Separase cooperates with Zds1 and Zds2 to activate Cdc14 phosphatase in early anaphase

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Completion of mitotic exit and cytokinesis requires the inactivation of mitotic cyclin-dependent kinase (Cdk) activity. A key enzyme that counteracts Cdk during budding yeast mitotic exit is the Cdc14 phosphatase. Cdc14 is inactive for much of the cell cycle, sequestered by its inhibitor Net1 in the nucleolus. At anaphase onset, separase-dependent down-regulation of PP2A^{Cdc55} allows phosphorylation of Net1 and consequent Cdc14 release. How separase causes PP2A^{Cdc55} down-regulation is not known. Here, we show that two Cdc55-interacting proteins, Zds1 and Zds2, contribute to timely Cdc14 activation during mitotic exit. Zds1 and Zds2 are required downstream of separase to facilitate nucleolar Cdc14 release. Ectopic Zds1 expression in turn is sufficient to down-regulate PP2A^{Cdc55} and promote Net1 phosphorylation. These findings identify Zds1 and Zds2 as new components of the mitotic exit machinery, involved in activation of the Cdc14 phosphatase at anaphase onset. Our results suggest that these proteins may act as separase-regulated PP2A^{Cdc55} inhibitors.

Introduction

Complexes between cyclins and cyclin-dependent kinases (Cdk) govern the progression through the cell division cycle in all eukaryotes. Entry into mitosis occurs when cyclin B/Cdk (Clb2/Cdk in budding yeast) reaches a peak of kinase activity. This leads to formation of the mitotic spindle, condensation of the chromosomes, and their alignment on the spindle. Successful bipolar attachment of the chromosomes releases an inhibitory mitotic checkpoint signal, allowing the activation of the anaphase-promoting complex (APC) by its coactivator Cdc20 (APC^{Cdc20}; for review see Morgan, 2007; Peters, 2006). APC^{Cdc20} is a ubiquitin ligase that ubiquitinates securin, an inhibitor of the protease separase. Ubiquitinated securin is targeted for degradation by the proteasome, thus freeing separase. Sister chromatid separation, and thereby anaphase onset, is now triggered when separase cleaves the Scc1 subunit of the chromosomal cohesin complex (Uhlmann et al., 2000). At this time, APC^{Cdc20} also targets mitotic cyclins for degradation to down-regulate mitotic Cdk activity. However, APC^{Cdc20} is not sufficient for complete cyclin destruction and Cdk inactivation. In addition, activation of the mitotic phosphatase Cdc14 is essential to promote exit

from mitosis (for review see Stegmeier and Amon, 2004). Cdc14 contributes to the down-regulation of Cdk activity by dephosphorylating and thereby activating a second coactivator of the APC, Cdh1, which completes the destruction of mitotic cyclins, as well as the Cdk inhibitor Sic1. Cdc14 furthermore directly counteracts Cdk activity by dephosphorylating mitotic Cdk targets (Visintin et al., 1998).

For much of the cell cycle, Cdc14 is kept inactive in the nucleolus by binding to its nucleolar inhibitor Net1 (also called Cfi1; Shou et al., 1999; Visintin et al., 1999). During anaphase, mitotic kinase-dependent phosphorylation of Net1 releases active Cdc14. Phosphorylated Net1 shows reduced affinity for, and looses its ability to inhibit Cdc14 (Shou et al., 2002; Yoshida and Toh-e, 2002; Azzam et al., 2004). Two complementary regulatory pathways are essential for Cdc14 release from the nucleolus. During early anaphase, separase initiates Net1 phosphorylation and Cdc14 release in conjunction with a series of proteins, Slk19, Spo12, and Fob1. The action of these proteins has been summarized as the Cdc fourteen early anaphase release (FEAR) pathway (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003; Azzam et al., 2004; Queralt et al., 2006). Initial Cdc14 release is promoted by Cdk-dependent Net1 phosphorylation

