Adaptation to the spindle checkpoint is regulated by the interplay between Cdc28/Clbs and PP2A<sup>Cdc55</sup>

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The spindle checkpoint arrests cells in metaphase until all chromosomes are properly attached to the chromosome segregation machinery. Thereafter, the anaphase promoting complex (APC/C) is activated and chromosome segregation can take place. Cells remain arrested in mitosis for hours in response to checkpoint activation, but not indefinitely. Eventually, they adapt to the checkpoint and proceed along the cell cycle. In yeast, adaptation requires the phosphorylation of APC/C. Here, we show that the protein phosphatase PP2A<sup>Cdc55</sup> dephosphorylates APC/C, thereby counteracting the activity of the mitotic kinase Cdc28. We also observe that the key regulator of Cdc28, the mitotic cyclin Clb2, increases before cells adapt and is then abruptly degraded at adaptation. Adaptation is highly asynchronous and takes place over a range of several hours. Our data suggest the presence of a double negative loop between PP2A<sup>Cdc55</sup> and APC/C<sup>Cdc20</sup> (i.e., a positive feedback loop) that controls APC/C<sup>Cdc20</sup> activity. The circuit could guarantee sustained APC/C<sup>Cdc20</sup> activity after Clb2 starts to be degraded.

Introduction

During metaphase, chromosomes consist of pairs of sister chromatids held together by the cohesin complex. Cohesin cleavage by the protease separase is the essential step that starts the physical separation of sister chromatids and drives cells into anaphase (Nasmyth, 2002). For separase to cleave cohesin, cells need to degrade securin (Pds1 in Saccharomyces cerevisiae), the stoichiometric inhibitor of the protease. Degradation of Pds1 is mediated by the proteasome after ubiquitination by the essential E3 ubiquitin ligase anaphase promoting complex or cyclosome (APC/C) bound to its cofactor Cdc20 (Primorac and Musacchio, 2013). Degradation of Pds1 is insufficient, however, to guarantee exit from mitosis, which also requires the inactivation of the mitotic kinase cyclin-dependent kinase 1 (CDK1; Cdc28 in S. cerevisiae; Drapkin et al., 2009). Cdc28 inhibition occurs via the degradation of mitotic cyclins (Clbs, primarily Clb2 in budding yeast), an event that is initiated by APC/C<sup>Cdc20</sup> and is completed by another complex APC/C<sup>Cdb1</sup>, where Cdh1 is a second cofactor that replaces Cdc20 as cells enter anaphase (Primorac and Musacchio, 2013).

A surveillance pathway known as the spindle checkpoint prevents APC/C<sup>Cdc20</sup> activation in the presence of as few as one kinetochore unattached to spindle microtubules (Rieder et al., 1995; Lara-Gonzalez et al., 2012). As a consequence of checkpoint activation, Cdc20 is sequestered in the mitotic checkpoint complex (MCC), which is composed of Cdc20 itself and three components of the checkpoint pathway: Mad2, Mad3 (BubR1 in mammals), and Bub3. Cdc20 in the MCC binds to APC/C, but APC/C<sup>MCC</sup> is unable to recognize Pds1 and Clb2 (Lara-Gonzalez et al., 2012). After all kinetochores are properly attached to microtubules, the checkpoint is disengaged, MCC dissociates, APC/C<sup>Cdc20</sup> is activated, and cells can transit into anaphase.

Remarkably, the lifting of checkpoint-inducing stimuli is not strictly required for cells to enter anaphase. When the checkpoint is constantly induced, cells can adapt to the checkpoint and progress into the cell cycle after several hours of metaphase arrest (Rudner et al., 2000; Brito and Rieder, 2006; Rossio et al., 2010). How APC/C is activated in the presence of stimuli that induce Cdc20 sequestration, and thus APC/C inhibition, is still not completely understood.

In vertebrates, the mechanism of adaptation has been ascribed to a slow, APC/C-dependent degradation of cyclin B (thus the phenomenon is also known as slippage) taking place in cells arrested by the spindle checkpoint (Brito and Rieder, 2006). When cyclin B drops below a critical level, CDK1 is

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inactivated, and cells can no longer sustain the mitotic state leading to exit from mitosis.

The molecular mechanisms by which Cdc28/CDK1 inhibition could induce APC/C\(^{Cdc20}\) activation, and transition into anaphase and adaptation, are not entirely clear. In mammals, CDK1 sustains the checkpoint (D’Angiolella et al., 2003; Morin et al., 2012) and thus indirectly inhibits APC/C\(^{Cdc20}\). It has therefore been proposed that CDK1 inactivation relieves the inhibition of APC/C\(^{Cdc20}\) exerted by the checkpoint and facilitates the transition to anaphase (Zeng et al., 2010; He et al., 2011).

A role for Cdc28 in maintenance of the spindle checkpoint, and thus in the inhibition of APC/C\(^{Cdc20}\), has also been proposed in budding yeast (Li and Cai, 1997; Kitazono et al., 2003). However, in this organism Cdc28 has also a role in promoting APC/C\(^{Cdc20}\) activity. Cdc28 activates APC/C by phosphorylating two of its subunits (Cdc16 and Cdc27), and thus favoring APC/C binding with Cdc20 (Rudner and Murray, 2000). Moreover, Cdc28 activity is required for the expression of Cdc20 during metaphase arrest (Liang et al., 2012). In summary, in yeast Cdc28 is reported to contribute to both APC/C\(^{Cdc20}\) inhibition, indirectly via the checkpoint, and to APC/C\(^{Cdc20}\) activation via APC phosphorylation and production of Cdc20 (Fig. 1 A). It is thus unclear whether Cdc28 activity promotes or inhibits metaphase arrest in cells subjected to a prolonged checkpoint stimulus.

Similarly, the relationship between APC/C\(^{Cdc20}\) activation and phosphatases during adaptation to the spindle checkpoint is not fully understood. Based on the premise that Cdc28 is involved in spindle checkpoint maintenance, Cdc14, its main opposing protein phosphatase (Rock and Amon, 2009), should favor checkpoint silencing and APC/C\(^{Cdc20}\) formation. Indeed, the overexpression of Cdc14 silences the checkpoint in a Cdc20-dependent manner, and Cdc14 prevents checkpoint reactivation in anaphase (Mirchenko and Uhlmann, 2010). It is unknown, however, whether physiological levels of Cdc14 have a role in promoting adaptation. This is not obvious, as Cdc14 is primarily required for events that follow anaphase and it is only fully activated well after APC/C\(^{Cdc20}\) has been activated.

Phosphatase PP2A\(^{Cdc55}\) (i.e., the type 2A phosphatase bound to its regulatory domain Cdc55) instead inhibits metaphase-to-anaphase transition: PP2A\(^{Cdc55}\) activity drops when cells enter anaphase (Queralt et al., 2006), whereas cdc55Δ mutants are checkpoint deficient (Minshull et al., 1996). During an unperturbed cell cycle, PP2A\(^{Cdc55}\) delays anaphase onset by inhibiting the release of Cdc14 from the nucleolus where it is bound to Net1 (Queralt et al., 2006; Wang and Ng, 2006; Yellman and Burke, 2006) and by inhibiting separase (Clift et al., 2009). Whether PP2A\(^{Cdc55}\) has a similar role in inhibiting adaptation is unknown.

In this manuscript, we explore possible mechanisms of APC/C activation in cells arrested by the spindle checkpoint in budding yeast. We show that transition to anaphase in adaptation: (1) requires APC/C phosphorylation and Cdc28/Cls activity; (2) is opposed by PP2A\(^{Cdc55}\) via APC/C dephosphorylation; (3) is variable in length; and (4) does not require Cdc14. Finally, we suggest the presence of a positive feedback loop that allows a rapid transition from metaphase arrest to anaphase during adaptation to the spindle checkpoint.

