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Downregulation of PP2A^{Cdc55} Phosphatase by Separase Initiates Mitotic Exit in Budding Yeast

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SUMMARY

After anaphase, the high mitotic cyclin-dependent kinase (Cdk) activity is downregulated to promote exit from mitosis. To this end, in the budding yeast *S. cerevisiae*, the Cdk counteracting phosphatase Cdc14 is activated. In metaphase, Cdc14 is kept inactive in the nucleolus by its inhibitor Net1. During anaphase, Cdk- and Polo-dependent phosphorylation of Net1 is thought to release active Cdc14. How Net1 is phosphorylated specifically in anaphase, when mitotic kinase activity starts to decline, has remained unexplained. Here, we show that PP2A^{Cdc55} phosphatase keeps Net1 underphosphorylated in metaphase. The sister chromatid-separating protease separase, activated at anaphase onset, interacts with and downregulates PP2A^{Cdc55}, thereby facilitating Cdk-dependent Net1 phosphorylation. PP2A^{Cdc55} downregulation also promotes phosphorylation of Bfa1, contributing to activation of the "mitotic exit network" that sustains Cdc14 as Cdk activity declines. These findings allow us to present a new quantitative model for mitotic exit in budding yeast.

INTRODUCTION

Entry into mitosis in the eukaryotic cell cycle is promoted by the increasing activity of mitotic kinases, including Cdk and Polo. Cells in metaphase reach a peak in kinase activity. Provided that all chromosomes are correctly aligned on the mitotic spindle, this will lead to activation of the anaphase-promoting complex (APC), a multisubunit ubiquitin ligase. The APC ubiquitylates securin, an inhibitor of the protease separase, thereby targeting securin for destruction by the proteasome. Free separase in turn cleaves the cohesive link between sister chromatids to trigger anaphase. At the same time, the APC starts to ubiquitylate cyclins and later also Polo to downregulate mitotic kinases (Morgan, 1999; Zachariae and Nasmyth, 1999).

Activation of the APC at anaphase onset occurs through an activatory subunit, called Cdc20. In budding yeast, this form of the APC (APC^{Cdc20}) initiates cyclin destruction but is not by itself sufficient to complete mitotic exit. A second essential step during mitotic exit is activation of the Cdk counteracting phosphatase Cdc14. Cdc14 contributes to Cdk downregulation by dephosphorylating both a second APC activatory subunit, Cdh1, that sustains APC activity at low Cdk levels, as well as the Cdk inhibitor Sic1 (Visintin et al., 1998). Cdc14 also directly counteracts Cdk activity by reversing phosphorylation of Cdk substrates.

Cdc14 phosphatase is sequestered and kept inactive in the nucleolus for most of the cell cycle by binding to the nucleolar protein Net1 (also called Cfi1) (Shou et al., 1999; Visintin et al., 1999). Release from the nucleolus and activation of Cdc14 in anaphase occurs after phosphorylation of Net1, depending on both Cdk and Polo. Phosphorylated Net1 shows reduced affinity for and in vitro no longer inhibits Cdc14 (Azzam et al., 2004; Shou et al., 2002; Yoshida and Toh-e, 2002). A G protein signaling cascade that involves Polo, the mitotic exit network (MEN), is essential in budding yeast for Cdc14 activation (Jaspersen et al., 1998; Lee et al., 2001; Stegmeier and Amon, 2004).

At anaphase onset Cdc14 is initially activated independently of MEN, in a reaction that has been called Cdc*14* *e*arly *a*naphase *r*elease (FEAR; Stegmeier et al., 2002). A number of proteins, including Cdk, Polo, Slk19, Spo12, and separase have been implicated in this early release, and it requires Net1 phosphorylation at Cdk consensus sites (Azzam et al., 2004). It is not known how and which of these FEAR components conveys an anaphase-specific Cdc14 release signal. A good candidate is separase, which is released from its inhibitor securin at this time. Ectopic expression of separase in metaphase is indeed sufficient to trigger Cdc14 activation, in a reaction independent of its proteolytic activity (Sullivan and Uhlmann, 2003). The

physiological trigger for Cdc14 activation has remained unknown. FEAR is thought to be nonessential, and likewise, an essential signal that activates MEN in anaphase has so far not been identified.

Type 2A phosphatases (PP2A) are a group of abundant protein phosphatases involved in several essential aspects of cell growth and proliferation (Stark, 1996). These phosphatases consist of a scaffold protein (Tpd3 in budding yeast) that associates with a catalytic subunit (one of the closely related Pph21 or Pph22) and one of three regulatory subunits (Rts1, Rts3, or Cdc55). The latter is thought to provide substrate specificity. We refer to $PP2A^{Cdc55}$ as a complex consisting of Tpd3, either Pph21 or Pph22, and Cdc55. Previous studies have shown that Cdc55 regulates inhibitory Cdk tyrosine phosphorylation and is required for stable mitotic arrest in response to spindle damage. Recently, a role of Cdc55 in mitotic exit has been described (Minshull et al., 1996; Wang and Ng, 2006; Yellman and Burke, 2006).

Here we show that in metaphase, $PP2A^{Cdc55}$ prevents premature Net1 phosphorylation and Cdc14 activation. At anaphase onset, $PP2A^{Cdc55}$ activity is downregulated in a separase-dependent manner, triggering a first wave of Cdk-dependent Net1 phosphorylation and Cdc14 release. Utilizing a new conditional allele of separase we show that separase is the essential trigger for Cdc14 activation in anaphase. We present a quantitative model of how $PP2A^{Cdc55}$ downregulation, together with Cdc14 activation and cyclin proteolysis, promotes mitotic exit.

RESULTS

Separase Activation Is Essential for Mitotic Exit

Conditional mutants in budding yeast separase have been described that block chromosome segregation by largely reducing, but not eliminating, cohesin cleavage. Such mutants introduce a delay to the nucleolar release of Cdc14 (McGrew et al., 1992; Stegmeier et al., 2002; Uhlmann et al., 1999). This suggested that separase contributes to but is not essential for mitotic exit. We were prompted to revisit the possibility that separase plays a more decisive role in mitotic exit by three considerations: (1) The separase alleles so far studied likely present a hypomorphic and not the true null phenotype. (2) An undegradable version of the separase inhibitor securin, carrying a mutated destruction box motif ($securin^{mdb}$), not only prevents chromosome segregation but also blocks mitotic exit (Cohen-Fix and Koshland, 1999). (3) Ectopic separase expression in metaphase is sufficient for Cdc14 activation and triggers mitotic exit if cyclin levels are reduced (Sullivan and Uhlmann, 2003).

We first asked by which mechanism $securin^{mdb}$ blocks mitotic exit. Securin could inhibit Cdc14 activation or prevent mitotic exit in a different way. Cells were arrested in metaphase by depletion of the APC activator Cdc20 under control of the methionine-repressible *MET3* promoter, and $securin^{mdb}$ was induced from the galactose-inducible *GAL1* promoter. Then cells were released into synchronous anaphase by reinduction of Cdc20. Endogenous securin was rapidly degraded, and in control cells without $securin^{mdb}$ Cdc14 was released from the nucleolus at the same time (Figure 1A). The presence of $securin^{mdb}$ prevented both Cdc14 release and mitotic exit, suggesting that securin blocks mitotic progression by inhibiting Cdc14 activation.

We next wanted to know whether securin blocks Cdc14 release through inhibiting separase. If this was the case, inactivation of separase should also lead to a mitotic block. We generated a new conditional allele of separase (*esp1-2*^{td}) by fusing a degron cassette to the thermosensitive *esp1-2* allele, expressed from the native *ESP1* promoter. Then we compared cell-cycle progression in an allelic series of wild-type, *esp1-1*, *esp1-2*, and *esp1-2*^{td} cells, released from an α factor block in G1 (Figure 1B). As reported, Cdc14 nucleolar release and mitotic exit are delayed in *esp1-1* mutant cells, when compared to wild-type (Stegmeier et al., 2002). Cdc14 release was further reduced and delayed in the *esp1-2* mutant and almost entirely blocked in the *esp1-2*^{td} strain. After defective anaphase, *esp1-1* mutant cells undergo delayed cytokinesis to produce aneuploid daughter cells. Cytokinesis was further delayed by the *esp1-2* mutation, and *esp1-2*^{td} cells failed to undergo cytokinesis and remained arrested in mitosis. As a control that the arrest was not due to a failure of APC^{Cdc20} activation, we analyzed securin that was degraded in all strains with similar timing (Figure 1B). The mitotic arrest after $securin^{mdb}$ expression or separase inactivation could be overcome by ectopic Cdc14 activation using *net1-1* or the dominant-active Cdc14 allele *CDC14-TAB6* (Figure S1; Shou and Deshaies, 2002). This suggests that separase inactivation in budding yeast leads to a cell-cycle block in mitosis, caused by the failure to activate Cdc14.