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(Azzam et al., 2004), which is counteracted in metaphase by the type 2A protein phosphatase (PP2A) in conjunction with its Cdc55 activatory subunit (PP2A^{Cdc55}; Queralt et al., 2006; Yellman and Burke, 2006). Separase-dependent PP2A^{Cdc55} down-regulation at anaphase onset allows Net1 phosphorylation and Cdc14 release. PP2A^{Cdc55} down-regulation, declining Cdk activity, and released Cdc14 now activate a G protein–coupled kinase signaling cascade, the mitotic exit network (MEN; Jaspersen et al., 1998; Lee et al., 2001). The MEN maintains Cdc14 active during the later stages of anaphase when mitotic Cdk activity declines (Jaspersen and Morgan, 2000; Stegmeier et al., 2002; Queralt et al., 2006). Kinases of the MEN, including Polo kinase, may act to sustain Net1 phosphorylation at this time (Lee et al., 2001; Shou et al., 2002; Yoshida and Toh-e, 2002; Visintin et al., 2008).

PP2A is a family of widely conserved protein serine/threonine phosphatases, with roles in a multitude of cellular processes (Stark, 1996). They function as a trimeric complex, a catalytic subunit (in budding yeast one of the two closely related proteins Pph21 or Pph22), a scaffold subunit (Tpd3 in budding yeast), and a regulatory subunit (one of the three Rts1, Rts3, or Cdc55). With PP2A^{Cdc55}, we refer to a complex consisting of Pph21 or Pph22, Tpd3, and Cdc55. The mechanistic basis for separase-dependent PP2A^{Cdc55} down-regulation at anaphase onset is not known. Recently Zds1 and Zds2, two sequence paralogues in budding yeast, have been identified as putative interactors of Cdc55 by large-scale affinity purification analyses (Gavin et al., 2002; Krogan et al., 2006). Zds1 and Zds2 (zillion different screens) have also been isolated in numerous genetic screens, relating to regulators of the Cdc42 GTPase (Bi and Pringle, 1996), the RNA polymerase II mediator complex (Yu et al., 1996), sister chromatid cohesion (Heo et al., 1999), chromatin silencing (Roy and Runge, 2000), cell wall integrity signaling (Griffioen et al., 2003), cell polarity (Zanelli and Valentini, 2005), and other cellular processes. Contributions of Zds1 and Zds2 to cell cycle progression have been noted (Bi and Pringle, 1996; Ma et al., 1996; Yu et al., 1996; Mizunuma et al., 1998). Both Zds1 and separase have been found to act as high copy suppressors of the *cdc28-1N* mutation, suggesting a possible functional link between the two proteins, although the mechanism by which either Zds1 or separase act in *cdc28-1N* suppression has not yet been elucidated (Yu et al., 1996; Jones et al., 2000). The molecular function of Zds1 and Zds2 thus remains poorly understood. No sequence motifs have been detected in their primary amino acid sequence that could yield hints as to their activity. Orthologues of Zds1 and Zds2 can be found in most ascomycetes, with the strongest similarity between them confined to a C-terminal "Zds1 C" motif.

Here, we confirm a physical interaction of Zds1 and Zds2 with PP2A^{Cdc55}, and show that Zds1 and Zds2 are required for timely nucleolar release of Cdc14 in early anaphase. Separase-induced Cdc14 activation requires Zds1 and Zds2, and ectopic Zds1 expression in metaphase down-regulates PP2A^{Cdc55} and releases Cdc14 in a Net1 phosphorylation-dependent reaction. This implies that Zds1 and Zds2 act downstream of separase to initiate Cdc14 activation at anaphase onset, and suggests that Zds1 and Zds2 may exert their biological function as PP2A regulators.
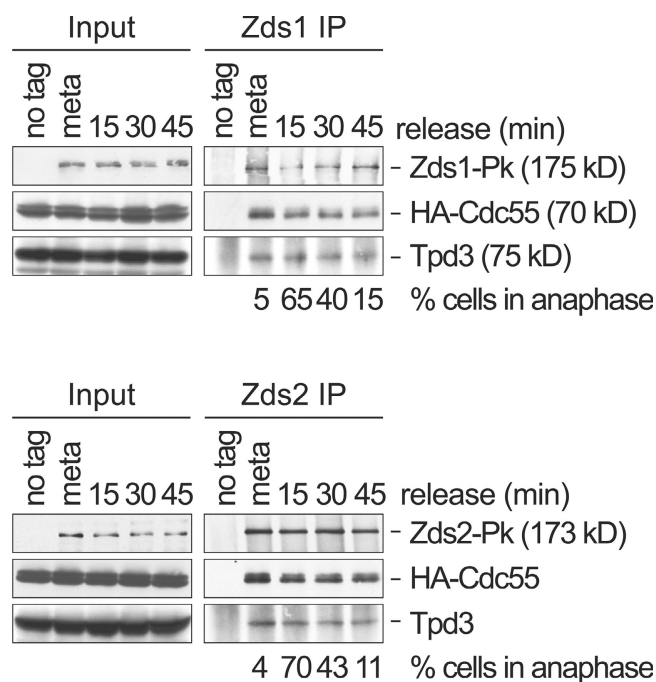