### Results

#### Cdc20 and APC/C phosphorylation, but not Cdh1, are required for adaptation to the spindle checkpoint

APC/C is activated both by posttranslational modifications and by association with the cofactors Cdc20 and Cdh1. We asked which of these mechanisms is required for budding yeast cells to adapt to a prolonged checkpoint stimulus. To this aim, we compared the kinetics of the metaphase-to-anaphase transition in adapting cells (1) expressing nonphosphorylatable mutants for two subunits of the APC/C, Cdc27 (cdc27-5A) and Cdc16 (cdc16-6A); (2) deleted for Cdh1; and (3) depleted of Cdc20.

In principle, we could have induced the spindle checkpoint by treating cells with tubulin-depolymerizing drugs like nocodazole. However, we observed that the effects of nocodazole begin to wear off after 5–6 h, and thus transition to anaphase after tubulin repolymerization and microtubule/kinetochore attachment can easily be mistaken for adaptation. We thus induced checkpoint activation by overexpressing one of its essential components, Mad2. We used yeast cells containing three copies of the MAD2 gene under the control of the inducible GAL1 promoter (GAL1-MAD2 (3X); Rossio et al., 2010). Addition of galactose to the medium of these cells switches on the GAL1 promoter and causes Mad2 to accumulate to a level that ectopically induces MCC formation, similarly to nocodazole (Mariani et al., 2012).

**GAL1-MAD2 (3X)** cells adapted to the checkpoint after several hours of growth in galactose. Adaptation was marked by the degradation of Clb2 and Pds1, and by the elongation of mitotic spindles, in agreement with previously published data (Rossio et al., 2010; Fig. 1, B and C). The presence of nonphosphorylatable subunits of APC/C prevented adaptation, with **GAL1-MAD2 (3X) cdc16-7A cdc27-5A** cells displaying persistently high levels of Clb2 and Pds1 (Fig. 1 D), and a large majority of metaphase spindles after several hours (Fig. 1 C). When grown in glucose, **GAL1-MAD2 (3X) cdc16-7A cdc27-5A** completed one cell cycle with kinetics that were only slightly delayed compared with **GAL1-MAD2 (3X)** cells (Fig. S1 A). These results are in agreement with data from Rudner and Murray (2000), who analyzed the growth of APC mutants in solid media under checkpoint-inducing conditions.

We next assessed the requirement of the cofactors Cdc20 and Cdh1 in adaptation to the spindle checkpoint. Given the essential role of Cdc20 in the metaphase-to-anaphase transition, it was not surprising that depletion of Cdc20 impaired adaptation to the checkpoint (Fig. S1 B). The role of CDH1, a nonessential gene in the metaphase-to-anaphase transition, was harder to predict. Because it was difficult to synchronize **GAL1-MAD2 (3X) cdh1Δ** cells in G1, we tested their ability to adapt to the spindle checkpoint using a cycling population. Our results show that **GAL1-MAD2 (3X) cdh1Δ** cells assembled and disassembled metaphase spindles very similarly to **GAL1-MAD2 (3X)** cells, thus excluding a fundamental role for Cdh1 in adaptation (Fig. 1 E).

We conclude that although Cdh1 is dispensable for adaptation to the spindle checkpoint, both Cdc20 and phosphorylation of the APC/C subunits Cdc16 and Cdc27 are essential.
It is thus plausible that the slow degradation of Clb2 by decreasing Cdc28 activity leads to checkpoint inactivation, Cdc20 release from the MCC, and ultimately to adaptation via the formation of APC/CCdc20.

To verify this hypothesis, we asked whether it is possible to overcome the spindle checkpoint arrest in yeast by inhibiting Cdc28. To modulate Cdc28 activity, we used a mutant of CDC28, cdc28-as1, that can be inhibited by the ATP analogue 1-NMPP1 (Bishop et al., 2000). APC/CCdc20 activity was monitored by the degradation of Pds1 and Clb2. Depending on the...
amount of 1-NMPP1, Cdc28-as1 can be fully or partially inactivated (Bishop et al., 2000; Liang et al., 2012). We reasoned that partial inactivation would better mimic the initial stages of Cdc28 inactivation after Clb2 degradation in adaptation. For this reason, we used 500 nM 1-NMPP1, a concentration that allows DNA replication but arrests cells in a premitotic state (Bishop et al., 2000; Liang et al., 2012). We confirmed this by adding 500 nM 1-NMPP1 to cdc28-as1 cells released from G1 arrest and measuring their DNA content by FACS and mitotic spindle assembly (Fig. S1, C and D).

cdc28-as1 cells were arrested in prometaphase by nocodazole for 3 h, and then treated with 500 nM 1-NMPP1. Having confirmed that nocodazole depolymerized the mitotic spindle throughout the experiment (Fig. S1 E) and that cells arrested with a 2C DNA content (Fig. S1 F), we monitored cells for a further 2 h. We observed that APC/C was not reactivated toward Pds1 and Clb2, which were not destabilized, but Cdc20 levels rapidly decreased when compared with DMSO control (Fig. 2, A and B). The reduction in Cdc20 could be caused by its decreased synthesis or increased degradation. We favor the former hypothesis because Cdc28 inhibition arrests CDC20 transcription (Liang et al., 2012), whereas Cdc20 is quickly degraded during checkpoint arrest, its degradation being dependent on APC/C (Pan and Chen, 2004).

Our results suggest that the checkpoint is still active, and thus APC/C^{Cdc28} is unable to target Pds1 and Clb2 after the partial inhibition of Cdc28. To confirm that this is the case, we repeated the experiment with a yeast strain carrying Mad2-GFP, which is recruited at kinetochores during a checkpoint-induced arrest (Gillett et al., 2004). Our results confirmed that the checkpoint pathway was still active at the kinetochores after Cdc28 inhibition, as Mad2-GFP colocalized with a component of the outer kinetochore, Nuf2, 2 h after treatment with 500 nM 1-NMPP1 (Fig. 2 E), like in the DMSO control. Cells released from nocodazole, which repolymerized mitotic spindles (Fig. S1 G), did not show kinetochore localization of Mad2 as expected.

In summary, our results show that the inhibition of Cdc28 with 500 nM 1-NMPP1 is sufficient to keep the upstream spindle checkpoint pathway active, but unable to support the production of Cdc20. As a consequence, APC/C^{Cdc28} could not be activated and cells remained arrested in metaphase.
Cdh1 is required for Clb2 and Pds1 degradation upon strong Cdc28 inhibition

We asked whether the degradation of Pds1 and Clb2 after strong inhibition of Cdc28 was caused by APC/CCdh1. This possibility is supported by evidence that Cdc28 inhibits Cdh1 (Zachariae et al., 1998). Unfortunately, we could not use \textit{cdh1} \textsuperscript{-} cells that carry the \textit{cdc28as-1} allele because the two mutations are synthetic lethal. We thus inhibited Cdc28 activity by overexpressing the stoichiometric inhibitor Sic1 (Schwob et al., 1994), expressed under the \textit{GAL1} promoter (Amon, 1997). We reasoned that the discrepancy between these published data and our results is most likely caused by the level of Cdc28 inhibition. Therefore, we increased the dose of 1-NMPP1 from 500 nM to 5 \mu M, a concentration that is known to also inhibit DNA replication (Bishop et al., 2000). To test the efficacy of the drug, we synchronized cells in G1 and released them in media supplemented with 5 \mu M 1-NMPP1. We confirmed that under these conditions, cells arrested with IC DNA content for 2.5 h without forming metaphase spindles (Fig. S1, C and D). We then added 5 \mu M 1-NMPP1 to \textit{MAD2-GFP NUF2-mCherry cdc28as-1} cells arrested in prometaphase by nocodazole. Similar to the results obtained with 500 nM 1-NMPP1, we observed that Mad2-GFP was largely localized at the kinetochores up to 2 h after the addition of 5 \mu M 1-NMPP1 (Fig. 2 E), which suggests that the checkpoint was not dismantled. However, at variance with results obtained with 500 nM 1-NMPP1, Pds1 and Clb2 levels decreased markedly after 1.5 h of treatment (Fig. 2 D), Cdc20 levels decreased even more rapidly after treatment with 5 \mu M 1-NMPP1, which implies that the reduction in Pds1 and Clb2 was not caused by APC/C\textsuperscript{Cdc20} (Fig. 2, C and D).