Separase Promotes Net1 Phosphorylation

Cdc14 activation in early anaphase depends on Cdk-dependent Net1 phosphorylation (Azzam et al., 2004). We tested whether this phosphorylation is controlled by separase, using an antibody that recognizes a Cdk-dependent, anaphase-specific Net1 phospho-epitope. Cells were arrested in metaphase by depletion of Cdc20, and separase expression was induced from the *GAL1* promoter (Figure 2A). The Net1 phospho-epitope became detectable coincidentally with Cdc14 release. To analyze whether Net1 phosphorylation was responsible for separase-induced Cdc14 release, we repeated the experiment using the *net1-6Cdk* mutant that lacks six Cdk phosphorylation sites required for early Cdc14 release (Azzam et al., 2004). After separase induction, Cdc14 nucleolar release was strongly reduced by the *net1-6Cdk* mutation (Figure 2B). This suggests that separase induces Cdc14 activation by promoting Net1 phosphorylation.

It was important to know whether Net1 phosphorylation during normal anaphase also depends on separase. Therefore, wild-type and *esp1-2* mutant cells were synchronized in metaphase by Cdc20 depletion and released into

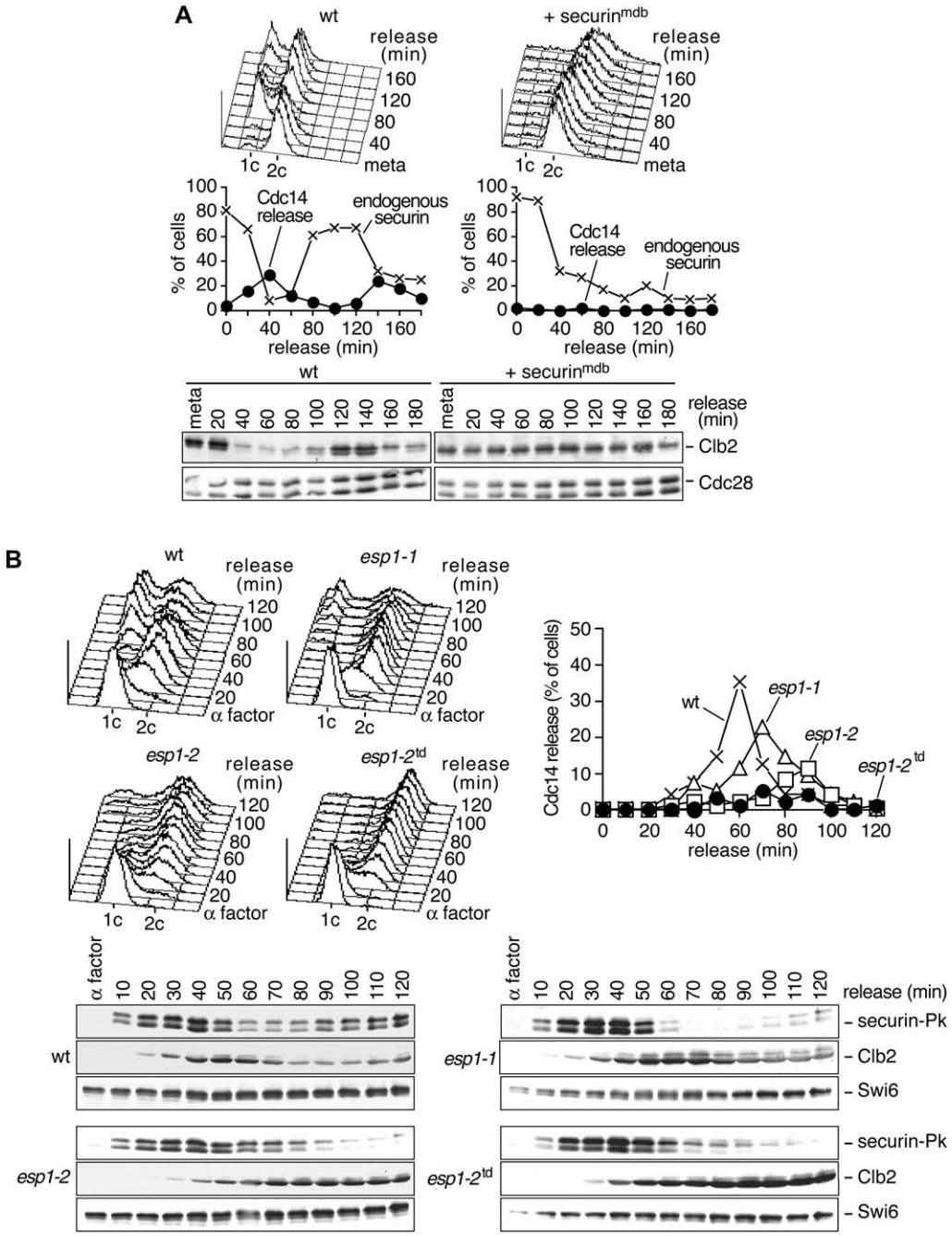

Figure 1. Separase Activation Is Essential for Cdc14 Nucleolar Release

(A) Securin blocks Cdc14 release. Strain Y1851 (*MAT*a *MET-CDC20 CDC14-HA6 PDS1-myc18*), with or without *GAL-PDS1*^{mdb} (encoding securin^{mdb}), was arrested in metaphase (meta) by Cdc20 depletion, and securin^{mdb} expression was induced. Cells were released into synchronous anaphase by Cdc20 readdition. Cdc14 nucleolar release and the presence of endogenous securin were visualized by indirect immunofluorescence. Clb2 levels were analyzed by Western blotting, using Cdc28 as loading control.

(B) The separase allele *esp1-2*^{td} blocks mitotic exit. Strains Y1504 (*MAT*a *CDC14-HA6 PDS1-Pk3 GAL-UBR1*), Y1825 (as Y1504, but *esp1-1*), Y1505 (as Y1504, but *esp1-2*), and Y1507 (as Y1504, but *esp1-2*^{td}) were arrested in G1 with α factor. Ubr1 was induced by galactose addition and cultures shifted to 37°C for 2 hr before release into a synchronous cell cycle. Swi6 served as a loading control for Clb2 and securin.

synchronous anaphase by Cdc20 readdition (Figure 2C). In wild-type cells, phospho-Net1 appeared concomitant with Cdc14 release. The phospho-epitope was not detectable after release of *esp1-2* cells. This indicates that separase activation at anaphase onset promotes Cdc14 release by facilitating Cdk-dependent Net1 phosphorylation.

