19Δ cdc15-2* cells is because cells fail to attain bi-orientation in the absence of Cdk1 activity despite having assembled a short bipolar spindle. Therefore, we examined the establishment of bi-orientation using 'tetracyclin operator and repressor system' which allows visualization of chromosome V centromeres (*CENV*) with GFP (Michaelis *et al*, 1997). *cdc28-as1 slk19Δ cdc15-2* cells with GFP-labelled *CENV* were arrested in metaphase by nocodazole and then released in the absence or presence of 1NM-PP1. Without 1NM-PP1, cells established bi-orientation and proceeded to anaphase with 60% of cells showing two, well-separated GFP dots near each SPB at 120 min after nocodazole removal (Figure 1D). In contrast, only 21% of cells exhibited bi-orientation at 120 min in the presence of 1NM-PP1. The percentage of cells with two GFP dots increases sluggishly over time, suggestive of an inability to establish bi-orientation efficiently in the absence of Cdk1 activity (Figure 1D). To ensure that compromised bi-orientation efficiency is due to Cdk1 inhibition, 1NM-PP1 was removed after 120 min of treatment to allow the restoration of Cdk1 activity. A surge in the proportion of bi-oriented cells (~60%) can be seen within 60 min after 1NM-PP1 removal, which subsequently progress into anaphase at 120 min (Supplementary Figure S3A). Consistent with this, overexpression of wild-type Cdk1 in 1NM-PP1-treated *cdc28-as1 slk19Δ cdc15-2* cells can restore bi-orientation to a similar extent (Supplementary Figure S3B). Hence, the inability of 1NM-PP1-treated *cdc28-as1* cells to switch off the SAC after

Figure 1 Recovery of *cdc28-as1* cells from nocodazole-induced SAC arrest. **(A)** G1 synchronized *cdc28-as1 slk19Δ cdc15-2* cells expressing Pds1-myc₁₈ were released into nocodazole-containing medium at 30°C. Pds1-myc₁₈, Mad1, G6PD levels and photomicrograph of cells arrested in nocodazole are shown. **(B)** Nocodazole-arrested cells as described in **(A)** were released in fresh medium with or without 1NM-PP1. Pds1-myc₁₈, Mad1, Clb2-HA₃, G6PD levels, DNA content and spindles were monitored. Bar scale: 5 μm. **(C)** Graph showing proportion of cells that recovered from nocodazole arrest as determined by their entry into anaphase. **(D)** *cdc28-as1 slk19Δ cdc15-2* cells arrested with nocodazole treatment were released into medium with or without 1NM-PP1. Photomicrograph shows Spc42-RFP (marking the SPBs) and CENV-GFP staining at 120 min. The graph shows the bi-orientation kinetics. In all, 100–200 cells were analysed for each sample. Each experiment was performed at least twice and one representative experiment is shown here. Bar scale: 5 μm. Figure source data can be found in Supplementary data.

nocodazole removal is due to their failure to establish bi-orientation efficiently in the absence of Cdk1 activity.

Microtubule capture by kinetochores per se does not require Cdk1 activity

The re-establishment of bi-orientation after nocodazole removal requires the capture of one member of the sister

kinetochore pair by microtubules emanating from one SPB followed by attachment of the second member to the other SPB (Tanaka *et al*, 2005). It is possible that the inability of cells to re-establish bi-orientation efficiently during recovery without Cdk1 activity (Figure 2A) is because the kinetochores are unable to capture any microtubules at all. Alternatively, while one sister kinetochore may be able to

Figure 2 Cdk1 inhibition does not affect kinetochore capturing. (A) *cdc28-as1 GAL-CENIII-GFP SPC42-RFP MET-CDC20* cells were incubated in nocodazole-containing YEP + Raff + Gal + Met medium and then released into three different media conditions: (1) YEP + Gal + Met + 1NM-PP1 medium, (2) YEP + Glu + Met + 1NM-PP1 medium and (3) YEP + Glu + Met -1NM-PP1 medium. Photomicrographs depict the positions of *CENIII-GFP* and *SpC42-RFP* 30 min after release. Bar scale: 5 μ m. Bottom panels: bar charts showing proportion of cells with distances between *CENIII-GFP* and *SpC42-RFP* after release from nocodazole arrest. Approximately 100 cells were analysed for each sample. (B) Bi-orientation in *cdc28-as1 slk19Δ cdc15-2* cells after release from HU arrest in absence or presence of 1NM-PP1. Photomicrographs show the state of nuclear division, spindle staining and *CENV-GFP* signal 60 and 120 min after release from HU arrest. Bar scale: 5 μ m. Graph depicts bi-orientation kinetics after the release. Experiments were performed twice and >200 cells were analysed for each sample.

capture microtubules from one pole, the other sister fails to connect to the opposite pole in the absence of Cdk1 activity.

To distinguish between these two possibilities, we determined the kinetics of kinetochore capture in cells recovering from nocodazole-induced arrest by measuring the distance between the GFP-labelled *CENIII* and its nearest SPB. In yeast, captured kinetochores (prior to bi-orientation) usually lie close to the SPBs as a result of pole-ward transportation along the nuclear microtubules. In contrast, the distance between totally unattached kinetochores and SPBs shows a random distribution (Tanaka *et al*, 2005). We used cells in which *GAL1* promoter had been introduced upstream of the chromosome III centromere (*CENIII*) (Tanaka *et al*, 2005). In galactose medium, transcription activity from *GAL1* across centromeric region prevents kinetochore assembly, precluding any attachment to microtubules. A shift to glucose medium, however, turns off the *GAL1* promoter allowing kinetochore assembly and its capture by microtubules emanating from the SPBs. To prevent cells from entering anaphase during the experiment, we replaced endogenous *CDC20* by a methionine promoter-driven *CDC20* and incubated cells in methionine medium (+Met) to turn off *Cdc20* expression.

cdc28-as1 cells carrying GFP-detectable *GAL1-CENIII*, *SPC42-RFP* (SPB component) and *MET-CDC20* were arrested in YEP + Gal + Met medium containing nocodazole. Once the cells were arrested, nocodazole was removed, the culture was washed and cells were re-suspended in YEP + Gal + Met medium containing 1NM-PP1, YEP + Glu + Met medium containing 1NM-PP1 or YEP + Glu + Met medium free of 1NM-PP1. The relative positions of *CENIII* and SPB were determined. In YEP + Gal + Met medium containing 1NM-PP1, *CENIII* remained >1 µm away from its nearest SPB in ~80% of the cells. However, *CENIII* was located very close to the SPB (<1 µm) in 70% cells in YEP + Glu + Met medium regardless of 1NM-PP1 addition (Figure 2A). This implies that cells are capable of capturing the kinetochores in the absence of Cdk1 activity and dragging them pole-ward, making establishment of bi-orientation difficult. However, it is noteworthy that over-expression of bi-orientation promoting proteins, such as *Ipl1* and *Sgo1*, does not rescue the bi-orientation defect in cells lacking Cdk1 activity (our unpublished observations), suggesting that Cdk1 may not directly regulate bi-orientation via these proteins.