We conclude that although APC/C can be activated by strong inhibition of Cdc28, its activation does not require Cdc20 nor the inactivation of the upstream checkpoint pathway at the kinetochores.

\textbf{Figure 3.} \textit{Cdh1 is responsible for Pds1 and Clb2 degradation upon strong Cdc28 inhibition.} (A and B) \textit{GAL-SIC1} (yAC2025) and \textit{GAL-SIC1 cdh1} \textsuperscript{-} (yAC2023) cells were grown in either raffinose [A] or glucose [B] at 23°C. When in log phase, cells were either supplemented with galactose [A] or kept in glucose [B]. Samples were analyzed by Western blotting (top) and FACS (bottom). The data are from a single representative experiment out of three repeats.

\textbf{Cdh1 is required for Clb2 and Pds1 degradation upon strong Cdc28 inhibition}

We asked whether the degradation of Pds1 and Clb2 after strong inhibition of Cdc28 was caused by APC/C\textsuperscript{Cdh1}. This possibility is supported by evidence that Cdc28 inhibits Cdh1 (Zachariae et al., 1998). Unfortunately, we could not use \textit{cdh1} \textsuperscript{-} cells that carry the \textit{cdc28as-1} allele because the two mutations are synthetic lethal. We thus inhibited Cdc28 activity by overexpressing the stoichiometric inhibitor Sic1 (Schwob et al., 1994), expressed under the \textit{GAL1} promoter (Amon, 1997). We first confirmed that Sic1 overexpression efficiently inhibited Cdc28 activity in vivo (Fig. S2 D). We then arrested cells in prometaphase with nocodazole for 2.5 h, and afterward we induced Sic1 overexpression with galactose. Crucially, Clb2 and Pds1 were stable in \textit{cdh1} \textsuperscript{-} cells but were quickly degraded in cells carrying \textit{CDH1} wild type (Fig. 3 A). Notice that cells are under nocodazole and thus unable to form microtubules: that is why they cannot undergo cytokinesis and exit mitosis regardless of the degradation of Pds1 and Clb2 (Fig. 3 A). This result confirms the hypothesis that APC/C\textsuperscript{Cdh1} is activated when Cdc28 is strongly inhibited. As expected, with physiological levels of Sic1, cells maintained the mitotic arrest with stable Pds1 and Clb2 independently of the presence of Cdh1 (Fig. 3 B). The degradation of Cdc20 in \textit{cdh1} \textsuperscript{-} cells is likely caused by the inhibition of its transcription after Cdc28 inactivation.
We thus conclude that when Cdc28 activity is greatly reduced, APC/C<sup>Cdh1</sup> is activated and degrades both Pds1 and Clb2. Although Pds1 and Clb2 were degraded, it would be wrong to conclude from these data that adaptation requires APC/C<sup>Cdh1</sup> activation mediated by a strong inactivation of Cdc28, as we have previously shown Cdh1 to be dispensable for adaptation (Fig. 1 E).

**Cdc28 activity is required for cells to adapt**

The experiments performed on cells arrested in prometaphase by nocodazole suggest that cells do not adapt to the checkpoint by inhibiting Cdc28 activity. We thus decided to test the opposite scenario, where Cdc28 activity is required for adaptation. In this scenario, it would be possible to inhibit adaptation by inhibiting Cdc28 with a minimal amount of ATP analogue sensitivity. To test this prediction, we assessed the effect of as little as 50 nM 1-NMPP1 on adaptation to a checkpoint arrest induced by Mad2 overexpression. **GAL1-MAD2 (3X) cdc28-as1** cells were synchronized in G1 by α-factor and released in galactose to induce Mad2 overexpression. Under control conditions (DMSO), the majority of cells adapted within 5 h (Fig. 4 A, left; and Fig. 4 B); however, in the presence of 50 nM 1-NMPP1, adaptation was inhibited in the vast majority of cells (Fig. 4 A, right; and Fig. 4 B). At this low concentration of 1-NMPP1, no effects were observed on cells expressing wild-type CDC28 (Fig. S3, A and B). Moreover, when **GAL1-MAD2 (3X) cdc28-as1** cells were grown in a medium lacking galactose and therefore were not checkpoint arrested, 50 nM 1-NMPP1 induced only a small delay in the transition to anaphase as detected by spindle dynamics and FACS (Fig. S3 C).

We conclude therefore that in budding yeast Cdc28 activity is required for adaptation to the spindle checkpoint.

**Clb2 levels increase constantly during a checkpoint-induced arrest and are rapidly reduced during the transition to anaphase**

The notion that active Cdc28 is required for adaptation prompted us to reconsider the observation that Clb2, which is required for Cdc28 activity, is slowly degraded as cells adapt (Fig. 1 B). We reasoned that Western blots might mask the real nature of adaptation taking place in individual cells. We thus resorted to single cell analysis and followed the dynamics of Clb2 in yeast strains in which the mitotic cyclin was tagged with GFP (Hood et al., 2001).

Asynchronous **GAL1-MAD2 CLB2-GFP TUB2-Cherry** cells were loaded in flow chambers in the presence of galactose, and after a short time they arrested as budded cells in metaphase. We then followed adaptation in individual cells (Fig. 5 A), and the results were doubly surprising. Not only did we observe that Clb2 increased constantly during metaphase arrest, but the mitotic cyclin was also degraded very rapidly when cells...
sudden and stochastic activation of APC/C, and not its residual activity, drives the transition to anaphase during adaptation. The PP2A phosphatase opposes Cdc16 phosphorylation during checkpoint arrest

We next asked why Cdc28 activity is necessary for adaptation (Fig. 4 A). We have already shown that phosphorylation of the APC/C subunits, Cdc16 and Cdc27, is required for adaptation (Fig. 1 D); therefore, it is possible that Cdc28 is needed to phosphorylate APC/C during adaptation. This notion is supported by published data showing that the two APC/C subunits are phosphorylated by Cdc28 when cells enter anaphase (Rudner and Murray, 2000). We confirmed that before adaptation Cdc16 is progressively phosphorylated, as detected by Western blotting using Phos-tag reagent (Fig. S5, A and B).