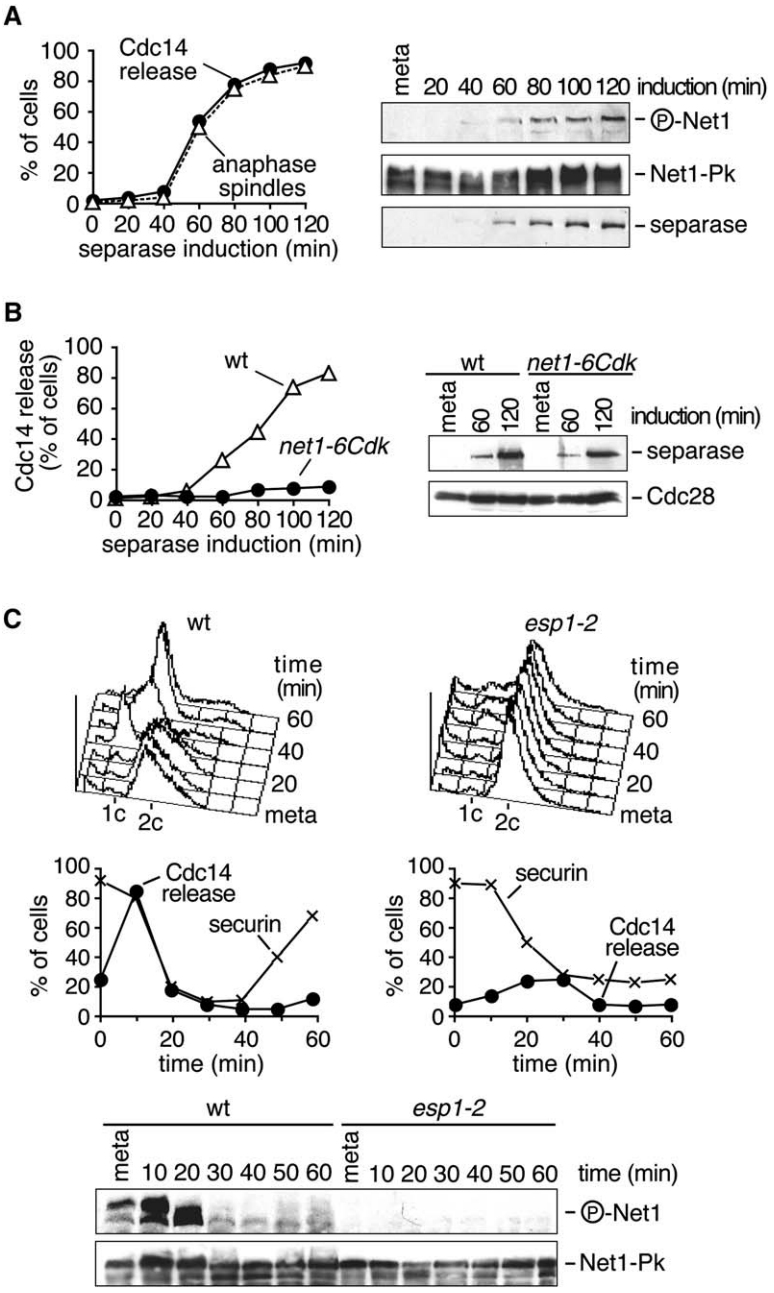

Figure 2. Separase Promotes Net1 Phosphorylation

(A) Ectopic separase induces a Cdk-dependent Net1 phospho-epitope. Strain Y2170 (*MAT*a *MET-CDC20 GAL-Flag-ESP1 CDC14-HA6 NET1-Pk3*) was arrested in metaphase (meta) and separase expression induced. Cdc14 nucleolar release and spindle elongation were monitored by immunofluorescence. Net1 phosphorylation was detected using the phospho-Net1-specific antibody PP-B (Azzam et al., 2004). Total Net1 was detected by the Pk epitope tag.

(B) Cdk phosphorylation sites are required for separase-induced Cdc14 release. Strains Y2807 (*MAT*a *GAL-Flag-ESP1 CDC14-Pk9*) and Y2808 (as Y2807, but *net1-6Cdk*) were arrested in metaphase by nocodazole treatment and separase expression induced. The rate of spindle elongation was similar in both strains (not shown).

(C) Separase-dependent Net1 phosphorylation in anaphase. Strains Y2639 (*MAT*a *MET-CDC20 CDC14-HA6 PDS1-myc18 NET1-Pk3*) and Y2640 (as Y2639, but *esp1-2*) were arrested in metaphase by Cdc20 depletion and shifted to the restrictive temperature (37°C) 3 hr before release into anaphase.

Cdk Is Responsible for Separase-Induced Cdc14 Activation

We next confirmed that Cdk itself, and not another kinase that might recognize the Cdk consensus sites on Net1, was responsible for separase-induced Cdc14 activation. Cells carrying the ATP analog (1NM-PP1)-sensitive Cdk allele *cdc28-as1* (Bishop et al., 2000) were arrested in metaphase by nocodazole treatment, then 1NM-PP1 was added and separase expression induced (Figure 3A). Cells containing wild-type Cdc28 released Cdc14 from the nucleolus as expected, but inhibition of Cdc28-as1 by 1NM-PP1 efficiently blocked Cdc14 release. This demonstrates that separase-induced Cdc14 release depends on Cdk activity.

Polo has also been implicated in Cdc14 activation at anaphase onset. We therefore repeated the experiment but inactivated Polo using the tight temperature-sensitive *cdc5-4* allele (Park et al., 2003; Figure 3B). Cdc14 release occurred with the same kinetics in *cdc5-4* and wild-type cells. This suggests that mainly Cdk, but not Polo, is responsible for separase-induced Net1 phosphorylation. These results are consistent with an essential role for Polo later in mitotic exit as part of MEN. After release from a metaphase block, *cdc5-4* cells only transiently

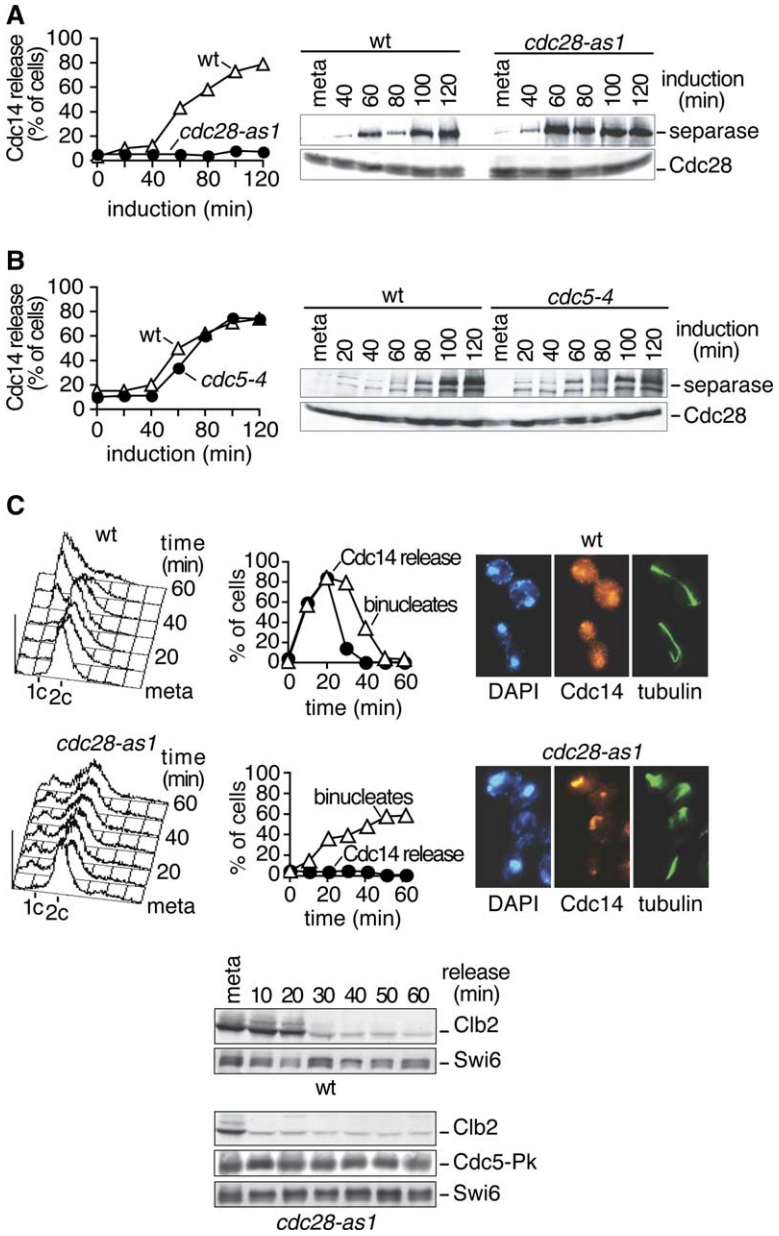

Figure 3. Cdk Confers Separase-Induced Net1 Phosphorylation

(A) Cdk activity is required for separase-induced Cdc14 release. Strains Y828 (*MAT*a *GAL-Flag-ESP1 CDC14-HA6)* and Y2178 (as Y828, but *cdc28-as1*) were arrested in metaphase by nocodazole treatment. One micromolar 1NM-PP1 was added to inhibit Cdc28-as1 and separase was induced 15 min later.

(B) Polo is not required for separase-induced Cdc14 release. Strains Y2286 (*MAT*a *GAL-Flag-ESP1 CDC14-myc18)* and Y2288 (as Y2286, but *cdc5-4*) were arrested in metaphase with nocodazole and shifted to 37°C for 60 min before separase induction.

(C) Cdk is required for Cdc14 activation at anaphase onset. Strains Y2298 (*MAT*a *GAL-CDC20 CDC14-HA6*) and Y2428 (as Y2298, but *cdc28-as1 CDC5-Pk3*) were arrested in metaphase by depletion of Cdc20; 1 μM 1NM-PP1 was added 15 min before Cdc20 readdition. Photographs are shown of cells 20 min after release.

released Cdc14, reminiscent of MEN mutants, and were unable to complete mitotic exit (Figure S2).

To explicitly test the role of Cdk-mediated Net1 phosphorylation during mitotic exit, we analyzed whether cells can exit from mitosis without Cdk activity. Cells were synchronized in metaphase by Cdc20 depletion, then Cdc28-as1 was inhibited with 1NM-PP1, and Cdc20 reinduced (Figure 3C). Control cells containing wild-type Cdc28 underwent anaphase, activated Cdc14, and completed mitotic exit. In contrast, *cdc28-as1* cells kept Cdc14 sequestered in the nucleolus and did not exit. This was in spite of the fact that Clb2 was degraded and chromosome segregation occurred in both cultures, indicating that APC^{Cdc20} was active. Binucleate formation was less efficient in *cdc28-as1* cells, most likely due to spindle malfunction. APC^{Cdh1} remained inactive, as levels of its substrate Polo remained high. This suggests that Cdk downregulation is not sufficient for mitotic exit if Cdk has not first fulfilled its role in the activation of Cdc14.