To investigate whether Cdk1 plays a direct role in bi-orientation outside the SAC context, we examined bi-orientation during S phase in *cdc28-as1* cells recovering from hydroxyurea (HU) imposed arrest. HU inhibits DNA replication and causes cells to arrest in early S phase with a short spindle and a single kinetochore on the majority of the chromosomes (Krishnan *et al*, 2004; Bachant *et al*, 2005). *cdc28-as1* cells carrying GFP-detectable *CENV* were treated with HU and then released into HU-free medium with or without 1NM-PP1. Following HU removal, bi-orientation in cells lacking Cdk1 activity is not significantly delayed compared with cells recovering without Cdk1 activity (Figure 2B, graph). Notably, the GFP dots remained juxtaposed and spindle remained short since nuclear division fails in *cdc28-as1* cells (Figure 2B). Moreover, when spindle assembly was disrupted by nocodazole treatment of HU-arrested *cdc28-as1* cells, subsequent establishment of bi-orientation following the removal of HU and nocodazole was not significantly

delayed by inhibition of Cdk1 activity (Supplementary Figure S4). These observations indicate that: (1) the dependency of bi-orientation on Cdk1 following nocodazole removal is not an artefact due to spindle disruption; (2) the dependency of bi-orientation on Cdk1 seems cell-cycle stage specific in that Cdk1 is required for efficient bi-orientation during recovery from SAC-induced metaphase arrest but is dispensable for establishment of bi-orientation during S phase.

Premature spindle extension impedes bi-orientation in the absence of Cdk1 activity during recovery from SAC arrest

We noticed that while *cdc28-as1* cells recovering from SAC arrest in the absence of 1NM-PP1 formed bi-polar spindles with a length <3 µm within 20 min, spindles assembled in a large proportion (59%) of 1NM-PP1 (500 nM) treated cells were >5 µm (Figure 3A and B) and lacked bi-orientation (Figure 3A, left panel). Upon removal of 1NM-PP1, an increasing proportion of these cells shortened their spindle length (<3 µm) before nuclear division (Figure 3A, right panel; Figure 3B, right-most graph) raising the possibility that premature lengthening of mitotic spindles in the absence of Cdk1 activity renders bi-orientation process inefficient in cells recovering from nocodazole arrest. The premature extension of the spindles is also seen in cells treated with higher concentrations of 1NM-PP1 (1 and 2 µM), thus ruling out the possibility of this effect being due to insufficient inhibition of Cdk1 (Supplementary Figure S5A and B).

A previous study (Liu *et al*, 2008) has hinted that increasing the distance between spindle poles may reduce the efficiency of bi-orientation. To determine if *Cdc28* deficiency promotes spindle extension in the absence of bi-orientation, we used *cdc28-as1 ndc10-1 cdc15-2* strain. At restrictive temperature, the *ndc10-1* cells are defective in kinetochore assembly and SAC signalling; therefore, spindle extension in these cells occurs in the absence of kinetochore bi-orientation. *cdc28-as1 ndc10-1* cells were first arrested at metaphase by nocodazole treatment. *Ndc10* was then inactivated by incubation at 36°C for 30 min before nocodazole removal and cells were released in the absence or presence of 1NM-PP1. Interestingly, 75% of cells released in the presence of 1NM-PP1 assembled spindles longer than 4 µm within 10 min after nocodazole removal, whereas only 38% of cells with similarly long spindles were found without 1NM-PP1 treatment (Supplementary Figure S6B). Since cells in both cultures lack bi-orientation due to the lack of *Ndc10* function, this experiment implies that mitotic spindles extend faster in absence of Cdk1 activity. Next, we asked if the inhibition of Cdk1 activity causes spindle elongation even when the bi-orientation is preserved during SAC-induced arrest. To test this, we arrested cells in early mitosis with bi-orientated kinetochore by activating SAC signalling via *Mps1* overexpression and determined the effect of Cdk1 inhibition on the bi-orientated, short spindles. Being the pivotal kinase for SAC signalling, *Mps1* overexpression hyperphosphorylates downstream effectors such as *Mad1*, 'forcibly' activates SAC signalling and arrests cells in mitosis even when kinetochores are bi-orientated (Hardwick and Murray, 1995; Hardwick *et al*, 1996). Consistent with the above findings, we find that the bi-oriented, short metaphase spindles formed in *Mps1* overexpressing cells also tend to lengthen upon inhibition of Cdk1 activity (Supplementary Figure S6A). Collectively, these

Figure 3 Spindle length and bi-orientation in *cdc28-as1* cells after release from nocodazole. (A) *cdc28-as1 slk19Δ cdc15-2* cells were arrested in nocodazole and subsequently released into 1NM-PP1 containing medium for 120 min. 1NM-PP1 was then removed to allow recovery. Photomicrographs show the state of nuclear division, spindle staining and CENV-GFP in 1NM-PP1 medium (left panel) and after removal of 1NM-PP1 (right panel). Bar scale: 5 μ m. (B) Nocodazole-arrested *cdc28-as1 slk19Δ cdc15-2* cells were released in the absence or presence of 1NM-PP1. One half of 1NM-PP1-treated culture was further allowed to recover in 1NM-PP1-free medium to restore Cdk1 activity. The bar charts represent percentage of cells with different spindle lengths. (C–E) *cdc28-as1 slk19Δ cdc15-2* and *cdc28-as1 cin8Δ slk19Δ cdc15-2* cells were released from nocodazole arrest into medium containing 1NM-PP1. (C) Graphs shows the proportion of cells with different spindle lengths. (D) Spindle staining and CENV-GFP bi-orientation. Bar scale: 5 μ m. (E) Kinetics of CENV-GFP bi-orientation. Approximately 100 cells were analysed for every sample in each experiment.

results indicate that cells lacking Cdk1 activity are prone to premature spindle extension.