Figure 1. **Zds1 and Zds2 interact with PP2A^{Cdc55}.** Cell extracts were prepared from strains Y2541 (*MATa GAL-CDC20 HA$_3$-CDC55*), Y3131 (as Y2541, but also *ZDS1-Pk$_9$*), and Y3200 (as Y2541, but also *ZDS2-Pk$_9$*), which were synchronized at the metaphase to anaphase transition, and coimmunoprecipitation of subunits of the PP2A^{Cdc55} complex with Zds1 and Zds2 was analyzed by Western blotting. The apparent molecular weights of the subunits, relative to marker proteins during gel electrophoresis, are indicated. Anaphase progression was monitored by tubulin staining.

Results

Zds1 and Zds2 interact with PP2A^{Cdc55}

We have previously shown that separase activation at anaphase onset causes down-regulation of the PP2A^{Cdc55} phosphatase to facilitate Net1 phosphorylation and Cdc14 release. Separase and PP2A^{Cdc55} physically interact (Queralt et al., 2006). However, PP2A^{Cdc55} is present in cells in stoichiometric excess over separase (Ghaemmaghami et al., 2003), making a mechanism of direct PP2A^{Cdc55} inhibition by separase binding unlikely. This suggests that additional factors are involved downstream of separase in PP2A^{Cdc55} regulation. High throughput affinity purification of budding yeast protein complexes has detected interactions between the subunits of the PP2A^{Cdc55} complex and both Zds1 and Zds2 (Gavin et al., 2002; Krogan et al., 2006). This prompted us to confirm a possible interaction between Zds1, Zds2, and PP2A^{Cdc55}, and ask whether the interaction occurs in a cell cycle–regulated manner. We arrested cells in metaphase by depletion of the APC coactivator Cdc20 under the control of the galactose-inducible *GAL1* promoter, and released them into synchronous anaphase progression by reintroduction of Cdc20. In immunoprecipitates of Zds1 from metaphase-arrested cells, we detected Cdc55 as well as the PP2A scaffold subunit, Tpd3 by Western blotting (Fig. 1). This suggests that Zds1 and PP2A^{Cdc55} interact at this stage in the cell cycle. We were unable to analyze coprecipitation of the catalytic subunit Pph21/Pph22, as epitope tags at either terminus of these proteins interfere with