Cdk Activity against Net1 Is Downregulated at the Time of Cdc14 Release

The above results suggest that separase induces Cdk-dependent Net1 phosphorylation in anaphase. This presents a paradox, as at this time overall Cdk activity starts to decline. One possible explanation could be a separase-dependent specificity change of Cdk. Cyclin Clb2-associated Cdk activity promotes Net1 phosphorylation during

anaphase (Azzam et al., 2004), therefore we measured its in vitro activity against Net1 after purification from synchronous cells. The substrate was a recombinant fragment of Net1, spanning amino acids 1–600, that in vivo is sufficient for cell cycle-regulated Cdc14 inhibition (Azzam et al., 2004; Traverso et al., 2001). As control substrate we used histone H1, against which Cdk activity is expected to decline during anaphase. Clb2-associated Cdk activity against both Net1 (1–600) and H1 started to decline as soon as cells entered anaphase and Cdc14 was released (Figure S3). While this in vitro assay may not fully recapitulate possible regulation of Cdk specificity in vivo, it did not reveal evidence for increased Net1 phosphorylation in anaphase.

PP2A^{Cdc55} Prevents Cdc14 Release in Metaphase

Instead, we considered whether a phosphatase might limit Net1 phosphorylation in metaphase, as has been suggested based on theoretical considerations (Chen et al., 2004). Defects in mitotic progression have been reported due to mutations in the budding yeast type 2A phosphatase (Evans and Stark, 1997; Minshull et al., 1996), therefore we analyzed its possible influence on Net1 phosphorylation. We individually deleted each of the three PP2A regulatory subunits, Rts1, Rts3, and Cdc55, and analyzed cell-cycle progression after synchronous release from G1 (Figure S4). *rts3Δ* cells behaved identical to the wild-type control, while both *rts1Δ* and *cdc55Δ* cells showed a noticeable delay in progression through mitosis. Release of Cdc14 from the nucleolus coincided with anaphase in *rts1Δ* cells but was delayed by 10 min in *cdc55Δ* cells.

To further analyze the roles of Rts1 and Cdc55, we synchronized cells at the metaphase to anaphase transition. *cdc55Δ* cells cannot be stably arrested in metaphase by nocodazole treatment (Minshull et al., 1996), but Cdc20 depletion allowed a robust block. After release into anaphase by Cdc20 readdition, the kinetics of Cdc14 nucleolar release was indistinguishable between *rts1Δ* and wild-type cells (Figure S4). In contrast, Cdc14 was prematurely released from the nucleolus of *cdc55Δ* cells arrested in metaphase (Figure 4A). Furthermore, resequestration of Cdc14 after release into anaphase occurred with a delay of at least 20 min. Premature Cdc14 release in *cdc55Δ* cells was accompanied by the appearance of the Cdk-specific Net1 phospho-epitope. As a consequence of hyperphosphorylation, the interaction between Net1 and Cdc14 was weakened, and less Cdc14 coprecipitated with Net1 from *cdc55Δ* metaphase cells compared to wild-type (Figure 4B). These findings suggest that PP2A^{Cdc55} prevents Cdc14 release in metaphase by counteracting Net1 phosphorylation. These results are consistent with premature nucleolar release of Cdc14 in nocodazole-treated *cdc55Δ* cells, as recently described (Wang and Ng, 2006; Yellman and Burke, 2006).

If PP2A^{Cdc55} counteracts Net1 phosphorylation in metaphase, why was Cdc14 release delayed in *cdc55Δ* cells released after synchronization in G1? *cdc55Δ* cells enter mitosis with compromised Cdk activity because of inhibitory Cdc28-Y19 phosphorylation (Minshull et al., 1996). To correct for this difference we introduced the *CDC28*^{Y19F} allele that is refractory to inhibition. Compared to both *cdc55Δ* and *CDC28*^{Y19F} single mutants, the *cdc55Δ CDC28*^{Y19F} strain showed significantly advanced Cdc14 release from the nucleolus. Cdc14 was released in many cells that judged by their short mitotic spindle were still in metaphase (Figure 4C), confirming that Cdc55 counteracts Cdc14 release.

PP2A regulatory subunits are not always acting as substrate-specific activators. To analyze whether indeed PP2A phosphatase activity opposes Net1 phosphorylation, we used a strain carrying a deletion of *PPH21* and a temperature-sensitive *pph22-172* allele to inactivate the two PP2A catalytic subunits. Pph3 phosphatase can partly compensate for PP2A function in budding yeast and was therefore also deleted from this strain (Evans and Stark, 1997) (Figure 4D). Net1 phosphorylation was detectable in *pph3Δpph21Δpph22-172* cells, arrested in metaphase by nocodazole treatment, already at permissive conditions. Phosphorylation increased after shift to the restrictive temperature, and Cdc14 was released from the nucleolus. Sic1 started to accumulate, confirming that Cdc14 was activated. This suggests that PP2A phosphatase activity is required to keep Net1 underphosphorylated in metaphase.

Separase-Dependent Downregulation of PP2A^{Cdc55} at Anaphase Onset

If PP2A^{Cdc55} counteracts Net1 phosphorylation in metaphase, does its activity change during anaphase to allow Net1 phosphorylation? We first visualized Cdc55 localization at the metaphase to anaphase transition. Cdc55 shows abundant nuclear localization throughout the cell cycle (Gentry and Hallberg, 2002 and data not shown). To detect nucleolar localization, we immunostained spread chromosomes from which nucleoplasmic protein has been washed away (Figure 5A). Colocalization with Net1 revealed nucleolar enrichment of Cdc55 in metaphase. Cdc55 was still enriched in the nucleolus in cells released into synchronous anaphase. Thus, while Cdc55 is at the right place to keep Net1 underphosphorylated in metaphase, no localization change is obvious during anaphase.

We then measured the in vitro phosphatase activity of PP2A^{Cdc55} after immunopurification from synchronous cultures. As substrates, we prepared in vitro ^{32}P-phosphorylated histone H1 and Net1 (1–600). Cdc55 immunoprecipitates from cultures undergoing synchronous metaphase to anaphase transition were incubated with these substrates, and release of acid soluble ^{32}P-phosphate was quantified by scintillation counting (Figure 5B). After the reaction, the yield of Cdc55 in the immunoprecipitates was quantified by Western blotting. In metaphase, significant Cdc55-associated phosphatase activity could be measured using both substrates. During anaphase, protein levels of Cdc55 remained unchanged but Cdc55