If inefficient bi-orientation in Cdk1-deficient cells recovering from nocodazole arrest is caused by untimely spindle

extension, then abrogation of premature spindle extension should restore bi-orientation. The kinesin Cin8 is known to contribute to the extension and stability of spindles during anaphase (Gheber *et al*, 1998). Hence, *cdc28-as1* and

cdc28-as1 cin8Δ cells were arrested in metaphase by nocodazole treatment and then released into 1NM-PP1 containing medium. While *cdc28-as1* cells exhibited premature spindle extension with only 10–30% cells showing spindle length $<3\mu\text{m}$, $>90\%$ of *cdc28-as1 cin8Δ* cells exhibited spindle $<3\mu\text{m}$ (Figure 3C). Correspondingly, the proportion of cells with bi-orientation (as judged by *CENV*-GFP) increased to nearly 60% in *cdc28-as1 cin8Δ* mutant (Figure 3D and E). Thus, preventing premature spindle elongation caused by absence of Cdk1 activity largely restores cells' ability to re-establish kinetochore bi-orientation during recovery from nocodazole-induced arrest. These observations reveal a causal relationship between premature spindle extension

and the inability of Cdk1-deficient cells to establish bi-orientation efficiently during recovery.

Mechanism of premature spindle extension in Cdk1-deficient cells during checkpoint recovery

Spindle extension in anaphase is a coordinated process which requires a concerted action by microtubules and the motor proteins. The elongation-conductive kinesin Cin8 is recruited to the spindle mid-zone by dephosphorylated Ase1 (Khmelniskii *et al*, 2007, 2009). We find that Ase1 deficiency also prevents premature spindle extension seen in Cdk1-deficient cells released from SAC-induced arrest (Figure 4A and C). Correspondingly, *CENV* bi-orientation is

Figure 4 Ase1 deficiency prevents premature spindle extension in *cdc28-as1* cells after nocodazole release. (A–C) *cdc28-as1 slk19Δ cdc15-2* and *cdc28-as1 ase1Δ slk19Δ cdc15-2* cells were released from nocodazole into medium containing 1NM-PP1. (A) Spindle staining and *CENV*-GFP 20 and 80 min after the release from nocodazole. Bar scale: $5\mu\text{m}$. (B) Bi-orientation kinetics. (C) Graphs showing percentage of cells with different spindle lengths. Approximately 100 cells were analysed for every sample in the experiment. (D) Ase1 phosphorylation status correlated with spindle length dynamics in *cdc28-as1* cells. *cdc28-as1 slk19Δ cdc15-2* expressing Ase1-HA cells were released from nocodazole in the absence or presence of 1NM-PP1. Western blot shows Ase1-HA immunoprecipitated from whole-cell lysate, Ase1-HA immunoprecipitates treated with CIP (+ CIP) and G6PD from whole-cell lysate. The state of spindles is shown in the right panel. Bar scale: $5\mu\text{m}$. Figure source data can be found in Supplementary data.

restored in >60% of cells (Figure 4A and B). Since Ase1 is phosphorylated by Cdk1 and dephosphorylated by Cdc14 phosphatase (Khmelinskii *et al*, 2007, 2009), it is possible that premature spindle extension in 1NM-PP1 is a result of Ase1 misregulation in the absence of Cdk1 activity. Therefore, we examined Ase1 phosphorylation status in *cdc28-as1* cells expressing Ase1-HA released from nocodazole arrest. Nocodazole-arrested cells showed minimal Ase1 phosphorylation despite the presence of high Cdk1 kinase activity (Figure 4D). However, when cells were released from nocodazole arrest in the absence of 1NM-PP1 (high Cdk1 activity), Ase1 was prominently phosphorylated at 20–40 min, the time interval corresponding to short spindle formation. The phosphorylation declined from 60 min onwards, corresponding to the initiation of spindle extension and anaphase entry (Figure 4D, –1NM-PP1). As expected, Ase1 remains largely unphosphorylated in cells lacking Cdk1 activity (Figure 4D, +1NM-PP1). It is surprising that Ase1 is phosphorylated not during nocodazole arrest but during

release. We speculate that Cdk1 kinase predominantly phosphorylates Ase1 associated with spindle microtubules. Since microtubules disassemble in the presence of nocodazole, Ase1 would be poorly phosphorylated during nocodazole arrest despite high Cdk1 kinase activity.

These data suggest a correlation between the low level of Ase1 phosphorylation after nocodazole removal and the premature spindle extension in cells lacking Cdk1 activity. To test this causal relationship further, we employed Ase1 phosphomutants, *ase1-7A* and *ase1-7D*, in which seven Cdk1 phosphorylation sites are mutated to mimic the non-phosphorylated or phosphorylated form, respectively. After nocodazole removal, *ase1-7A* cells exhibited more severe premature spindle extension (Figure 5A), with nuclei pulled apart by the rapidly extending spindle even before the establishment of bi-orientation, causing unequal nuclear division. On the other hand, expression of *ase1-7D* in *cdc28-as1* cells significantly reduced spindle length after nocodazole release, resulting in spindles <3 µm in ~60% cells (Figure 5B and D)

Figure 5 Effect of phosphomimetic mutants of Ase1 on spindle elongation and bi-orientation. (A) Spindle staining in *cdc28-as1 slk19Δ cdc15-2 ase1-7A* cells after release from nocodazole arrest without Cdk1 inhibition. White arrows indicate cells with unequal nuclear division. Bar chart depicts percentage of cells with different spindle lengths. (B) Spc42-RFP and CENV-GFP signals in *cdc28-as1 slk19Δ cdc15-2* and *cdc28-as1 ase1-7D slk19Δ cdc15-2* cells after release from nocodazole arrest in the presence of 1NM-PP1. Bar scale: 5 µm. (C) Bi-orientation kinetics in cells described in (B). (D) The proportion of cells with various spindle lengths in the experiment described in (B). Approximately 100 cells were analysed for every sample in each experiment.

and restored bi-orientation significantly compared with *cdc28-as1* cells (Figure 5C).