We reasoned that a phosphatase might oppose the increasing Cdc28 activity during adaptation because cells took several hours to adapt despite accumulation of Cdc28 in the nucleus. If Cdc28-mediated phosphorylation of APC/C is a key molecular event in overcoming the checkpoint arrest, then the phosphatase that dephosphorylates APC/C should be required to maintain arrest. A plausible candidate that could be responsible for APC/C dephosphorylation is the phosphatase PP2ACdc55, as cdc55Δ cells are checkpoint deficient (Minshull et al., 1996). Indeed, we confirmed that GAL1-MAD2 (3X) cdc55Δ cells do not undergo spindle checkpoint–induced arrest triggered by Mad2 overexpression (Fig. S5 C). We thus decided to test whether PP2A Cdc55 controls the phosphorylation state of APC/C during nocodazole-induced arrest.
Figure 6. PP2A<sup>Cdc55</sup>-mediated dephosphorylation of APC subunits is essential for spindle checkpoint activity. (A) CDC16-myc<sup>6</sup> (yAC1936), CDC16-myc<sup>6</sup> cdc55<sup>Δ</sup> (yAC1994), CDC27-myc<sup>9</sup> (yAC1863), and CDC27-myc<sup>9</sup> cdc55<sup>Δ</sup> (yAC1888) cells were grown in YEPD at 30°C, arrested in G1 phase, and released in nocodazole. Samples were taken during the G1 arrest and after 2 and 3 h for Western blotting analysis of Cdc16 (left) and Cdc27 (right) using Phos-tag.
We compared the Western blot profiles of Cdc16 and Cdc27 in CDC55 wild-type and cdc55Δ cells after releasing them from a G1 arrest into nocodazole. Using acrylamide gels containing Phos-tag reagent (Kinosita et al., 2008), we observed that Cdc16 is already more highly phosphorylated in cdc55Δ cells compared with CDC55 wild-type strains during G1 arrest (indicated by the arrows in Fig. 6 A, left). This difference became more evident in cells arrested for 2 and 3 h in nocodazole: in cdc55Δ cells we observed phosphorylation-dependent shifts that were completely missing in CDC55 cells (Fig. 6 A, left), and that were sensitive to phosphatase treatment (not depicted). A shift that was sensitive to phosphatase treatment was also detected in standard Western blots of lysates from cdc55Δ cells (Fig. S5 D), confirming that Cdc16 underwent enhanced phosphorylation in the absence of PP2ACdc55. The same analysis with Cdc27 did not reveal major differences in the total level of phosphorylation between CDC55 wild type and cdc55Δ cells (Fig. 6 A, right).

These data indicate that the protein phosphatase PP2ACdc55 directly or indirectly dephosphorylates Cdc16 and that these events could be critical to maintenance of checkpoint arrest and inhibition of adaptation.

Together, our results support a scenario where Cdc28/Cib phosphorylates and PP2ACdc55 dephosphorylates Cdc16. The final output of these opposing activities could be net phosphorylation, leading to adaptation, or net dephosphorylation, leading to mitotic arrest. If this hypothesis were correct, we would expect the loss of checkpoint arrest in cdc55Δ cells to be reversed in the presence of nonphosphorylatable Cdc16.

Although Cdc27 is not dephosphorylated by PP2ACdc55, the simultaneous presence of nonphosphorylatable Cdc27 and Cdc16 strongly decreases the ability of the single mutants to adapt (Rudner and Murray, 2000). For this reason, we tested the hypothesis by assessing spindle checkpoint activity in the triple mutant cdc16-7A cdc27-5A cdc55Δ. By following the kinetics of mitotic spindles and the stability of Pds1, we observed that, in contrast to GAL1-MAD2 (3X) cdc55Δ cells, GAL1-MAD2 (3X) cdc16-6A cdc27-5A cdc55Δ were arrested by the checkpoint and in fact were severely impaired in adaptation to the spindle checkpoint induced by Mad2 overexpression (Fig. 6, B–E). The fact that a minority of triple mutant cells eventually adapted, whereas no cdc16-6A cdc27-5A cells did, could be due to the presence of additional phosphorylation sites on the APC/C recognized by PP2ACdc55. Alternatively, PP2ACdc55 might also inhibit APC/C activation via other pathways, which are nevertheless less relevant than APC/C dephosphorylation. When Mad2 was not overexpressed, all three strains progressed through the cell cycle with similar kinetics (Fig. S5 E).

We conclude that increased phosphorylation of Cdc16 causes the checkpoint deficiency of cdc55Δ cells.

PP2ACdc55 inhibits adaptation independently of Cdc14

Our data indicate that during adaptation, PP2ACdc55 inhibits the transition into anaphase by dephosphorylating Cdc16. Previous data showed that during a regular cycle PP2ACdc55 delays anaphase by preventing Cdc14 release from the nucleolus (Queralt et al., 2006; Wang and Ng, 2006; Yellman and Burke, 2006). It is thus possible that during mitotic arrest PP2ACdc55 not only prevents adaptation by dephosphorylating APC/C, but also by inhibiting the release of Cdc14, which could help cells to transit into anaphase by silencing the checkpoint (Mirchenko and Uhlmann, 2010).

To test this possibility, we determined whether constitutive Cdc14 activity is sufficient to inactivate a nocodazole-induced checkpoint. We used the net1Δ mutant, where Cdc14 is constitutively released throughout the cell cycle. Our results show that net1Δ cells are checkpoint proficient, as indicated by the stability of Pds1 (Fig. 7 A). Notably, cdc55Δ cells, which also release Cdc14 prematurely, are instead checkpoint deficient (Fig. 7 B). The metaphase-to-anaphase transition is similar in the two mutants during an unperturbed cell cycle (Fig. S5, F and G). These results indicate that Cdc14 release alone is not sufficient to inactivate the checkpoint, and imply that PP2ACdc55 activity does not arrest cells in metaphase by preventing Cdc14 release.

We then tested whether Cdc14 activity is essential for adapting cells to enter anaphase. If so, we would expect that inactivation of Cdc14 after checkpoint arrest would cause a permanent metaphase block. Because CDC14 is an essential gene, we used the temperature-sensitive mutant cdc14-1, which produces a phosphatase that is inactivated after ~30 min at 37°C (Akiyoshi and Biggins, 2010). In the absence of active Cdc14, cells arrest in anaphase with elongated spindles (Rock and Amon, 2009). To observe the effect of an incomplete activation of Cdc14, we also used a strain carrying a temperature-sensitive mutation of Cdc15, which causes partial release of Cdc14, but nevertheless arrests in anaphase (Rock and Amon, 2009). We activated the checkpoint in these temperature-sensitive mutants by overexpressing Mad2 from the GAL1 promoter.

Cells were synchronized in G1 at 23°C and released in galactose to activate the checkpoint. To inactivate cdc14-1 and cdc15-2, the temperature was increased to 37°C, 2 h after the release when most of the cells had large buds. The strains carrying cdc14-1 and cdc15-2 degraded Pds1 and Cib2 and disassembled metaphase spindles with only a small delay compared with GAL1-MAD2 (3X), and in contrast to the adaptation-deficient GAL1-MAD2 (3X) cdc27-5A cdc16-6A cells; Cdc14 and Cdc15 were clearly inactivated as cells accumulated in anaphase (Fig. 7, C and D). GAL1-MAD2 cdc15-2 cells were slightly delayed compared with GAL1-MAD2 cdc14-1 cells. As expected,
APC/C cofactor, is dispensable for adaptation to the spindle checkpoint, as also suggested by Rossio et al. (2010) and Rudner et al. (2000). A second option is that cells adapt because they switch off the checkpoint cascade. However, we did not obtain any evidence to support such a scenario. Previous studies in budding yeast suggest that the pathway upstream of MCC formation is switched off via a decrease of Mad1/Bub3 binding (Rossio et al., 2010), two components of the spindle checkpoint pathway that localize at the kinetochores. In our adaptation experiments, we have activated the checkpoint ectopically by overexpressing Mad2. We have shown previously that in this setting MCC formation occurs independently from Mad1 (Mariani et al., 2012) and thus the reduced binding of Mad1 with Bub3 is unlikely to lead to the release of Cdc20 from the MCC. It was also reported that Bub1 degradation helps adaptation (Goto et al., 2011), but we have previously shown that Bub1 does not localize to kinetochores in cells overexpressing Mad2 (Mariani et al., 2012). For this reason, we suspect that Bub1 inactivation does not underlie adaptation in our system. Another attractive candidate for checkpoint silencing is Cdc14, given its recently discovered role in preventing checkpoint reactivation in anaphase (Mirchenko and Uhlmann, 2010). However, we determined that Cdc14 was neither necessary nor sufficient for adapting cells to transit into anaphase. Cdc28 inactivation might when the same strains were released at 37°C in glucose (i.e., without switching on the GAL1 promoter and inducing Mad2 overexpression), cells carrying the mutations in CDC14 and CDC15 showed metaphase spindle kinetics similar to wild-type cells before arresting in anaphase, whereas the double APC/C cdc27-5A cdc16-6A mutant was delayed by ~20 min in the exit from metaphase (Fig. S5 H).