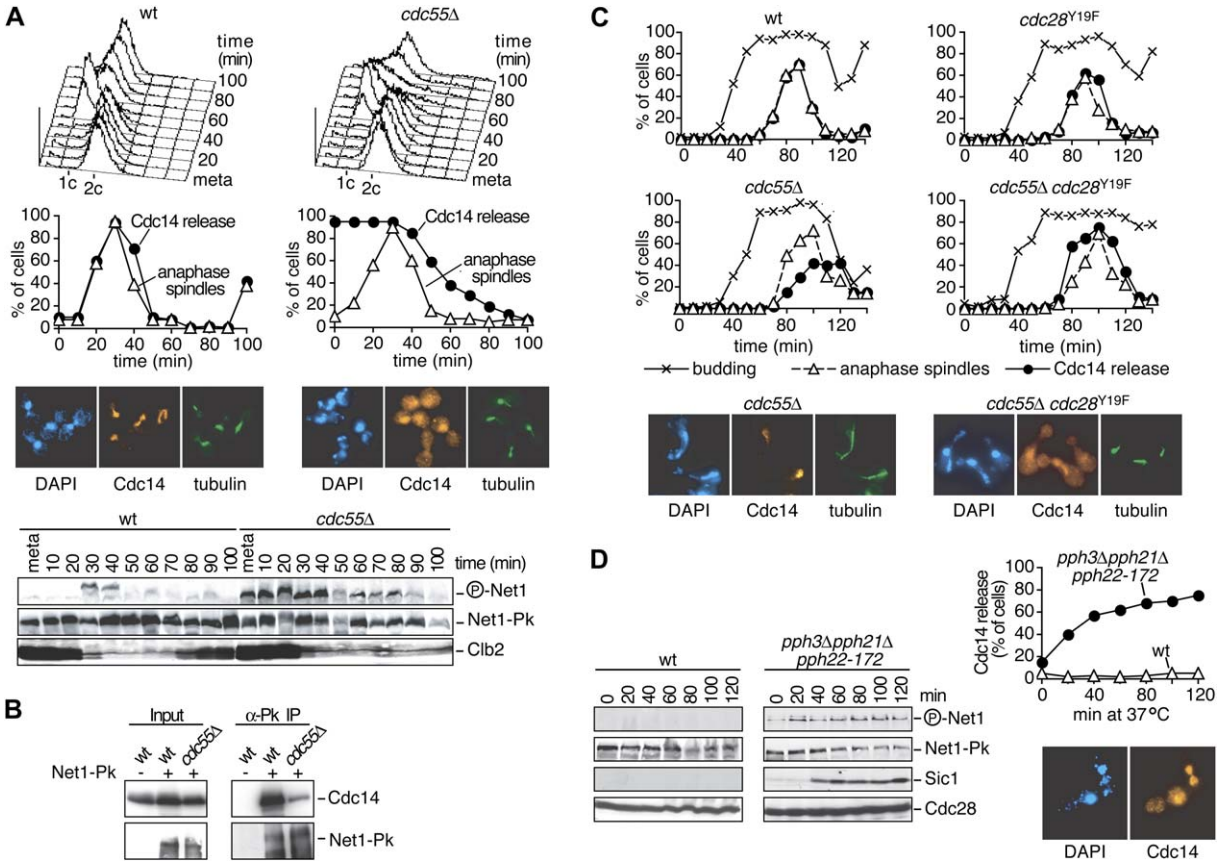

Figure 4. PP2A^{Cdc55} Prevents Net1 Phosphorylation in Metaphase

(A) Cdc55 deletion causes premature Net1 phosphorylation and Cdc14 nucleolar release in metaphase. Strains Y2118 (*MAT*a *MET-CDC20 NET1-Pk3 CDC14-HA6)* and Y2389 (as Y2118, but *cdc55Δ*) were arrested in metaphase and released into synchronous anaphase. Photographs are shown of cells in the metaphase arrest.

(B) Reduced interaction between Net1 and Cdc14 in the absence of Cdc55. Cell extracts were prepared from strains Y2299 (*MAT*a *MET-CDC20 CDC14-HA6*), Y2118 (as Y2299, but also *NET1-Pk3)*, and Y2389 (as 2118, but *cdc55Δ*), and coimmunoprecipitation of Cdc14 with Net1 was analyzed.

(C) Premature Cdc14 nucleolar release in *cdc55Δ CDC28*^{Y19F} cells. Strains Y808 (*MAT*a *CDC14-HA6)*, Y2411 (as Y808, but *CDC28*^{Y19F}), Y2426 (as Y808, but *cdc55Δ*), and Y2442 (as Y2426, but *CDC28*^{Y19F}) were arrested with α factor and released into a synchronous cell cycle. Cells shown are from the 80 min time point.

(D) Net1 phosphorylation and Cdc14 nucleolar release after inactivation of the PP2A catalytic subunit. Strains Y2498 (*MAT*a *NET1-Pk3 CDC14-myc18*) and Y2384 (as Y2498, but *pph3Δ pph21Δ pph22-172*) were arrested in metaphase by nocodazole treatment and shifted to 37°C. The photographs show cells 80 min after the temperature shift.

phosphatase-specific activity decreased to less than half of the value in metaphase. This suggests that PP2A^{Cdc55} activity is downregulated at anaphase onset.

To determine whether the decrease of Cdc55-associated phosphatase activity in anaphase depends on separase, we repeated the phosphatase assay using an *esp1-2* mutant strain. Cdc55 levels are reduced in *esp1* mutant strains (Figure S5). Phosphatase activity could nevertheless be readily measured after Cdc55 immunopurification from metaphase-arrested cells (Figure 5C). Strikingly, the phosphatase specific activity remained constant after release into anaphase. This suggests that separase is required to downregulate PP2A^{Cdc55} activity at anaphase onset.

To address how separase regulates PP2A^{Cdc55}, we asked whether separase and Cdc55 physically interact. Coimmunoprecipitation experiments showed that separase forms a complex with Cdc55 (Figure 5D). An interaction could be detected in metaphase cells that became stronger during anaphase when securin was degraded and Cdc14 released. Interaction studies using overexpressed separase and securin confirmed that separase interacts with Cdc55 both in the presence and absence of securin (data not shown). This opens the possibility that direct association of separase and Cdc55 is involved in regulating PP2A^{Cdc55} activity. The degradation of securin at anaphase onset might change the quality of this interaction, thereby initiating downregulation of PP2A^{Cdc55}.

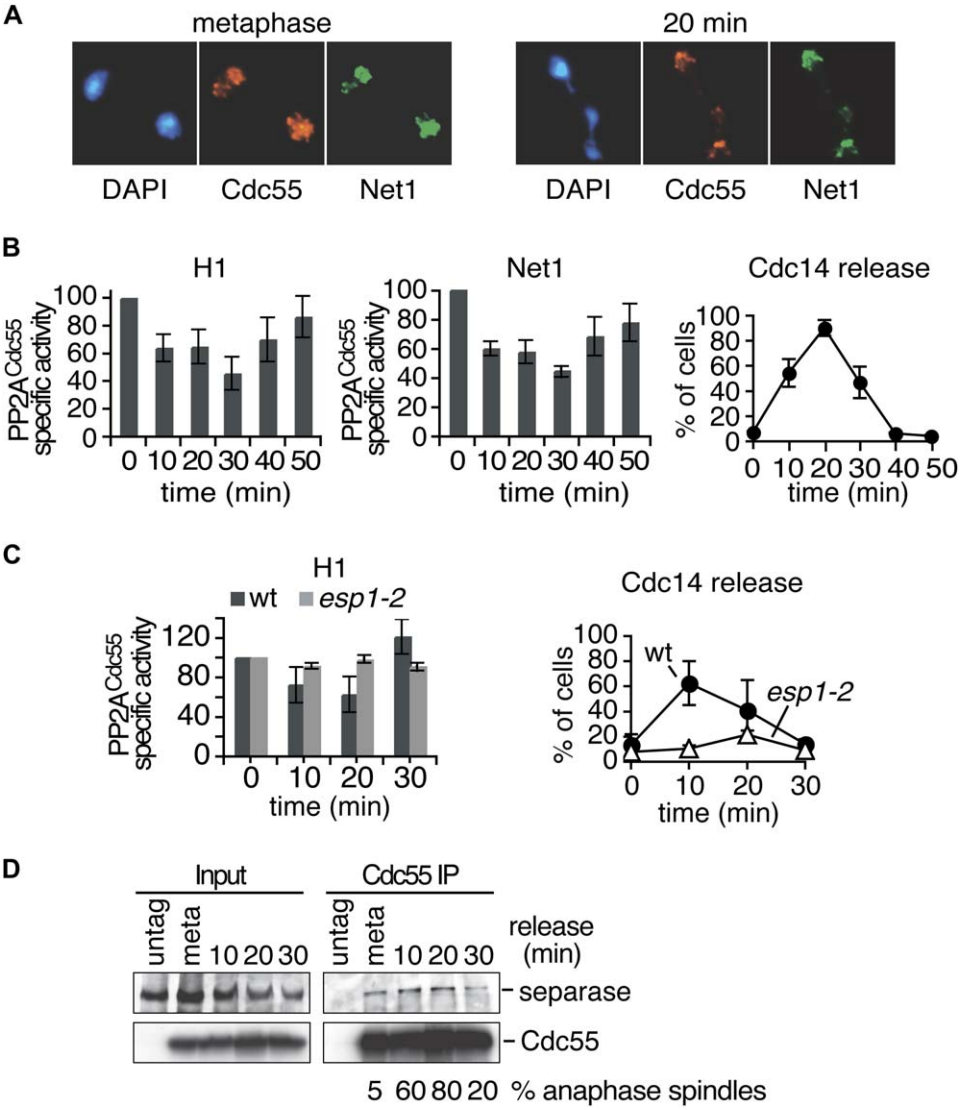

Figure 5. Separase-Dependent PP2A^{Cdc55} Downregulation at Anaphase Onset

(A) Cdc55 localization in the nucleolus. Strain Y2676 (*MAT*a *GAL-CDC20 HA3-CDC55 NET1-GFP*) was arrested in metaphase by the depletion of Cdc20 and released into synchronous anaphase. Chromosome spreading was performed in metaphase, and 20 min after release, when 85% of cells were in anaphase as seen by tubulin staining.

(B) PP2A^{Cdc55} downregulation at anaphase onset. Strain Y2521 (*MAT*a *GAL-CDC20 Pk3-CDC55 CDC14-HA6*) was synchronized at the metaphase to anaphase transition. Phosphatase activity of immunopurified Cdc55 was measured as described in Experimental Procedures. Mean and standard deviations of the phosphatase-specific activity relative to metaphase in three experiments are given.

(C) Separase-dependent downregulation of PP2A^{Cdc55} phosphatase activity. As (B), but strains Y2521 and Y2626 (as Y2521, but *esp1-2*) were synchronized at the metaphase to anaphase transition at 37°C.