Lack of Cdk1 activity during recovery from SAC-induced arrest also causes dramatic loss of Cdc20 protein

The absence of bi-orientation in cells lacking Cdk1 activity results in persistent SAC activity; thus, cells are unable to recover from nocodazole-induced SAC arrest. We asked if preserving bi-orientation during SAC-induced arrest would facilitate subsequent recovery from arrest and progression

through anaphase in the absence of Cdk1 activity. Overexpression of Mps1 ‘forcibly’ activate SAC signalling and arrest cells in metaphase with bi-orientated kinetochores. Once *MPS1* transcription is switched off, the protein is rapidly degraded, Mad1 phosphorylation declines, and the checkpoint is turned off.

G1-synchronized *cdc28-as1 GAL-MPS1 slk19Δ cdc15-2* cells with GFP-labelled *CENV* were released into YEP + Raff + Gal medium to induce Mps1 expression. Upon metaphase arrest (Figure 6A), cells were transferred to glucose medium with or without 1NM-PP1 and monitored for their ability to recover

Figure 6 Recovery of *cdc28-as1* cells from *GAL-MPS1*-induced SAC arrest. (A) *cdc28-as1 slk19Δ cdc15-2 GAL-MPS1* cells were arrested in metaphase by growing in YEP + Raff + Gal medium. Cells show short spindles and bi-orientated *CENV*-GFP. Bar scale: 5 μm. (B) Cells arrested in (A) were released into glucose medium with or without 1NM-PP1. Spindles and Pds1-myc₁₈, Mad1, G6PD levels were monitored. (C) Levels of Cdc20 protein in cells described in (B). (D) *cdc28-as1 slk19Δ cdc15-2 GAL-MPS1* cells arrested in galactose medium were released into glucose medium containing 1NM-PP1. The culture was divided into two portions: while one half remained in 1NM-PP1 medium; the cells from the other half were released into 1NM-PP1-free medium. Myc₈-Cdc20, Pds1-myc₁₈ and G6PD levels were monitored. (E) *cdc28-as1 slk19Δ cdc15-2* cells were arrested in nocodazole and then released into the medium with or without 1NM-PP1. Cdc20 levels were determined by western blotting. Figure source data can be found in Supplementary data.

from the arrest. Cells released into 1NM-PP1-free medium recovered normally from *GAL-MPS1*-induced arrest as indicated by Pds1 degradation (at 40 min), spindle extension and anaphase entry (60 min) (Figure 6B, left panel). In contrast, 1NM-PP1-treated cells (95%) remained arrested with a short spindle and showed stable Pds1 level at 60 min after the release despite Mad1 dephosphorylation (Figure 6B, right panel) and bi-orientated kinetochores (Supplementary Figure S6C). This implies that even when kinetochores are bi-oriented and SAC signalling has been silenced, cells are incapable of initiating anaphase in the absence of Cdk1 kinase activity.

Since Pds1 degradation is directly dependent on Cdc20, we monitored Cdc20 protein levels in these cells. Surprisingly, Cdc20 protein levels drop dramatically when *cdc28-as1* activity is inhibited by 1NM-PP1 (Figure 6C). This explains Pds1 stabilization in these cells and their inability to recover from metaphase arrest despite checkpoint silencing. Once 1NM-PP1 is removed, Cdc20 expression is restored, Pds1 is degraded and cells progress into anaphase (Figure 6D). The precipitous drop in Cdc20 level in the absence of Cdk1 activity is not specific to arrest imposed by Mps1 overexpression, but is also seen in cells released from nocodazole-induced arrest (Figure 6E). Moreover, Clb2, which like Cdc20 is a member of the *CLB2* gene cluster, does not exhibit a dramatic reduction upon Cdk1 inhibition (Figure 1B). Hence, Cdk1 activity is specifically required to maintain Cdc20 levels during SAC-induced arrest to subsequently mount an efficient recovery.

Cdk1 activity maintains CDC20 transcription during recovery from SAC-induced arrest via silencing of transcription repressor Yox1

Cdc20 levels remain largely constant during mitosis even though Cdc20 is highly unstable protein (D'Angiolella *et al*, 2003). Hence, a rapid decline in Cdc20 levels upon inhibition of Cdk1 activity (Figure 6C and E) could be either due to increased degradation of Cdc20 protein or due to decreased *de-novo* synthesis of Cdc20 in the absence of Cdk1. To test these possibilities, we performed a pulse-chase experiment using *cdc28-as1* cells carrying *GAL-myc₄-CDC20*. Inhibition of *cdc28-as1* by 1NM-PP1 resulted in only a very slight increase in Cdc20 degradation kinetics (Figure 7A). Hence, the drastic reduction in Cdc20 levels upon Cdk1 inhibition is not due to enhanced degradation. To test the effect of Cdk1 inhibition on *CDC20* mRNA level, *cdc28-as1* cells were first arrested by nocodazole treatment, re-suspended in medium containing nocodazole with or without 1NM-PP1 and *CDC20* transcription was monitored. *CDC20* mRNA levels showed a dramatic decline upon Cdk1 inhibition (Figure 7B). However, upon 1NM-PP1 removal, *CDC20* mRNA levels were restored rapidly (Figure 7B), suggesting that rapid dissipation of Cdc20 protein in Cdk1-deficient cells is due to the downregulation of *CDC20* mRNA. Interestingly, when the native *CDC20* promoter was replaced by *GAL1* promoter in *cdc28-as1* cells, Cdk1 inhibition no longer caused a decline in *CDC20* mRNA level (Figure 7C), implying that Cdk1 kinase activity may directly regulate *CDC20* transcription via the promoter region.

It has been reported that the activation of *CDC20* transcription requires displacement of Cdk1 by Cks1 from the promoter (Morris *et al*, 2003). It is plausible that 1NM-PP1 bound *cdc28-as1* kinase has poor affinity to Cks1 and cannot be

displaced from *CDC20* promoter region. However, Cks1 overexpression in cells lacking Cdk1 activity does not restore *CDC20* mRNA levels (our unpublished data), suggesting that Cks1 is perhaps not involved. *CDC20* promoter region is a hybrid consisting of FKH binding site (positive regulatory element to which transcription activator Ndd1 is also recruited) and YOX binding site (negative regulatory element) (Pramila *et al*, 2002). While overexpression of Ndd1 does not restore *CDC20* mRNA level in cells lacking Cdk1 activity (data not included), deletion of transcription repressor Yox1 restores *CDC20* mRNA expression (Figure 7D) and consequently, the Cdc20 protein level (Figure 7E). Yox1 contains multiple Cdk1 phosphorylation sites and is reported to be phosphorylated by Cdk1 *in vitro* (Ubersax *et al*, 2003); however, it is not known whether Yox1 is functionally regulated by Cdk1.