We conclude that Cdc14 activation is not necessary for cells to transit into anaphase when the checkpoint is activated.

**Discussion**

When cells are exposed to prolonged checkpoint-inducing stimuli, they are arrested in metaphase by the sequestration of the APC/C cofactor, Cdc20, in the MCC. After several hours, cells eventually adapt to the stimulus, resulting in segregation of their DNA material and exit from mitosis. Because Cdc20 is sequestered in the MCC during a checkpoint arrest, it is unclear how APC/C is activated during adaptation to drive cells into anaphase.

**Molecular mechanisms for adaptation to the spindle checkpoint**

One possibility is that APC/C bypasses the requirement for Cdc20 during adaptation. Our data show that Cdhl, the other APC/C cofactor, is dispensable for adaptation to the spindle checkpoint, as also suggested by Rossio et al. (2010) and Rudner et al. (2000). A second option is that cells adapt because they switch off the checkpoint cascade. However, we did not obtain any evidence to support such a scenario. Previous studies in budding yeast suggest that the pathway upstream of MCC formation is switched off via a decrease of Mad1/Bub3 binding (Rossio et al., 2010), two components of the spindle checkpoint pathway that localize at the kinetochores. In our adaptation experiments, we have activated the checkpoint ectopically by overexpressing Mad2. We have shown previously that in this setting MCC formation occurs independently from Mad1 (Mariani et al., 2012) and thus the reduced binding of Mad1 with Bub3 is unlikely to lead to the release of Cdc20 from the MCC. It was also reported that Bub1 degradation helps adaptation (Goto et al., 2011), but we have previously shown that Bub1 does not localize to kinetochores in cells overexpressing Mad2 (Mariani et al., 2012). For this reason, we suspect that Bub1 inactivation does not underlie adaptation in our system. Another attractive candidate for checkpoint silencing is Cdc14, given its recently discovered role in preventing checkpoint reactivation in anaphase (Mirchenko and Uhlmann, 2010). However, we determined that Cdc14 was neither necessary nor sufficient for adapting cells to transit into anaphase. Cdc28 inactivation might...
also underlie checkpoint inactivation at the time of adaptation, as has been proposed to occur during the transition to anaphase in vertebrates (Zeng et al., 2010; He et al., 2011). However, we observed that the inhibition of Cdc28 does not delocalize Mad2 from the kinetochores in yeast cells arrested in prometaphase by the checkpoint, which suggests that in yeast there is not such a strong link between Cdc28 activity and checkpoint maintenance. Moreover, we demonstrated that inhibition of Cdc28 activity in checkpoint-arrested cells is incompatible with transition into anaphase. In conclusion, our data support the notion that adaptation takes place when the checkpoint is fully functional as originally proposed by Brito and Rieder (2006).

In this scenario, adaptation would be driven by a mechanism that competes with the checkpoint and eventually overrides it through the formation of a critical amount of APC/C\(^{\text{Cdc20}}\) that drives cells into anaphase. Here, we propose that the phosphorylation of APC/C, favored by Cdc28 and opposed by PP2A\(^{\text{Cdc55}}\), plays a critical role in the adaptation process. The observation that the mitotic cyclin Clb2, a key regulator of Cdc28, accumulates steadily before cells adapt, supports the idea that a threshold level of active APC/C phosphorylation needs to be reached before cells can overcome checkpoint arrest. Possibly, APC/C phosphorylation drives the formation of APC/C\(^{\text{Cdc20}}\) because it increases the affinity of APC/C for Cdc20 (Rudner and Murray, 2000). Although we demonstrate that APC/C phosphorylation is necessary for adaptation, we cannot conclude that it is the ultimate event that causes it. Further work is needed to clarify this point, and particularly to study the regulation of PP2A\(^{\text{Cdc55}}\) in adapting cells. Whatever the mechanism that triggers adaptation, the high variability in adaptation times complicates the analysis of this process.

It is worthwhile to note that in mammals the molecular mechanism that drives adaptation is likely to differ from that in yeast. Indeed, it has been shown that during adaptation in single mammalian cells cyclin B is slowly degraded (Brito and Rieder, 2006), which is at variance with our results in single budding yeast cells.

The interplay of PP2A\(^{\text{Cdc55}}\) and Cdc28/Clb2 controls checkpoint proficiency

Our data provide a new explanation for the old observation that the regulatory subunit of the PP2A phosphatase, Cdc55, is required for the spindle checkpoint. We suggest that PP2A\(^{\text{Cdc55}}\) contributes to checkpoint arrest by inhibiting the formation of APC/C\(^{\text{Cdc20}}\) through the dephosphorylation of the APC/C subunit, Cdc16. In this scenario, cells lacking Cdc55 would “always be adapted” because the inability to dephosphorylate APC/C would render cells unable to mount a checkpoint arrest. Accordingly, cdc55A cells become checkpoint proficient again in a strain where the phosphorylation sites of Cdc16 and Cdc27 recognized by Cdc28/Clb2 are mutated to alanine. This result sheds light on a previous observation that a Cdc28 mutant with decreased kinase activity (Cdc28-VF) rescues the checkpoint defect of cdc55A (Minshull et al., 1996). In our interpretation, the rescue is caused by a balancing out of APC/C phosphorylation and dephosphorylation, achieved by the simultaneous decrease of Cdc28 kinase and PP2A phosphatase activity.

Our results showing that PP2A\(^{\text{Cdc55}}\) dephosphorylates Cdc16, which are in agreement with recent findings (Lianga et al., 2013), do not exclude the existence of phosphatases other than PP2A\(^{\text{Cdc55}}\) that could dephosphorylate APC/C. The phosphorylation state of Cdc27 is not affected by the deletion of CDC55, and yet the alanine substitution of the phosphorylation sites on Cdc27 decreases the capability of cells to adapt (Rudner and Murray, 2000). Moreover, the previous observation that the deletion of CDC55 is fully balanced by the partial inhibition of Cdc28 (Minshull et al., 1996) could be explained by the presence of overlapping phosphatases.

A positive feedback loop controlling APC/C\(^{\text{Cdc20}}\) activation during adaptation

During adaptation, activation of APC/C\(^{\text{Cdc20}}\) requires Cdc28/Clbhs. Consistently, Clb2 accumulates in the nucleus steadily for several hours until cells adapt. However, as soon as APC/C\(^{\text{Cdc20}}\) is activated it rapidly degrades Clb2: the high level of Cdc28/Clbs that cells accumulated in several hours is lost in a few minutes (Fig. 5 A). The later stages of Clb2 degradation are likely due to APC/C\(^{\text{Cdh1}}\), but the initial stages are APC/C\(^{\text{Cdc20}}\) dependent, and are aided by APC/C phosphorylation. It is therefore surprising that the rapidly diminishing levels of Cdc28/Clbs are able to sustain efficient phosphorylation of APC/C, in the initial stages after APC/C\(^{\text{Cdc20}}\) activation, in the presence of the counteracting phosphatase PP2A\(^{\text{Cdc55}}\).