(D) Separase and Cdc55 interact. Cell extracts were prepared from strains Y2063 (*MAT*a *GAL-CDC20 ESP1-HA6*) and Y2499 (as Y2063, but also *Pk3-CDC55*) synchronized at the metaphase to anaphase transition, and coimmunoprecipitation of separase with Cdc55 was analyzed. Anaphase progression was monitored by tubulin staining.

PP2A^{Cdc55} and the Mitotic Exit Network

It has recently been suggested that Cdc55 influences mitotic exit as part of MEN (Wang and Ng, 2006). In contrast, our results suggest that downregulation of PP2A^{Cdc55} allows Cdk-dependent Net1 phosphorylation in metaphase before MEN becomes active. Furthermore, Cdc14 activation by ectopic separase expression is independent of the MEN kinase Cdc15 (Sullivan and Uhlmann, 2003). To clarify whether MEN is required for Cdc14 nucleolar release in *cdc55Δ* cells, we synchronized *cdc55Δ* as well as *cdc55Δ cdc15-2* double mutant cells in G1 and followed them into a metaphase block at the restrictive temperature for *cdc15-2* (Figure 6A). Cdc14 was released from the nucleolus with similar timing in both strains. This indicates that

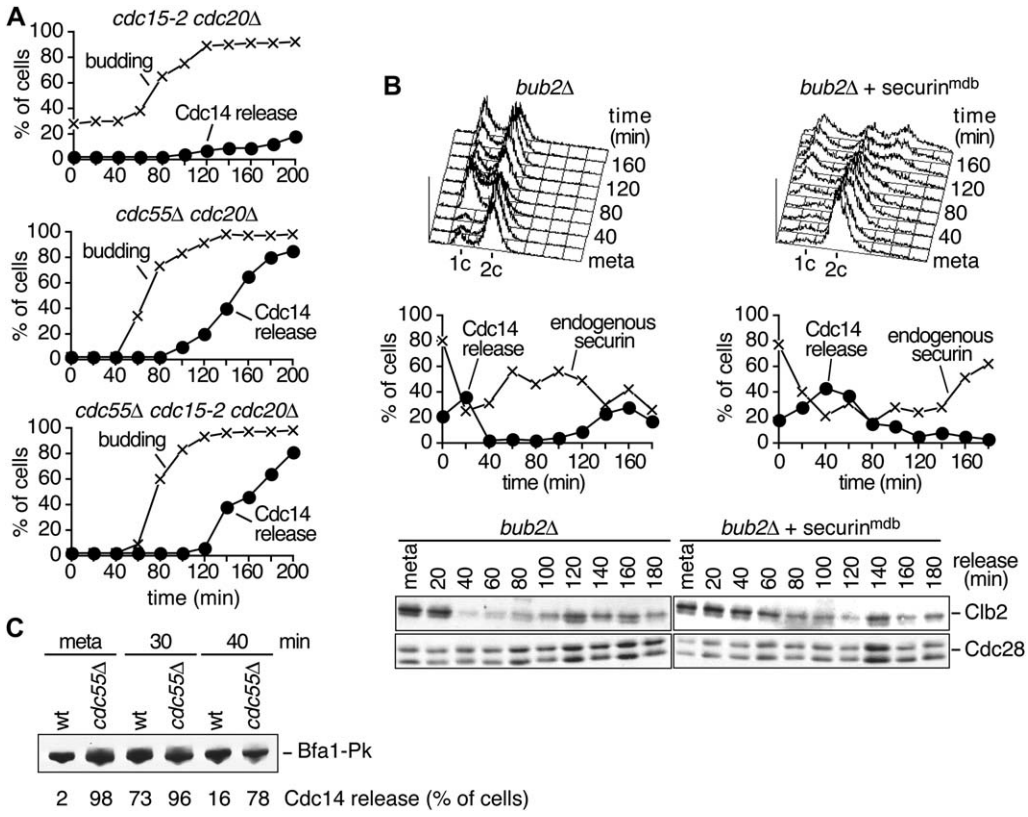

Figure 6. PP2A^{Cdc55} and the Mitotic Exit Network

(A) Cdc14 activation in metaphase by *cdc55Δ* is independent of MEN. Strains Y2687 (*MAT*a *MET-CDC20 CDC14-HA6 cdc15-2*), Y2389 (*MAT*a *MET-CDC20 CDC14-HA6 cdc55Δ*), and Y2688 (as Y2687, but *cdc55Δ*) were released from α factor block into a synchronous cell cycle at 37°C and arrested in metaphase by depletion of Cdc20.

(B) Securin cannot block mitotic exit in the absence of Bub2. As Figure 1A, but using strain Y1852 (as Y1851, but *bub2Δ*).

(C) Cdc55 counteracts Bfa1 phosphorylation. Strains Y2638 (*MAT*a *MET-CDC20 BFA1-Pk6 CDC14-HA6*) and Y2605 (as Y2638, but *cdc55Δ*) were synchronized at the metaphase to anaphase transition. Bfa1 electrophoretic mobility and Cdc14 release were analyzed in metaphase (meta), and 30 and 40 min after release.

Cdc15, and therefore MEN, is not required for Cdc14 nucleolar release in response to Cdc55 inactivation. We confirmed that MEN was inactive in this experiment using the *cdc15-2* control strain that remained arrested in telophase upon release from the metaphase block (data not shown).

We next wanted to know whether MEN can activate Cdc14 independently of separase. We ectopically activated MEN by deletion of Bub2, an inhibitor of the G protein Tem1 at the top of the MEN cascade. In metaphase arrest by Cdc20 depletion, about 20% of *bub2Δ* cells showed nucleolar release of Cdc14, compared to less than 5% in wild-type cells under the same conditions (Figure 6B, compare Figure 1A). This suggests that MEN can activate Cdc14 in metaphase, but not very efficiently. We then expressed securin^{mdb} before adding back Cdc20, to keep separase inhibited while activating the APC. Clb2 started to be degraded, Cdc14 was released, and cells exited from mitosis, albeit with a delay compared to cells without securin^{mdb}. Cdc14 release upon APC^{Cdc20} activation suggests that MEN is inhibited by high Cdk activity and becomes active as mitotic cyclins start to be degraded. Mitotic exit in the presence of securin^{mdb} furthermore suggests that Cdc14 activation by separase is no longer needed if MEN is activated by deletion of Bub2.

In wild-type cells containing Bub2, however, separase is indispensable for mitotic exit. This suggests that MEN activation depends on separase. It has been proposed that spindle elongation, as a consequence of separase-triggered cohesin cleavage, activates MEN by bringing spindle pole-associated Tem1 into contact with its activator Lte1 in the daughter cell (Bardin et al., 2000). However, spindle elongation by TEV-protease-mediated chromosome segregation does not cause detectable Cdc14 nucleolar release (Sullivan and Uhlmann, 2003). Phosphorylation of Bfa1, that together with Bub2 inhibits Tem1, also contributes to MEN activation (Hu et al., 2001; Pereira et al., 2002). Bfa1 phosphorylation depends on Polo, and, as with Net1, phosphorylation occurs in an anaphase-specific manner. As we could not find evidence for upregulation of Polo activity during anaphase (data not shown),