During normal cell cycle, Yox1 protein accumulates in early S phase but its levels decline rapidly upon cells' entry into mitosis (Supplementary Figure S7A). Thus, Yox1 expression pattern is just opposite to that of Cdc20 protein. Interestingly, although Yox1 level is barely detectable in nocodazole-arrested *cdc28-as1* cells, inhibition of Cdk1 activity results in dramatic accumulation of Yox1 (Figure 7F, left panel), suggesting that the intracellular level of Yox1 may be regulated by Cdk1. Yox1 is highly unstable in mitosis, and its degradation is only moderately reduced upon inhibition of Cdk1 (Supplementary Figure S7B and C). However, while the nocodazole-arrested *cdc28-as1* cells show very low levels of *YOX1* mRNA, *YOX1* transcript rapidly accumulates upon inhibition of Cdk1 activity (Figure 7F, right). Moreover, overexpression of Yox1 is sufficient to repress Cdc20 expression in metaphase-arrested cells (Supplementary Figure S7D). Hence, high Cdk1 activity functions to reduce both the protein stability and *YOX1* transcription in SAC-arrested cells so as to maintain a high level of Cdc20 for efficient recovery.

Discussion

High Cdk1 activity is a conspicuous feature of cells arrested in response to SAC activation. It has been suggested that the high Cdk1 activity is required for the maintenance of SAC-imposed arrest (Li and Cai, 1997; D'Angiolella *et al*, 2003). However, in this study we show that high Cdk1 activity in SAC-arrested cells is also necessary for efficient recovery, via two previously unrecognized mechanisms: (i) efficient re-establishment of kinetochore bi-orientation once the bi-polar spindles have been assembled after nocodazole removal (ii) maintenance of adequate levels of Cdc20 protein, critical for the dissolution of sister-chromatid cohesion following SAC silencing. The requirement of high Cdk1 activity for the establishment of bi-orientation appears to be specific to the recovery from SAC-induced metaphase arrest since high Cdk1 activity is dispensable for kinetochore bi-orientation normally established in late S phase (Figure 2B; Supplementary Figure S4). However, the involvement of Cdk1 in the maintenance of Cdc20 levels is mediated via effectors regulating Cdc20 expression during the unperturbed division cycle. Thus, we suggest that cells utilize a combination of context-specific and general mechanisms to establish the cellular conditions most conducive to mounting an efficient recovery from SAC arrest.

Figure 7 Cdk1 kinase activity regulates *CDC20* transcription in mitosis. (A) *cdc28-as1* cells with *GAL-myc₄-CDC20* were arrested in nocodazole-containing raffinose medium. Cells were induced to express Cdc20 for 60 min in YEP + Raff + Gal medium and then shifted to YEP + Glu + cycloheximide medium in absence or presence of 1NM-PP1. The status of myc-tagged Cdc20 protein was monitored by western blotting. (B) *cdc28-as1* *slk19Δ* *cdc15-2* cells were arrested in nocodazole. The culture was divided into two halves and cells were re-suspended in medium with or without 1NM-PP1. For cells with 1NM-PP1, 1NM-PP1 was removed from one half of the culture after 60 min to allow restoration of Cdk1 kinase activity. (C) Endogenous *CDC20* in *cdc28-as1* *slk19Δ* *cdc15-2* cells was replaced by *GAL-CDC20*. Cells were arrested in nocodazole + Gal medium and then re-suspended in nocodazole-containing + Gal medium without or with 1NM-PP1. (D) *CDC20* mRNA levels in *cdc28-as1* *slk19Δ* *cdc15-2* and *cdc28-as1* *yox1Δ* *slk19Δ* *cdc15-2* cells arrested in nocodazole and subsequently treated with 1NM-PP1. (E) Cdc20 protein levels in *cdc28-as1* *slk19Δ* *cdc15-2* and *cdc28-as1* *yox1Δ* *slk19Δ* *cdc15-2* cells arrested in nocodazole and then treated with 1NM-PP1. (F) Nocodazole-arrested *cdc28-as1* *slk19Δ* *cdc15-2* cells were divided into two portions. Cells from one half of the culture were re-suspended in nocodazole-containing medium without 1NM-PP1 and from the other half with 1NM-PP1. Yox1-HA₆ protein level (left panel) and *YOX1* mRNA level (right panel) are shown. Figure source data can be found in Supplementary data.

Our results suggest that the absence of Cdk1 activity during recovery causes premature extension of the mitotic spindle resulting in an inability to establish kinetochore bi-orientation efficiently (Figure 3). This is consistent with the suggestion from a previous study that short spindles may be conducive to bi-orientation (Liu *et al*, 2008). Thus, high Cdk1 activity is necessary to restrain spindle extension during recovery from SAC-induced arrest. Premature spindle elongation in cells deficient in Cdk1 activity, lack of Ase1 phosphorylation (Figure 4D) and inhibition of premature spindle elongation by *ase1-7D* in Cdk1-deficient cells (Figure 5B and D) imply that Cdk1 prevents untimely spindle extension by dampening Ase1 activity via phosphorylation. Since Ase1 is involved in Cin8 recruitment to the spindle mid-zone and Ase1 phosphorylation by Cdk1 reduces its ability to recruit Cin8 (Khmelnitskii *et al*, 2007, 2009), insufficient Ase1 phosphorylation in the absence of Cdk1 activity (Figure 4D) can result in enhanced recruitment of proteins such as Cin8 to the

spindle mid-zone. This would result in a much higher rate of spindle extension, severely compromising the efficiency of bi-orientation. However, bi-orientation is not dependent on the high Cdk1 activity in S phase, since low levels of motor proteins (Cin8 and Ase1) preclude the need for Cdk1-mediated phosphorylation to maintain a short spindle (Supplementary Figure S4). This is consistent with our observation that overexpression of Cin8 during S phase results in premature spindle elongation and delays bi-orientation (Supplementary Figure S4). Taken together, these results underscore the importance of high Cdk1 kinase activity in cells recovering from SAC-induced arrest and suggest a mechanism wherein Cdk1 promotes re-establishment of kinetochore bi-orientation and extinction of the SAC.