This conundrum does not concern only adaptation in budding yeast. In cycling extracts of Xenopus laevis, it was recently shown that this paradox is resolved by a time delay between CDK1 and APC/C\(^{\text{Cdc20}}\) activation, which guarantees high levels of APC/C\(^{\text{Cdc20}}\) after it starts to degrade Cyclin B (Yang et al., 2013). Our data suggest a possible molecular mechanism to explain the establishment of the time delay. During a regular transition to anaphase, PP2A\(^{\text{Cdc55}}\) is inhibited by separase (Queralt et al., 2006), which is in turn activated by APC/C\(^{\text{Cdc20}}\). Together, these proteins form a positive feedback loop (APC/C\(^{\text{Cdc20}}\) ⊣ Securin ⊣ separase ⊣ PP2A\(^{\text{Cdc55}}\) ⊣ APC/C\(^{\text{Cdc20}}\)) that is coupled to the negative feedback loop, whereby APC/C\(^{\text{Cdc20}}\) inactivates Cdc28/Clbhs that in turn activates APC/C (Fig. 8 A). A simple model based on these ideas (see Materials and methods) shows that Clb2 increases steadily until APC/C is activated, and then the negative feedback loop comes into action (Fig. 8 B). After this point, APC/C\(^{\text{Cdc20}}\) locks APC/C in the phosphorylated state by inhibiting the protein phosphatase responsible for APC/C dephosphorylation. Hereafter, the decrease of Cdc28/Clb2 activity becomes irrelevant, as the phosphatase that could oppose Cdc28 activity has been inactivated. In fact, APC/C\(^{\text{Cdc20}}\) activity stays high after Clb2 is long gone (in reality it will likely be removed, together with the remaining Clb2, by APC/C\(^{\text{Cdh1}}\), which is missing in the model).

Our model is based on the idea that the positive feedback loop creates two states (i.e., it is bistable): a metaphase state with APC/C\(^{\text{Cdc20}}\) inactive and PP2A\(^{\text{Cdc55}}\) active, and an anaphase state that follows adaptation where the activation states are reversed. The idea that a bistable switch controls transition into anaphase is not new. It was proposed for the first time after the observation that APC/C\(^{\text{Cdc20}}\) degrades Mps1, a component of
Strains, media, and reagents

Strains used in this study are listed in Table S1. All yeast strains were derivatives of or were backcrossed at least three times with W303 (ade2-1, trp1-1, leu2-3, 112, his3-11, 15, ura3-52, and ssd1), cdc27-5A and cdc16-6A mutants were obtained from A. Murray (Department of Molecular and Cellular Biology and Center for Systems Biology, Harvard University, Cambridge, MA); cdc28-as1 and yox1Δ were received from U. Surana (Institute of Molecular and Cell Biology, Agency for Science, Technology and Research [A*STAR], Singapore); cdc14-1, cdc15-2, and GAL1-SIC1 were from R. Visintin (IEO, Milan, Italy); and the original construct for GAL1-MAD2 was developed in the lab of S. Pietti (Centre de Recherche en Biochimie Macromoléculaire, Montpellier, France).

The population experiment in Fig. S1 B was performed in synthetic medium lacking methionine. All other population experiments were performed using YEP medium (1% yeast extract, 2% Bacto Peptone, and 50 mg/liter adenine) supplemented with 2% glucose (YEPD), 2% raffinose (YEPR), or 2% galactose (YEPG). Single cell adaptation experiments under Mad2 overexpression were performed using synthetic complete medium supplemented with ammonium sulfate.

α-Factor and nocodazole were used at 5 µg/ml and 15 µg/ml, respectively. α-Factor readdition was at 20 µg/ml. Methionine was added at a final concentration of 20 mM to repress the MET3 promoter. Unless otherwise specified, all experiments were performed at 30°C. In population experiments of adaptation, galactose was added 1 h before the release from α-factor (20 min for Fig. S1 B), while α-factor was re-added 1.5–2 h after the release from G1, unless otherwise stated.

Western blot analysis

In all Western blot experiments, samples were collected at the indicated time points, and cells were pelleted by centrifugation for 2 min at room temperatures. Then 100 µl TCA was added to the pellets to precipitate proteins. TCA protein extracts were prepared according to Fraschini et al. (1999). Protein samples were loaded and separated in 10% or 12.5% polyacrylamide gels (with a bis-acrylamide/acrylamide ratio of 1:29) with the voltage of separation apparatuses set to 140 V. For detection of Cdc16 and Cdc27 phosphorylation-specific bands, the Phos-tag system (50 µM Phos-tag reagent) was used (Kinoshiba et al., 2008). To visualize phosphorylation shifts of Cdc16 on normal gels, we prepared 7.5% polyacrylamide gels with a bis-acrylamide/acrylamide ratio of 1:80. Proteins were transferred from gels to Protran membranes for 1 h, with transfer apparatuses set at 100 V. For the detection of Myc-tagged proteins (Pds1-Myc18, Cdc16-Myc6, and Cdc27-Myc9), the last two strains were a gift from J.-M. Peters, Research Institute of Molecular Pathology (IMP, Vienna, Austria) and of Mad2, membranes were probed, respectively, with 9E10 anti-Myc and anti-ScMsd2 antibodies produced at the Monoclonal Antibodies Facility at the IFOM-IEO Campus (1:1,000 dilution). Commercial antibodies were used as follows: Cdc20 (yC-20; Santa Cruz Biotechnology, Inc.) at 1:1,000, Cdc2 (y180; Santa Cruz Biotechnology, Inc.) at 1:1,000, and Pgk1 (D660; Invitrogen) at 1:5,000. Secondary antibodies were from Bio-Rad Laboratories and proteins were detected by an enhanced chemiluminescence system (Pierce ECL; Thermo Fischer Scientific) according to the manufacturer’s instructions. Blots were acquired as digitalized images by a Chemidoc XR+System (Bio-Rad Laboratories) and the software Imagelab was used to quantify the signals. Molecular weights in the Western blot panels refer to Prestained Protein Marker (New England Biolabs, Inc.) bands run on the same blot.

To quantify Cdc16 and Cdc27 phospho-specific bands on P-Tag gels, the signal corresponding to the phosphorylation shift (above the second band visible during the G1-arrest) was normalized over the total amount of protein in the same gel at the same time point.

Other techniques

Flow cytometric DNA quantitation was determined using a flow cytometer (FACScan or FACSscalibur; BD) and analyzed with FlowJo Software. For each sample, we scored 10,000 events.

Metaphase and anaphase spindle formation (i.e., immunofluorescence [IF]) analysis was detected with α-tubulin immunostaining with the YOL34 monoclonal antibody (ABD Serotec) followed by indirect IF using FITC-conjugated anti-rat antibody (Jackson ImmunoResearch Laboratories, Inc.).

Single cell analysis was performed using microfluidic chambers (CELLASIC), with cells growing at 30°C in synthetic medium containing raffinose. Time-lapse movies were recorded using a DeltaVision Elite imaging system (Applied Precision) based on an inverted microscope (IX71; Olympus) with a camera (CoolSNAP HQ2; Photometrix) and a UPlan-Apochromat 60x (1.4 NA) oil immersion objective lens (Olympus). Cells expressing Mad2-3GFP (gift from T. Tanaka, Centre for Gene Regulation and Expression, College of Life Sciences, University of Dundee, Scotland, UK) and Nuf2-mCherry (gift from J.-M. Peters, Research Institute of Molecular Pathology [IMP, Vienna, Austria]) were fixed in cold 100% ethanol, and images were acquired on the DeltaVision Elite imaging system using a UPlan-Apochromat 100x (1.4 NA) oil immersion objective lens (Olympus). We counted cells as colocalized where the maximum of Mad2-GFP signal was localized with one Nuf2-mCherry dot.

To perform the phosphatase assay on protein samples extracted with TCA, Laemmli buffer was exchanged with Buffer3 (10 mM MgCl2, 100 mM NaCl, 50 mM TRIS, and 1 mM DTT, pH 7.9) using Amicon Ultra centrifugal filters (Millipore). The extracts were then incubated for 30 min at 37°C with buffer alone, calf intestinal phosphatase (CIP; New England Biolabs, Inc.), or CIP plus 10 mM sodium orthovanadate (to inhibit the CIP).