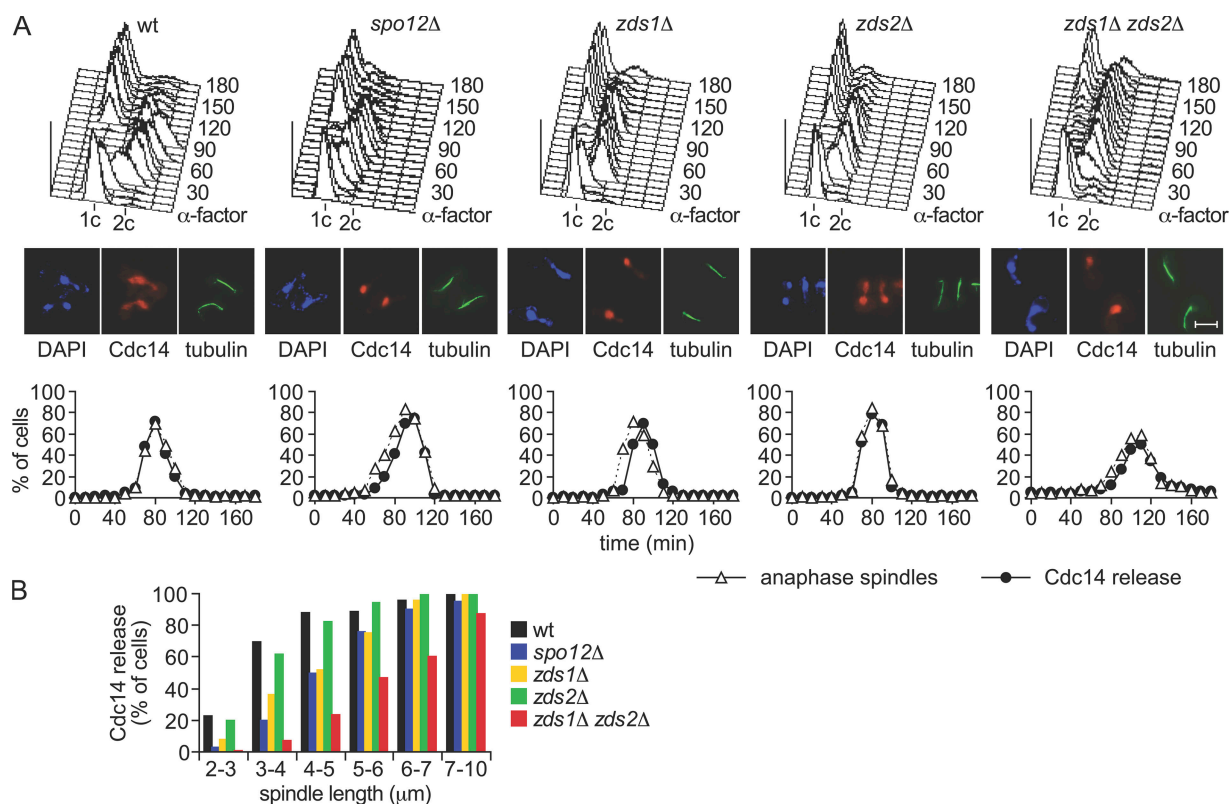
Figure 2. Delayed Cdc14 nucleolar release in the absence of Zds1 and Zds2. (A) Analysis of mitotic progression in the absence of Zds1 and Zds2. Strains Y2786 (*MATa CDC14-Pk9*), Y1016 (as Y2786, but *spo12Δ*), Y3013 (as Y2786, but *zds1Δ*), Y3036 (as Y2786, but *zds2Δ*), and Y3117 (as Y2786, but *zds1Δ zds2Δ*) were arrested with α-factor and released into a synchronous cell cycle. FACS analysis of DNA content was used to monitor cell cycle progression. Cdc14 nucleolar release and anaphase spindle elongation was analyzed by in situ immunofluorescence. At least 100 cells were scored at each time point. Photographs are shown of cells in early anaphase with a spindle length of 3–5 μm. Bar, 5 μm. (B) Cdc14 nucleolar release was analyzed in samples of the experiment in A as a function of spindle length.

PP2A^Cdc55 function (unpublished data). Tpd3 is the limiting subunit for PP2A holocomplex assembly, and Cdc55 is thought to associate with a preassembled Tpd3-Pph21/Pph22 dimer (Hombauer et al., 2007). It is therefore likely that also Pph21 or Pph22 subunits are present in the Zds1-PP2A^Cdc55 complex.

Zds1 immunoprecipitates from cells released into synchronous anaphase contained similar amounts of Cdc55 and Tpd3 compared with immunoprecipitates in metaphase (Fig. 1). This indicates that the interaction between Zds1 and PP2A^Cdc55 is constitutive throughout progression through mitosis and mitotic exit. We also analyzed the interaction between Zds2 and PP2A^Cdc55 in similar immunoprecipitation experiments. Zds1 and Zds2 share 37.5% sequence identity, concentrated in 6 regions of homology (Bi and Pringle, 1996). The two paralogues show largely similar, but not identical, behavior in the reported genetic interactions (Roy and Runge, 2000). Again, coimmunoprecipitation demonstrated interaction of Zds2 with Cdc55 and Tpd3 in metaphase as well as during synchronous progression through anaphase and mitotic exit (Fig. 1). This suggests that both Zds1 and Zds2 interact with PP2A^Cdc55 throughout mitosis.