Our conclusion that mitotic spindle undergoes premature elongation in cells recovering from SAC-induced arrest in the absence of Cdk1 kinase activity appears to contradict a previous study (Rahal and Amon, 2008), suggesting

that Cdk1/Clb is required for spindle elongation in normal anaphase, although the exact mechanism underlying this requirement remains unclear. Our investigations are set in the context of recovery from SAC arrest, specifically within a time period when cells have assembled a bi-polar spindle but have not established kinetochore bi-orientation. Therefore, while Cdk1 may have a role in spindle elongation during anaphase in normal cycle, our results suggest that Cdk1 activity is critical for restraining premature spindle extension to allow kinetochore bi-orientation during cells' recovery from SAC-induced arrest. Recently, Cdc2/Cdc13 (Cdk1/CycB) is reported to have a role in kinetochore bi-orientation in fission yeast via phosphorylation of chromosome passenger complex component Bir1 (Sli15 in budding yeast) (Tsukahara *et al*, 2010). However, we do not observe a significant defect in bi-orientation due to lack of Cdk1 activity during normal cell cycle (Figure 2). The reason for this difference is presently unclear.

Our study also reveals a requirement for Cdk1 in another critical aspect of the recovery process. We find that Cdc20 levels drop precipitously in cells recovering from SAC-induced metaphase arrest in the absence of Cdk1 activity (Figure 6C and E), making it virtually impossible for cells to proceed to anaphase. Since Cdc20 protein is unstable during mitosis, its level is sustained during metaphase arrest by Cdk1-dependent maintenance of *CDC20* transcription (Figure 7B and C). However, unlike *CLB2* (Supplementary Figure S8), the maintenance of *CDC20* transcription seems to be very sensitive to the level of Cdk1 activity. This is due to the presence of a binding site for transcription repressor Yox1 in the *CDC20* promoter region (Pramila *et al*, 2002). Binding of the repressors Yox1/Yph1 to the *YOX* site precludes the activation of MCM by Fkh1/2 complex (Darieva *et al*, 2010). Such a hybrid promoter containing inhibitory *YOX* site together with activating FKH/MCM site is found in promoters of a subset of *CLB2* cluster genes that are expressed somewhat later in mitosis (Pramila *et al*, 2002). Our observations suggest that the dramatic decline in *CDC20* transcription upon inhibition of Cdk1 activity is mediated by Yox1 since *YOX1* deletion largely restores both *CDC20* mRNA and Cdc20 protein levels in *cdc28-as1* cells treated with 1NM-PP1 (Figure 7D and E). Interestingly, we find that *YOX1* transcription is negatively regulated by Cdk1 (Figure 7F). Thus, Cdc20 abundance in mitosis is maintained via regulation of *CDC20* expression by what appears to be a Cdk1-dependent, sequential system for transcriptional control. Cdk1 downregulates the expression of the transcription repressor Yox1 which in turn allows transcription of *CDC20*. However, regulation of *YOX1* transcription by Cdk1 remains to be investigated. *YOX1* promoter contains putative binding sites for SBF/MBF (Harbison *et al*, 2004), raising the possibility of modulation by Cdk1/Clb activity.

Premature extension of the spindle and the inability of cells to undergo anaphase in the absence of Cdk1 activity during recovery from SAC-induced arrest could be attributed to the low abundance of Cdc20, since Cdc20 levels decline dramatically upon Cdk1 inhibition (Figure 6C). However, cells supplied with constant levels of Cdc20 (either from *MET-CDC20* construct or due to *YOX1* deletion) during the recovery process also fail to undergo anaphase (Supplementary Figure S2). This strongly suggests that the two functions of Cdk1 described here, that is, coordination of

spindle dynamics and the maintenance of Cdc20 transcription, are regulated independently by Cdk1. This conclusion is further supported by our observations that (i) spindles prematurely extend in cells recovering from nocodazole-induced arrest in the absence of Cdk1 activity but in constant presence of Cdc20 (Supplementary Figure S2A), (ii) the kinetics of premature spindle extension ($>3\ \mu\text{m}$) is comparable to 1NM-PP1-treated *cdc28-as1 yox1Δ* and *cdc28-as1* cells (Supplementary Figure S2B) and (iii) cells are unable to maintain Cdc20 levels in the absence of Cdk1 activity with or without the kinetochore bi-orientation (Figure 6C and E).

If the coordination of spindle dynamics with bi-orientation and the maintenance of *CDC20* transcription are the only functions served by Cdk1 during recovery from SAC-induced arrest, then compensation of these defects by *ASE1* deletion and ectopic expression of Cdc20 would be expected to overcome the recovery defect in 1NM-PP1-treated *cdc28-as1*. When released from nocodazole arrest into +Gal+1NM-PP1, 32% of *ase1Δ cdc28-as1 GAL-CDC20* cells progress into anaphase (extended spindle with equally segregated *CENV-GFP*) compared with 16% of *cdc28-as1* cells (Supplementary Figure S9B). The modest rescue implies that Cdk1 may serve additional functions during recovery. This is also reflected in the observation that Cdc20 expression from a heterologous promoter fails to induce efficient recovery in 1NM-PP1-treated *cdc28-as1* cells released from *GAL-MPS1*-induced SAC arrest (Supplementary Figure S9A). Nevertheless, given the general conservation of SAC between yeast and vertebrates, it will be interesting to determine if the regulatory controls and the physiological context necessary in yeast for efficient recovery from SAC-induced arrest have parallels in vertebrates. The dynamics of the recovery process has potential implications for cancer therapy with anti-mitotic agents. Recent studies on mammalian cell lines (Tao *et al*, 2005; Gascoigne and Taylor, 2008; Huang *et al*, 2009) suggest that the fate of cancer cells upon anti-mitotic drug treatment depends on the balance between Cdk1 activity and apoptotic activity, and that the time cells spend in mitotic arrest subsequently determines how susceptible they are to apoptosis. Here, we have shown that in budding yeast, reduction in Cdk1 activity hinders efficient recovery from SAC, therefore prolonging the time cells spent in a SAC-arrested state. Interestingly, *cdc28-as1* cells, even without 1NM-PP1 treatment, exhibit increased sensitivity to benomyl treatment compared with wild-type cells (our unpublished observation) implying that even a slight perturbation of Cdk1 activity may render cells more sensitive to spindle poisons. These considerations may have interesting and important parallels in mammalian cells that warrant further investigation in future studies.