Methods for image analysis

Segmentation and whole-cell signal analysis. Image analysis was performed with software written in MATLiab (MathWorks). For the segmentation and tracking of yeast cells we used the program phyloCell, written by G. Charvin (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France). From the fluorescent signal of the whole cell we subtracted a background signal that we obtained from cells not carrying the fluorescent markers grown in the same conditions. We observed a slight increase in autofluorescence during adaptation and corrected our measurements accordingly.

Nuclear signal analysis. The calculation of the concentration of fluorescence in the nucleus required us to know the area of the nucleus. Because our cells do not carry a nuclear marker, we used the Clb2-GFP signal itself, as Clb2 accumulates in the nucleus during mitosis. The identification of the nucleus was thus successful only when the intensity of the Clb2 nuclear signal was significantly higher than in the rest of the cell. The size of the nucleus could then be identified by k-means clustering, using k = 3 (the clusters corresponding to nucleus, cytoplasm, and extracellular region; see Video 1). In contrast, when the intensities in the nucleus and in the cytoplasm were comparable, the classification of the nuclear signal was not sufficient to separate the nucleus from the cytoplasm.
similar, the clustering gave unrealistically high values for the size of the nucleus. Therefore, the moment in which the measurement becomes reliable is marked by a drop in the ratio of the size of the nucleus to the size of the whole cell (Fig. S4 B, top). We considered the clustering to be reliable when this ratio was below a value of 30%. We used the nuclear signal for our calculations only in this region (Fig. S4 B, bottom, solid line), after smoothing using a moving average. For the sake of representation, in Fig. S5 A and Fig. S4 C we also plotted the nuclear concentrations when the definition of the nucleus was unreliable (see “Nuclear signal”).

The signal before interpolation is visible in Video 1.

Expression rate. We observed that the increase in nuclear Cdc2, both in cycling cells and in the initial phase of adaptation, was approximately linear. Therefore, to quantify the expression rates (Fig. 5 B, top right), we performed a linear fit and compared the resulting slopes for the two conditions. We only used the part of the curve where the increase was sufficiently linear and for which the measurement of the nuclear region was reliable (see “Nuclear signal”).

Degradation rate. It was not possible to measure the rate of Cdc2 degradation in adapting cells directly in the nucleus because the measurement of the nuclear region was not reliable during the degradation phase. For this reason we analyzed Cdc2 degradation using the mean fluorescence in the whole cell (Fig. 5 B, top left). We assumed that this degradation can be described by a simple exponential decay (Fig. S4 A, top) and that at the end of the process Cdc2 is fully degraded.

Maxima. The maxima of fluorescence (Fig. 5 B, bottom left) were measured by taking the local maxima of fluorescence in the case of cycling cells. In the case of the adapting cells, we took the highest fluorescence level after smoothing that was reached during the adaptation process.

Adaptation time. Adaptation time (Fig. 5 B, bottom right) was measured in cells adapting to Mad2 overexpression as the time that elapses between the moment Clb2 starts to accumulate to the time the mitotic spindle elongates. If the time of spindle elongation was uncertain, we chose the beginning of Cdc2 degradation. When we analyzed adaptation of cells treated with nocodazole (Fig. S4 C, top), we obviously chose only cells that had not formed clusters of tubulin by the time of Cdc2 degradation, and thus we did not take into account cells that degraded Cdc2 after tubulin reappeared (Fig. S4 C, bottom).

Mathematical model

Because Cdc28 is present in excess over Cdc2, both in cycling and in the initial phase of adaptation, we can use the mean fluorescence in the whole cell to calculate the expression rate. As for APC/C, we did not explicitly introduce APC/C in our model; we explicitly modeled APC/C phosphorylation and inactivation, because APC/C is known to be regulated by phosphorylation and inactivation.

Parameter values. Parameter values were refined to fit our observations of mitotic checkpoint activation and deactivation. Initial conditions were as follows.

\[ Cdc28 = 0.9 \]
\[ Japci = 0.01 \]
\[ kph = 0.6 \] (checkpoint ON), \[ kph = 1.2 \] (checkpoint OFF)
\[ PP2A^{kdeg} \] activation: \[ ka = 1.2 \]
\[ PP2A^{kdeg} \] inactivation: \[ ki = 1.2 \]
\[ Cdc20 synthesis: \] ksyn = 0.02
\[ Cdc20 degradation: \] kdeg = 0.055

Dimensionless parameters:

- Michaelis constant for APC/C activation: Japc = 0.01
- Michaelis constant for APC/C inactivation: Japci = 0.01
- Total APC/C: APCtot = 1
- Total Cdc55: Cdc55tot = 0.9

Initial conditions (dimensionless). Initial conditions were as follows.

- APCP = 0.012, Cdc55A = 0.875, Cdc28 = 0.965

Online supplemental material

Fig. S1 shows that Cdc28 inhibition arrests cells in mitosis with an active checkpoint. Fig. S2 shows that partial inhibition of Cdc28 does not destabilize Pds1 and Cdc2 in the presence of Cdc20. Fig. S3 shows that 50 nM of 1-NMPP1 only mildly delays cell cycle progression. Fig. S4 shows single cell analysis of Cdc2 accumulation during a checkpoint-arrest. Fig. S5 shows that Cdc16 phosphorylation induces a gel shift during a prolonged spindle checkpoint arrest, and that Cdc14 is not required for adaptation. Table S1 is a strain list: Video 1 shows the signal of Clb2-GFP and TUB2. Cherry in one adapting cell and the definition of the nuclear volume after clustering. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201303033/DC1.

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a different pathway from Mad2. *J. Cell Biol.* 145:979–991. http://dx.doi.org/10.1083/jcb.145.5.979