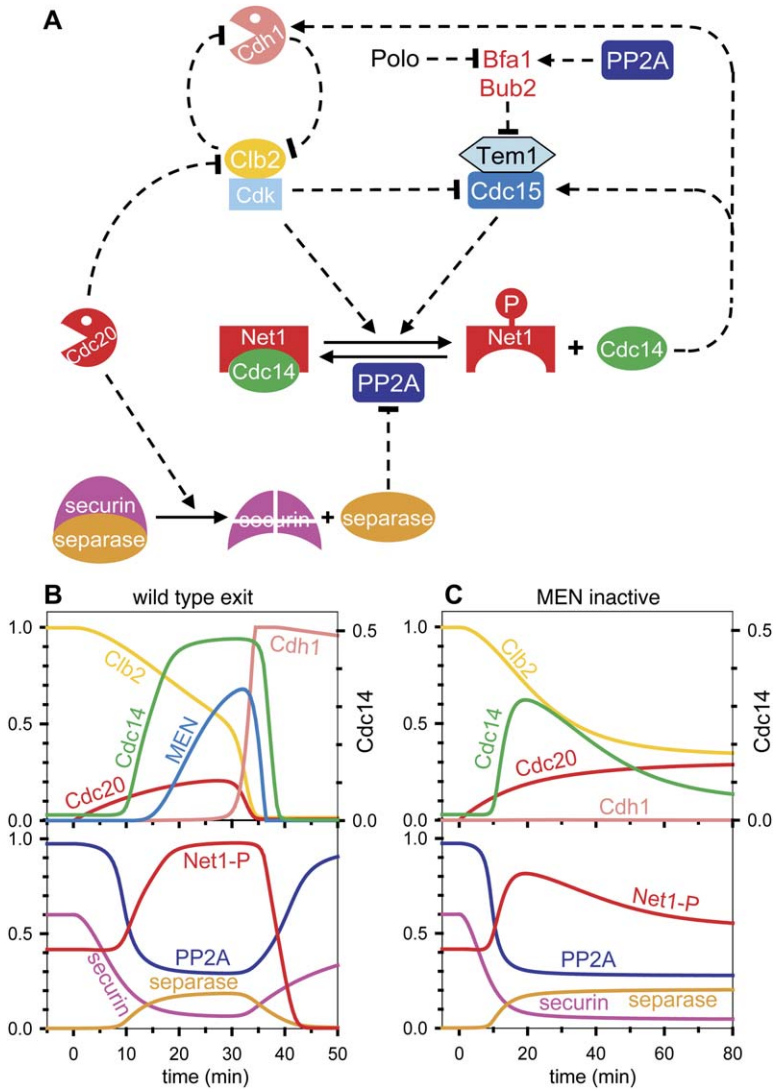

Figure 7. A Model for Mitotic Exit

(A) Wiring diagram showing biochemical reactions that control cell-cycle progression from metaphase into G1 in budding yeast. Cell-cycle regulators that do not make significant contribution at this stage of the cycle are not included. For simplicity, only one G1 regulator (Cdh1) is included, as its formal description is simpler than that of the Cdk inhibitor Sic1 that acts in parallel with Cdh1.

(B) Numerical simulation of mitotic exit in wild-type cells. The simulation starts in metaphase (Cdc20 deprivation), and Cdc20 synthesis is induced at time = 0.

(C) Simulation of mitotic progression of cells containing a MEN mutation. Cdc14 is transiently activated but is resequestered as Cdk activity declines (Stegmeier et al., 2002).

we tested whether downregulation of $PP2A^{Cdc55}$ could facilitate anaphase-specific phosphorylation of Bfa1. In *cdc55Δ* cells, Bfa1 showed an anaphase-like phosphorylation pattern already in metaphase (Figure 6C), consistent with its regulation by $PP2A^{Cdc55}$. Therefore, separase-dependent $PP2A^{Cdc55}$ downregulation at anaphase onset might contribute to MEN activation by facilitating Bfa1 phosphorylation.

A Quantitative Model for Mitotic Exit

At anaphase onset, separase is activated to trigger sister chromatid separation. At the same time the phosphatase Cdc14 is released from inhibition in the nucleolus. We now suggest a mechanism by which these two events are linked. Separase-dependent downregulation of $PP2A^{Cdc55}$ activity facilitates Cdk-dependent Net1 phosphorylation. This finding, together with results of previous studies, allows us to outline a quantitative model for mitotic exit in budding yeast (Figure 7A).

At the heart of the model lies reversible phosphorylation/dephosphorylation of Net1, catalyzed by Cdk and MEN kinases, and $PP2A^{Cdc55}$ and Cdc14 phosphatases (in addition to $PP2A^{Cdc55}$, Cdc14 itself contributes to Net1 dephosphorylation; Jaspersen and Morgan, 2000; Shou et al., 1999). Once active, Cdc14 dephosphorylates Cdc15, contributing to MEN activation. In a positive feedback loop, MEN causes further Net1 phosphorylation and Cdc14 release (Stegmeier et al., 2002). To avoid premature activation, this positive feedback loop is tightly regulated by Cdk and $PP2A^{Cdc55}$. While $PP2A^{Cdc55}$ inhibits Cdc14 release, Cdk has both activatory (Net1 phosphorylation) and inhibitory (Cdc15 phosphorylation) roles in Cdc14 release. The ratio of Cdc14 versus Cdk activity is read out by both MEN and the bistable Cdk-Cdh1 switch (Novak et al., 1998). The molecular network underlying this model is shown in Figure 7A (and Figure S6) and was converted into differential equations (Figure S6). Kinetic parameters were estimated by fitting numerical simulations

to experimental data. Bifurcation analysis shows that the model has three stable states corresponding to metaphase (high Cdk, low Cdc14), telophase (high Cdk and high Cdc14, Cdh1 inactive), and G1 (low Cdk and low Cdc14, Cdh1 active) for a wide range of parameters. The model accurately describes all our experimental data, as well as key observations in the published literature (Figures 7B and 7C and Figure S6).

Preparation for mitotic exit already starts in metaphase by Cdk-dependent phosphorylation of Net1. However, this is counteracted by $PP2A^{Cdc55}$. Absence of Cdc55 leads to premature nucleolar release and activation of Cdc14, yet exit from mitosis is blocked as long as APC^{Cdc20} is kept inactive. During a normal cell cycle Cdc14 is activated when APC^{Cdc20} promotes degradation of securin, releasing separase to downregulate $PP2A^{Cdc55}$ (Figure 7B).

Further progression of mitotic exit requires APC^{Cdc20}-dependent cyclin destruction. If cyclin is nondegradable, cells arrest in telophase, in a cell-cycle state similar to Cdc20-deprived *cdc55Δ* cells. Cdc14 is active but persisting Cdk activity blocks mitotic exit. If APC^{Cdc20} promotes degradation of Clb2, but MEN is inactive, Cdk activity is reduced to roughly half of the metaphase value (Yeong et al., 2000). In these cells, Cdc14 release becomes transient (Stegmeier et al., 2002), proportional to declining Cdk activity that is no longer sufficient to sustain Net1 phosphorylation (Figure 7C). Thereby, the ratio of Cdc14 to Cdk remains roughly constant. Cells reach a stable state, similar to the telophase state but with halfway lowered Clb2 levels, little Cdc14 released, and inactive Cdh1.

To complete mitotic exit, Cdc14 must remain active while Cdk activity is downregulated. This requires Cdk-independent Net1 phosphorylation, provided by MEN (Figure 7B). The mechanism of MEN activation, and whether MEN kinases directly phosphorylate Net1, is not fully understood. Cdc14 activates MEN at the level of Cdc15. We suggest that Bfa1 phosphorylation after $PP2A^{Cdc55}$ downregulation provides additional anaphase-specific activation of MEN. Once MEN is engaged, cells can lower Clb2 levels while maintaining Cdc14 activity, reaching the critical Cdc14/Cdk ratio for Cdh1 activation. This completely turns off Cdk activity, arriving at a stable G1 state that no longer requires Cdc14. Persisting Cdc14 activity would indeed compromise entry into the next cell cycle, so MEN and Cdc14 themselves must be switched off. This might be achieved by APC^{Cdh1}-dependent degradation of the MEN component Polo.

DISCUSSION

Cdc14 Early Anaphase Release

Separase-dependent nucleolar release of Cdc14 has first been described in context of the FEAR network to explain Cdc14 release in early anaphase (Stegmeier et al., 2002). The significance of separase in Cdc14 activation has been underestimated. This stems largely from the fact that the mutant alleles of separase so far employed did not reflect a complete loss of function. We now show that if separase activity is further reduced, an essential role in Cdc14 activation and mitotic exit is uncovered. Separase is required to reduce $PP2A^{Cdc55}$ phosphatase activity against Net1, thereby facilitating Cdk-dependent Net1 phosphorylation at anaphase onset. $PP2A^{Cdc55}$ downregulation may furthermore contribute to MEN activation. The mechanistic basis for separase-dependent $PP2A^{Cdc55}$ downregulation remains to be elucidated; it could involve a direct interaction between the two proteins. It also remains to be investigated whether Net1 is a direct target for $PP2A^{Cdc55}$. Its localization in the nucleolus is certainly consistent with this possibility.

Slk19 and Spo12 are two proteins that contribute to separase-induced Cdc14 activation in early anaphase and have been described as part of the FEAR network. Unlike separase, neither is essential. Slk19 influences separase localization during anaphase and could thus, in an indirect manner, alter separase's ability to promote $PP2A^{Cdc55}$ downregulation (Sullivan et al., 2001). Spo12 is present in the nucleolus where it interacts with the rDNA replication fork barrier protein Fob1 (Stegmeier et al., 2004). Fob1 binding overlaps with Net1 on the rDNA, so Spo12 or Fob1 could affect accessibility of Net1 to kinases or phosphatases.