Materials and methods

Yeast strains and growth conditions

All strains used in this study (the genetic background described in Supplementary Table 1) are congenic to wild-type strain W303. Standard molecular genetic techniques such as gene transplacement, gene disruption, tetrad dissection, PCR-based site-directed mutagenesis and tagging of endogenous genes were employed to construct plasmids and strains carrying various genotypes. Gene disruptions and transplacements were confirmed with PCR and western blotting. Cells were grown either in yeast extract peptone (YEP) or in synthetic medium supplemented with 2% glucose or 4% raffinose ± 2% galactose. Log-phase cells were synchronized in G1 by α -factor treatment (1 $\mu\text{g}/\mu\text{l}$ for *bar1Δ* and 5 $\mu\text{g}/\mu\text{l}$ for *BAR1*

strain) at 25°C for 2 h. Subsequently, cells were washed and released into appropriate medium. In experiments involving *cdc28-as1* mutant, cells were arrested with nocodazole (15 µg/ml) and released into medium containing 500 nM 1NM-PP1 (Cellular Genomics Inc).

Cell extract preparation and western blotting

Whole-cell extracts and protein precipitation by TCA for immunoblotting, and preparation of cell extracts for immunoprecipitations were carried out as described previously (Yeong *et al*, 2000). For western blot analysis, immunodetection was performed using anti-HA monoclonal antibody (Roche, 1:500 dilution), anti-myc polyclonal antibody (Santa Cruz, 1:500 dilution), anti-Mad1 antibodies (a gift from Dr Kevin Hardwick) or rabbit anti-G6PD (Sigma, 1:10 000). Enhanced chemiluminescence kit from Santa Cruz was used for signal detection. For detection of phosphoproteins, cells were lysed by acid-treated glass beads in lysis buffer with inhibitors (Surana *et al*, 1991). Goat anti-HA monoclonal antibody conjugated to agarose beads (Santa Cruz) were used to immunoprecipitate the target proteins before western blot analysis.

Anti-tubulin immunofluorescent staining and cell imaging

Spindles, CENV-GFP and Spc42-RFP were visualized by fluorescence microscopy as described previously (Zhang *et al*, 2009). Images were captured using a Zeiss AxioImager upright motorized microscope with Plan Apochromat × 100 objective and attached to a Photometrics Coolsnap HQ2 high sensitivity monochrome camera driven by the Metamorph software (Universal Imaging Corporation).

Northern blot analysis

Yeast cells were disrupted by acid-treated glass beads using the method described by Cross and Tinklenberg (1991) for total RNA

extraction. Northern blot analysis was performed according to Price *et al* (1991). Single stranded probes complementary to the mRNA sequence of *CDC20* and *YOX1* were prepared using Promega's Riboprobe *in vitro* Transcription System Kit and purified using ProbeQuant G50 microcolumns (Amersham, GE Healthcare). The blots were hybridized at 65°C with α -³²P rCTP-labelled probe (α -³²P rCTP at 10 µCi/µl from NEN). Subsequently, the blots were washed in 2 × SSC/0.1% SDS (3 × 15 min at room temperature) and then in 0.1 × SSC/0.1% SDS (3 × 30 min at 55°C).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: HL, HHL and US conceived and designed the experiments. HL and HHL performed the experiments. HL, AV and US analysed the data. HL, AV and US wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Amon A (1999) The spindle checkpoint. *Curr Opin Genet Dev* **9**: 69–75
- Bachant J, Jessen SR, Kavanaugh SE, Fielding CS (2005) The yeast S phase checkpoint enables replicating chromosomes to bi-orient and restrain spindle extension during S phase distress. *J Cell Biol* **168**: 999–1012
- Biggins RH, Murray AW (2001) The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate spindle checkpoint. *Genes Dev* **15**: 3118–3129
- Bishop AC, Ubersax JA, Petsch DT, Matheos DP, Gray NS, Blethrow J, Shimizu E, Tsien JZ, Schultz PG, Rose MD, Wood JL, Morgan DO, Shokat KM (2000) A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature* **407**: 395–401
- Cross F, Tinklenberg AH (1991) A positive feedback loop controlling CLN1 and CLN2 gene expression at the START of yeast cell cycle. *Cell* **65**: 875–883
- D'Angiolella V, Mari C, Nocera D, Rametti L, Grieco D (2003) The spindle checkpoint requires cyclin-dependent kinase activity. *Genes Dev* **17**: 2520–2525
- Dariva AD, Clancy A, Bulmer R, Williams E, Pic-Taylor A, Morgan BA (2010) A competitive transcription factor binding mechanism determines the time of late cell cycle-dependent gene expression. *Mol Cell* **38**: 29–40
- Farr KA, Hoyt MA (1998) Bub1p kinase activates the *Saccharomyces cerevisiae* spindle assembly checkpoint. *Mol Cell Biol* **18**: 2738–2747
- Gascoigne KE, Taylor SS (2008) Cancer cells display profound intra- and interline variation following prolonged exposure to anti-mitotic drugs. *Cancer Cell* **14**: 111–122
- Gheber L, Kuo SC, Hoyt MA (1998) Motile property of the kinesin-related Cin8p spindle motor extracted from *Saccharomyces cerevisiae* cells. *J Biol Chem* **274**: 9564–9572
- Harbison CT, Gordon DB, Lee TI, Rinaldi NJ, Macisaac KD, Danford TW, Hannett NM, Tagne JB, Reynolds DB, Yoo J, Jennings EG, Zeitlinger J, Pokholok DK, Kellis M, Rolfe PA, Takusagawa KT, Lander ES, Gifford DK, Fraenkel E, Young RA (2004) Transcriptional regulatory code of a eukaryotic genome. *Nature* **431**: 99–104
- Hardwick KG, Murray AW (1995) Mad1p, a phosphoprotein component of the spindle assembly checkpoint in budding yeast. *J Cell Biol* **131**: 709–720
- Hardwick KG, Weiss E, Luca FC, Winey M, Murray AW (1996) Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. *Science* **273**: 953–956
- Howell BJ, McEwen BF, Canman JC, Hoffman DB, Farrar EM, Rieder CL, Salmon ED (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
- Huang HC, Shi J, Orth JD, Mitchison TJ (2009) Evidence that mitotic exit is a better cancer therapeutic target than spindle assembly. *Cancer Cell* **16**: 347–358
- Khmelninskii A, Lawrence C, Roostalu J, Schiebel E (2007) Cdc14-regulated midzone assembly controls anaphase B. *J Cell Biol* **177**: 981–993
- Khmelninskii A, Roostalu J, Roque H, Antony C, Schiebel E (2009) Phosphorylation-dependent protein interactions at the spindle midzone mediates cell cycle regulation of spindle elongation. *Dev Cell* **17**: 244–256
- Kline-Smith SL, Sandall S, Desai A (2005) Kinetochore-spindle microtubule interactions during mitosis. *Curr Opin Cell Biol* **17**: 35–46
- Krishnan V, Nirantar S, Crasta K, Cheng AY, Surana U (2004) DNA replication checkpoint prevents precocious chromosome segregation by regulating spindle behavior. *Mol Cell* **16**: 687–700
- Li X, Cai M (1997) Inactivation of the cyclin dependent kinase Cdc28 abrogates cell cycle arrest induced by DNA damage and disassembly of mitotic spindles in *Saccharomyces cerevisiae*. *Mol Cell Biol* **17**: 2723–2734
- Li X, Nicklaus RB (1995) Mitotic forces control a cell cycle checkpoint. *Nature* **373**: 630–632
- Liu H, Liang F, Jin F, Wang Y (2008) The coordination of centromere replication, spindle formation, and kinetochore-microtubule interaction in budding yeast. *PLoS Genet* **4**: e1000262
- Mah AS, Jang J, Deshaies RJ (2001) Protein kinase Cdc15 activates the Dbf2-Mob1 kinase complex. *Proc Natl Acad Sci USA* **98**: 7325–7330
- Michaelis C, Ciosk R, Nasmyth K (1997) Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**: 35–45
- Morris MC, Kaiser P, Rudyak S, Baskerville C, Watson MH, Reed SI (2003) Cks1-dependent proteasome recruitment and