Figure S1.  
Cdc28 inhibition arrests cells in mitosis with an active checkpoint.  
(A) GAL1-MAD2 (3X) PDS1-MYC18 (yAC489) and GAL-MAD2 (3X) PDS1-MYC18 cdc27-5A cdc16-6A (yAC1675) cells were grown on YEPD, synchronized in G1 with α-factor, and released into the cell cycle; α-factor was re-added after 70 min. Samples were taken for IF analysis. The data are from a single representative experiment out of three repeats. For the experiment shown, n = 100.  
(B) GAL1-MAD2 (3X) PDS1-MYC18 MET3-CDC20-HA3 (yAC2138) cells were grown in Met synthetic medium at 30°C, synchronized in G1 with α-factor, and released into galactose. Samples were taken at the indicated times for IF analysis (left) and at 3 h after the release from α-factor for Western blotting of Cdc20 (right). The data shown are from a single representative experiment out of three repeats (for IF, n = 100).  
(C and D) cdc28-as1 PDS1-MYC18 (yAC779) cells were grown in YEPD and synchronized in G1 with α-factor. Then they were released into the cell cycle in the presence of the indicated concentrations of 1-NMPP1. Samples were collected for IF (C) and for FACS (D). The data shown are from a single representative experiment out of three repeats (for C, n = 100).  
(E) Left, a field of cdc28-as1 PDS1-MYC18 (yAC779) cells after 3 h in nocodazole from the experiment shown in Fig. 2 C. Right, an example of cycling cells.  
(F) PDS1-MYC18 (yAC339) and cdc28-as1 PDS1-MYC18 (yAC779) cells were treated as in Fig. 2 [A–D] and collected for FACS analysis of DNA content. The data shown are from a single representative experiment out of three repeats.  
(G) cdc28-as1 MAD2-3GFP NUF2-mCherry (yAC2182) cells from the experiment in Fig. 2 E were taken at the indicated times for IF analysis of mitotic spindles (i.e., metaphase and anaphase spindles). The data shown are from a single representative experiment out of three repeats (n = 100).
Partial inhibition of Cdc28 does not destabilize Pds1 and Clb2 in the presence of Cdc20. (A) cdc28-as1 PDS1-MYC18 yox1 (yAC791) cells were synchronized in G1 with α-factor and then released in nocodazole. After 2.5 h in nocodazole, either DMSO (left) or 500 nM 1-NMPP1 (right) were added. The levels of Pds1, Clb2, and Cdc20 were monitored by Western blotting. FACS analysis of the DNA content, performed in triplicate, was used to confirm the arrest induced by nocodazole. (B and C) The efficacy of cdc28-as1 inhibitor was tested by releasing cdc28-as1 PDS1-MYC18 yox1 (yAC791) from G1-arrested cells into the cell cycle supplemented with either DMSO or 500 nM 1-NMPP1. DNA content by FACS analysis (B) and percentages of metaphase spindles by IF (C) were used to follow cell cycle progression. The data shown are from a single representative experiment out of three repeats (for C, $n = 100$). (D) GAL-SIC1 PDS1-MYC18 (yAC2025) and GAL-SIC1 PDS1-MYC18 cdh1Δ (yAC2023) cells were grown in glucose (left) or raffinose (right) at 23°C and arrested in G1 by α-factor for 3 h. Cells grown in glucose were then released in the same medium. Cells grown in raffinose were supplemented with galactose for an additional 1.5 h during the arrest and then released in galactose. FACS analysis of DNA content was used to follow cell cycle progression of the two strains. The data shown are from a single representative experiment out of three repeats.
Figure S3. **50 nM of 1-NMPP1 only mildly delays cell cycle progression.** (A and B) The experiment in Fig. 4 was repeated with GAL1-MAD2 (3X) PDS1-MYC18 (yAC489) cells. The data shown are from a single representative experiment out of three repeats (for B, n = 100). (C) GAL1-MAD2 (3X) cdc28-as1 PDS1-MYC18 (yAC1788) cells were synchronized in G1 with α-factor and released in glucose. 65 min after the release, when 90% cells were small budded, either DMSO or 50 nM 1-NMPP1 were added to the medium, together with α-factor. Samples were collected for IF of mitotic spindles (i.e., metaphase and anaphase spindles; left) and for FACS analysis of DNA content (right). The data shown are from a single representative experiment out of three repeats (for the data on the left, n = 100).
Figure S4. **Single cell analysis of Clb2 accumulation during a checkpoint-arrest.** (A, top) The degradation rate (kdeg) was obtained by fitting the phase of Clb2 degradation in the whole cell with an exponential decay. (A, bottom) The expression rate was obtained by fitting the region of fast increase of Clb2 accumulation in the nucleus to a straight line. For the fitting, we only used the section of the dynamics where the nucleus is well defined (solid line). The same analysis was performed in cycling cells. (B, top) The ratio of nuclear area and whole cellular area during adaptation. The nuclear area is well defined only when Clb2 accumulates into the nucleus (between the gray vertical lines; see Materials and methods and Video 1). (B, bottom) We identify the dynamics of Clb2 accumulation when the area is well defined (solid line) as opposed to where it is not (broken line). The same analysis was performed for adapting and cycling cells (n = 50; Fig. 5 A). (C, top) Typical trajectories for Clb2 in cells adapting to nocodazole. (C, bottom) Formation of tubulin clusters during nocodazole treatment. Dots mark the time of budding after adaptation. The dark, broken line shows a case where Clb2 degradation occurred after tubulin clusters appeared. The data shown in C are from a single representative experiment out of two repeats (n = 27).
Figure S5. Cdc16 phosphorylation induces a gel shift during a prolonged spindle checkpoint arrest, and Cdc14 is not required for adaptation. (A and B) GAL1-MAD2 (3X) cdc16-MYC6 (yAC1933) cells were synchronized in G1 with α-factor and released into galactose. (A) We followed the mobility shift of Cdc16 during a prolonged checkpoint-induced arrest by Western blotting using Phos-tag reagent. (B) The timing of adaptation was followed by IF. The data shown are from a single representative experiment out of three repeats (n = 100). (C) GAL1-MAD2 (3X) PDS1-MYC18 (yAC489) and GAL1-MAD2 (3X) PDS1-MYC18 cdc55 Δ (yAC1823) cells were grown in YEPR, synchronized in G1 with α-factor, and released in galactose. 70 min after the release, α-factor was re-added. Protein levels were assessed by Western blotting. (D) CDC16-MYC6 (yAC1936) and CDC16-MYC6 cdc55 Δ (yAC1994) cells were arrested in G1 with α-factor and released in YEPR containing nocodazole. Extracts of both strains were treated for Western blotting (left), and only CDC16-MYC6 cdc55 Δ extracts collected at 2.5 h were treated for phosphatase-assay (right). (E) GAL1-MAD2 (3X) PDS1-MYC18 cdc55 Δ (yAC1823), GAL1-MAD2 (3X) PDS1-MYC18 cdc27-5A cdc16-6A (yAC1675), and GAL1-MAD2 (3X) PDS1-MYC18 cdc27-5A cdc16-6A cdc55 Δ (yAC1952) cells were grown in YEPR, synchronized in G1, and released in the cell cycle. Samples were taken for IF. The data shown are from a single representative experiment out of three repeats (n = 100). (F and G) net1 Δ PDS1-MYC18 (yAC1999) and cdc55 Δ PDS1-MYC18 (yAC1896) cells were grown at 25°C in YEPR, and synchronized in G1 with α-factor. 80 min after the release into the cell cycle, when 90% of the cells were budded, α-factor was added again to the medium. Samples were collected for Western blotting and FACS, which were repeated in triplicate. (H) GAL1-MAD2 (3X) PDS1-MYC18 (yAC489), GAL1-MAD2 (3X) cdc16-6A cdc27-5A PDS1-MYC18 (yAC1675), GAL1-MAD2 (3X) cdc14-1 PDS1-MYC18 (yAC1768), and GAL1-MAD2 (3X) cdc15-2 PDS1-MYC18 (yAC1780) cells were grown in glucose-containing medium at 23°C. They were synchronized in G1 with α-factor and released into the cell cycle at 37°C. After 80 min, α-factor was added again. At the indicated time points, samples were collected for IF. The data shown are from a single representative experiment out of three repeats (n = 100).

Video 1. Clb2 dynamics in adapting cells. CLB2-GFP TUB2-Cherry GAL1-MAD2 (3X) (yAC1732) cells were grown in synthetic medium containing raffinose and then shifted to galactose. We show Clb2-GFP accumulation signal in the nucleus after smoothing. Time-lapse movies were acquired every 10 min using a DeltaVision Elite imaging system (Applied Precision) based on an inverted microscope (IX71; Olympus).
Table S1. Strain list

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<th>Relevant genotype</th>
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<td>MATa, 18MYC-PDS1::LEU2, leu2-3::LEU2::GAL1-MAD2 (3X), cdc27::CDC27-5A::KanMX6, cdc16::CDC16-6A::TRP1</td>
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<td>yAC1732</td>
<td>MATa, leu2-3::LEU2::GAL1-MAD2 (3X), CLB2-GFP::LEU2, TUB2-Cherry::URA3</td>
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<td>MATa, CDC27-MYC9::::TRP1</td>
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<td>MATa, CDC27-MYC9::::TRP1, cdc55::::KanMX6</td>
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<td>yAC1896</td>
<td>MATa, 18MYC-PDS1::LEU2, cdc55::::KanMX6</td>
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<td>yAC1933</td>
<td>MATa, 6MYC-CDC16::URA3, leu2-3::LEU2::GAL1-MAD2 (3X)</td>
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<td>yAC1936</td>
<td>MATa, 6MYC-CDC16::URA3</td>
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<td>MATa, 6MYC-CDC16::URA3, cdc55::::KanMX6</td>
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<td>yAC1999</td>
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<td>yAC2023</td>
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<td>MATa, cdc28-as1::::TRP1, MAD2-3GFP::::KanMX6, NUF2-mCherry::::KanMX6</td>
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All strains were in W303 background.