Zds1 and Zds2 are required for Cdc14 nucleolar release in early anaphase

The interaction with Zds1 and Zds2 in mitosis opened the possibility that these proteins are involved in down-regulation of

PP2A^Cdc55 phosphatase activity and nucleolar release of Cdc14. To test this possibility, we constructed single and double deletion mutants of the *ZDS1* and *ZDS2* genes and analyzed cell cycle progression and Cdc14 release after synchronous release of these strain from α-factor block in G1. For comparison, we included a *spo12Δ* strain, in which Cdc14 nucleolar release is delayed due to the Spo12 requirement for Cdc14 early anaphase release (Stegmeier et al., 2002; Fig. 2 A). *zds2Δ* cells showed kinetics of cell cycle progression, and Cdc14 release at the time of anaphase onset, indistinguishable from the wild-type control. In contrast, in *zds1Δ* cells, Cdc14 release from the nucleolus was retarded by 10 min relative to the onset of spindle elongation, similar to the delay observed in *spo12Δ* cells. In *zds1Δ zds2Δ* double-mutant cells, Cdc14 release was also delayed relative to anaphase onset. The difference in timing was more difficult to establish, as *zds1Δ zds2Δ* cells entered anaphase with a delay, and less synchronously, compared with the control.

To analyze the delay in Cdc14 activation relative to an internal marker for mitotic progression, we analyzed Cdc14 nucleolar release with respect to the anaphase spindle length. Cdc14 was half-maximally released in *zds2Δ* cells at a spindle length of 3 μm, similar to wild-type cells (Fig. 2 B). In contrast, *zds1Δ* cells released Cdc14 only when the spindle reached lengths of 4–5 μm, similar to what is observed in *spo12Δ* cells.
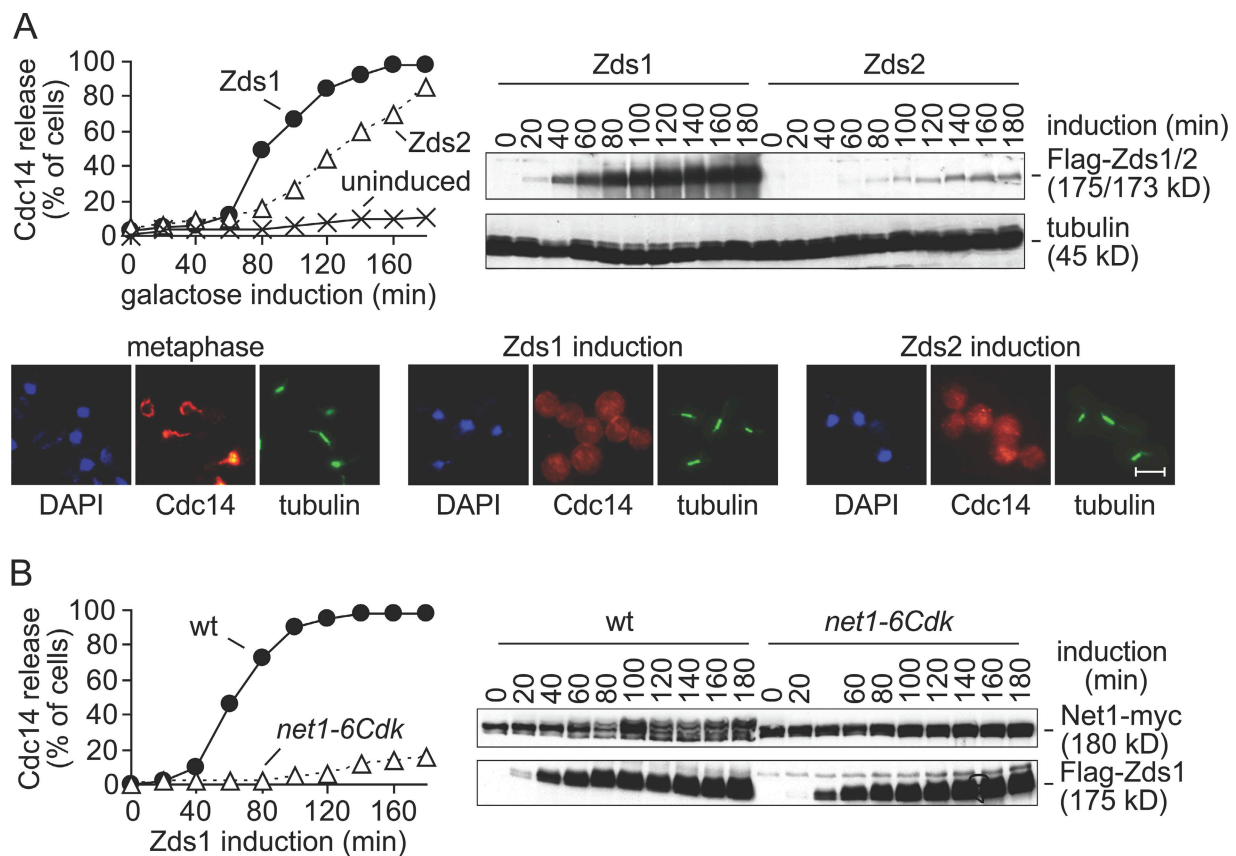
Figure 3. **Zds1 promotes Cdc14 nucleolar release by Net1 phosphorylation.** (A) Ectopic expression of Zds1 and Zds2 promote Cdc14 nucleolar release. Strains Y3288 (*MATa MET-CDC20 GAL-Flag-ZDS1 CDC14-Pk₉*) and Y2220 (*MATa MET-CDC20 GAL-Flag-ZDS2 CDC14-Pk₉*) were arrested in metaphase (meta) and Zds1 or Zds2 expression induced. Part of the culture of strain Y3288 was maintained without Zds1 induction during the course of the experiment. Cdc14 nucleolar release was monitored by immunofluorescence, and Zds1 and Zds2 expression levels were analyzed by Western blotting. Tubulin served as a loading control. Photographs are shown of cells in metaphase and 120 min after Zds1 or Zds2 induction. Bar, 5 μm. (B) Cdk phosphorylation sites are required for Zds1-induced Net1 phosphorylation and Cdc14 release. Strains Y1008 (*MATa MET-CDC20 GAL-Flag-ZDS1 CDC14-Pk₉ NET1-myc₉*) and Y1013 (as Y1008, but *net1-6Cdk-myc₉*) were arrested in metaphase by Cdc20 depletion and Zds1 expression was induced. Net1 phosphorylation was monitored by Western blotting against the myc epitope. Apparent relative molecular weights are indicated.