- activation of CDC20 transcription in budding yeast. *Nature* **423**: 1009–1013
- Musacchio A, Salmon ED (2007) The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol* **8**: 379–393
- Nilsson J, Yekezare M, Minshull J, Pines J (2008) The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction. *Nat Cell Biol* **10**: 1411–1420
- Pan J, Chen RH (2004) Spindle checkpoint regulates Cdc20p stability in *Saccharomyces cerevisiae*. *Genes Dev* **18**: 1439–1451
- Pramila T, Miles S, GuhaThakurta D, Jemiole D, Breeden LL (2002) Conserved homeodomain proteins interact with MADS box protein Mcm1 to restrict ECB-dependent transcription to the M/G1 phase of the cell cycle. *Genes Dev* **16**: 3034–3045
- Price C, Nasmyth K, Schuster T (1991) A general approach to the isolation of cell cycle-regulated genes in the budding yeast, *Saccharomyces cerevisiae*. *J Mol Biol* **218**: 543–556
- Prinz S, Hwang ES, Visintin R, Amon A (1998) The regulation of Cdc20 proteolysis reveals a role for APC components Cdc23 and Cdc27 during S phase and early mitosis. *Curr Biol* **8**: 750–760
- Rahal R, Amon A (2008) Mitotic CDKs control the metaphase-anaphase transition and trigger spindle elongation. *Genes Dev* **22**: 1534–1548
- Reddy SK, Rape M, Margansky WA, Kirschner MW (2007) Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. *Nature* **446**: 921–925
- Stegmeier F, Visintin R, Amon A (2002) Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. *Cell* **108**: 207–220
- Surana U, Robitsch H, Price C, Schuster T, Fitch I, Futcher AB, Nasmyth K (1991) The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell* **65**: 145–161
- Tanaka K, Mukae N, Dewar H, van Breugel M, James EK, Prescott AR, Antony C, Tanaka TU (2005) Molecular mechanisms of kinetochore capture by spindle microtubules. *Nature* **434**: 987–994
- Tao W, South VJ, Zhang Y, Davide JP, Farrell L, Kohl NE, Sepp-Lorenzino L, Lobell RB (2005) Induction of apoptosis by an inhibitor of the mitotic kinesin KSP requires both activation of the spindle assembly checkpoint and mitotic slippage. *Cancer Cell* **8**: 49–59
- Tsukahara T, Tanno Y, Watanabe Y (2010) Phosphorylation of the CPC by Cdk1 promotes chromosome bi-orientation. *Nature* **467**: 719–723
- Ubersax JA, Woodbury EL, Quang PN, Paraz M, Blethrow JD, Shah K, Shokat KM, Morgan DO (2003) Targets of the cyclin-dependent kinase Cdk1. *Nature* **425**: 859–864
- Wassmann K, Liberal V, Benezra R (2003) Mad2 phosphorylation regulates its association with Mad1 and the APC/C. *EMBO J* **22**: 797–806
- Weiss E, Winey M (1996) The *Saccharomyces cerevisiae* spindle pole body duplication gene MPS1 is part of a mitotic checkpoint. *J Cell Biol* **132**: 111–123
- Xia G, Luo X, Habu T, Rizo J, Matsumoto T, Yu H (2004) Conformation-specific binding of p31(comet) antagonizes the function of Mad2 in the spindle checkpoint. *EMBO J* **23**: 3133–3143
- Yeong FM, Lim HH, Padmashree CG, Surana U (2000) Exit from mitosis in budding yeast: biphasic inactivation of the Cdc28-Clb2 mitotic kinase and the role of Cdc20. *Mol Cell* **5**: 501–511
- Zhang T, Nirantar S, Lim HH, Sinha I, Surana U (2009) DNA damage checkpoint maintains Cdh1 in active state to inhibit anaphase progression. *Dev Cell* **17**: 541
- Zhu G, Spellman PT, Volpe T, Brown PO, Botstein D, Davis TN, Futcher B (2000) Two yeast forkhead genes regulate the cell cycle and pseudohyphal growth. *Nature* **406**: 90–94