Mutation of six Net1 Cdk phosphorylation sites also leads to a delay, but not block, of Cdc14 activation (Azzam et al., 2004). This indicates that Cdk-dependent Cdc14 release may be dispensable if MEN provides Cdk-independent Net1 phosphorylation. On the other hand, we found that Cdk activity is essential for exit from mitosis. This could be because the *net1-6Cdk* mutation did not abolish all Net1 Cdk phosphorylation and at least a low level of Cdk-dependent Cdc14 activation is necessary. Alternatively, Cdk could play an additional role in MEN activation, e.g., by maintaining the activity of Polo (Mortensen et al., 2005). The essential role of separase in mitotic exit could likewise have two reasons. Separase-induced, Cdk-dependent Cdc14 release could be essential to initiate the positive feedback loop of MEN activation. Alternatively, Cdk-dependent Net1 phosphorylation and early Cdc14 release is entirely dispensable, but in its absence separase-dependent MEN activation via Bfa1 phosphorylation may become essential. A possible contribution of separase-dependent spindle elongation has yet to be rigorously assessed. A separase mutant devoid of protease activity, which fails to promote cohesin cleavage and spindle elongation, is still proficient in promoting mitotic exit in the separase degron mutant background (data not shown).

Polo is an essential MEN component (Jaspersen et al., 1998; Lee et al., 2001) and has also been implicated in separase-induced early Cdc14 activation (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003). The contribution to early Cdc14 release was seen after Polo inactivation early in the cell cycle. When we inactivated Polo specifically at the time of anaphase onset we could no longer detect a contribution to separase-induced Cdc14 activation.

As Polo is thought to promote the build-up of Cdk activity during mitotic entry, it might be that early Polo inactivation leads to compromised early Cdc14 release as a consequence of attenuated Cdk activation.

Separase and Mitotic Exit in Higher Eukaryotes

While Cdc14 is tightly regulated and essential for mitotic exit in budding yeast, this is not true for its orthologs in fission yeast and *C. elegans* (Cueille et al., 2001; Saito et al., 2004). In principle, mitotic exit can be brought about by Cdk downregulation against a constant background of unregulated phosphatase(s), and we cannot exclude that this is the case in most organisms. An important difference in budding yeast concerns the regulation of mitotic entry, in particular in response to DNA damage. DNA damage activates checkpoint kinases that in most organisms prevent entry into mitosis by stabilizing inhibitory Cdk tyrosine phosphorylation. Cells arrest in G2, well before entry into mitosis. Budding yeast, in contrast, enter mitosis after DNA damage and arrest with active Cdk and active APC^{Cdc20}. Mitotic arrest depends on checkpoint kinase-dependent phosphorylation of securin, preventing its destruction by APC^{Cdc20} (Wang et al., 2001). Thus, securin maintains a stable mitotic state, and keeping Cdk counteracting phosphatase activity low is crucial for this arrest to be robust. While metaphase arrest in response to DNA damage is prominent in budding yeast, the underlying pathway may be preserved in other eukaryotes. A cryptic metaphase arrest has been documented after replication fork blockage during fission yeast meiosis (Murakami and Nurse, 1999).

Controlled phosphatase activation at anaphase onset may provide further advantages for mitotic regulation. For example, anaphase onset coincides with a sudden change in microtubule dynamics in many organisms, important for successful chromosome segregation (Higuchi and Uhlmann, 2005). In budding yeast, Cdc14 activation regulates this change. Release of a phosphatase has the potential to cause abrupt change in the phosphorylation status of a substrate, compared to the slower process of Cdk downregulation against constitutive phosphatases.

Mutations in *Drosophila* separase uncovered a distinct, albeit short, delay in mitotic exit (Pandey et al., 2005). Meanwhile human separase, after securin destruction, directly interacts with Cdk and inhibits the kinase molecules it has bound to (Gorr et al., 2005). This suggests that aspects of mitotic exit regulation by separase are conserved. Another similarity may exist in separase's interaction with PP2A. We found protein levels of Cdc55 reduced in separase mutant cells. Reduced $PP2A^{Cdc55}$ in turn leads to increased inhibitory Cdk phosphorylation by Swe1 kinase, a phenotype also observed in human cells containing mutant separase (Papi et al., 2005; Yang et al., 2000). Cdc55 instability in the absence of separase could be due to the missing protein interaction. It will be interesting to see whether separase/Cdc55 interaction and regulation during anaphase are conserved. Our study provides insight into how separase couples cohesin cleavage at anaphase onset with a second essential role in activation of Cdc14 phosphatase and mitotic exit in budding yeast.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids

All strains were derivatives of W303. Epitope tagging of endogenous genes and gene deletions were performed by gene targeting using polymerase chain reaction (PCR) products (Knop et al., 1999). The *esp1-2*^{td} allele was created by cloning a 525 nucleotide PCR fragment of the *ESP1* promoter and 400 nucleotides of the *ESP1* open reading frame into plasmid pPW66R (Dohmen et al., 1994). The plasmid was integrated after linearization with Msc I into *esp1-2* strains (Buonomo et al., 2000), harboring plasmid pKL59 (*GAL1-UBR1*, a gift from K. Labib). For N-terminal tagging of endogenous *CDC55*, fragments of the *CDC55* promoter from −500 and of the first 178 nucleotides of the *CDC55* open reading frame were generated by PCR and introduced into plasmid pRS306. Epitope tags were inserted in front of the open reading frame, and plasmids were integrated into the yeast genome after linearization with Msc I. *CDC28*^{Y19F} mutant strains were created by integration, and loop out by 5-FOA selection, of the linearized pJM1054 plasmid, a gift from D.J. Lew (McMillan et al., 1999). The securin^{mdb} strains were created by integration of plasmid pOC70, a gift from O. Cohen-Fix (Cohen-Fix and Koshland, 1999). The expression of separase to trigger anaphase in cells arrested in metaphase by Cdc20 depletion was described previously (Uhlmann et al., 2000). Cell synchronization using α factor and metaphase arrest by Cdc20 depletion and release into synchronous anaphase were as described (Uhlmann et al., 1999).

Immunoprecipitation, Clb2/Cdk Kinase Assay, and $PP2A^{Cdc55}$ Phosphatase Assay

Protein extracts were prepared after spheroplast lysis as described (Uhlmann et al., 1999). For immunoprecipitation, the clarified extracts were precleared with protein A sepharose beads (Amersham Biosciences) and incubated with antibody, and immunocomplexes were bound to protein A dynabeads (Dynal). Beads were washed and boiled with SDS-PAGE loading buffer. For the kinase assay, the beads were washed twice more with kinase buffer (50 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 5 mM β-glycero-phosphate). Beads were incubated with 20 μl reaction mix (kinase buffer supplemented with 50 μM ATP and 2.5 μg H1 [Roche], or 10 μM ATP and 1 μg of purified recombinant Net1 [1–600], both including 0.25 μCi/μl γ-^{32}P-ATP) for 20 min at 30°C and the reactions were stopped by adding SDS-PAGE loading buffer. H1 and Net1 phosphorylation was detected by autoradiography and quantified using a PhosphoImager (Molecular Dynamics). For the phosphatase assays, H1 and Net1 (1–600) were phosphorylated as above, but the incubation was extended to 1 hr. Substrates were purified away from unincorporated nucleotides by gel filtration using Sephadex G-50 Nick columns (Amersham Biosciences). N-terminally Pk epitope-tagged Cdc55 was immunopurified on protein A dynabeads as above, and the beads were washed with phosphatase buffer (50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 1 mg/ml bovine serum albumin [Sigma], 0.1% β-mercaptoethanol). Reaction mix (1 μg ^{32}P-phospho-H1, or 0.5 μg ^{32}P-phospho-Net1 [1–600], in 50 μl phosphatase buffer) was added to the beads and incubated at 30°C for 20 min. Reactions were terminated on ice, protein was precipitated from the supernatants with 16% trichloroacetic acid, and acid-soluble ^{32}P-phosphate was quantified in a scintillation counter. Cdc55 recovered on the beads was quantified by Western blotting using an IRdye 800 coupled secondary antibody and the Odyssey Infrared Imaging System (LI-COR Biosciences).

Other Techniques

Net1 (1–600) was prepared as described (Traverso et al., 2001). 1NM-PP1 (Toronto Research Chemicals) was prepared as 1 mM stock

solution in DMSO and diluted directly into the culture medium. Cell extracts for detection of the phospho-Net1 epitope were prepared as described (Azzam et al., 2004). Antibodies used for Western blotting were α-phospho-Net1 serum PP-B, a gift from R. Deshaies (Azzam et al., 2004), α-Sic1 serum, a gift from M. Tyers (Nash et al., 2001), α-Swi6 (a gift from K. Nasmyth), α-Pk clone SV5-Pk1 (Serotec), α-FLAG clone M2 (Sigma), and α-Clb2 serum sc-9071 and α-PSTAIRE serum recognizing Cdc28 sc-53 (both Santa Cruz Biotechnology). In situ immunofluorescence and chromosome spreading were performed as previously described (Michaelis et al., 1997). Antibodies used for cytology were α-HA clone 16B12 (Babco), α-myc clone 9E10 (Babco), and α-tubulin clone YOL1/34 (Serotec).

Supplemental Data

Supplemental Data include six figures and can be found with this article online at http://www.cell.com/cgi/content/full/125/4/719/DC1/.

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