More strikingly, most *zds1Δ zds2Δ* cells released Cdc14 only at spindle lengths of 6–7 μm and longer. These results indicate that Cdc14 activation is delayed in *zds1Δ* and further delayed in *zds1Δ zds2Δ* double-mutant cells. Thus, both Zds1 and Zds2 participate in timely Cdc14 activation in early anaphase. The contribution of Zds1 appeared to be stronger than that of Zds2, as *zds1Δ* but not *zds2Δ* single-mutant cells showed a discernible delay of Cdc14 release.

Zds1 and Zds2 promote Net1 phosphorylation

Ectopic expression of separase in metaphase is sufficient to cause Cdc14 nucleolar release (Sullivan and Uhlmann, 2003). We therefore also asked whether ectopic expression of Zds1 or Zds2 is sufficient to activate Cdc14. We arrested cells in metaphase by depletion of Cdc20, and induced ectopic Zds1 or Zds2 expression under control of the galactose-inducible *GAL1* promoter (Fig. 3 A). Both Zds1 and Zds2 induction led to Cdc14 nucleolar release. Even though similar expression constructs were used for both paralogues, Zds1 accumulated more rapidly and to higher levels after induction. We do not know the reason for this difference. Correlating with its faster accumulation,

Cdc14 release occurred earlier after induction of Zds1 expression, compared with Zds2 expression. At later time points, Cdc14 was efficiently released also in cells expressing Zds2. Cells expressing Zds1 or Zds2 remained arrested in mitosis despite the activation of Cdc14. This can be explained by the observation that Cdc14 activation is not sufficient to promote mitotic exit if not Cdk activity is down-regulated at the same time (Queralt et al., 2006). We conclude that ectopic expression of either Zds1 or Zds2 in metaphase-arrested cells is sufficient to cause nucleolar release of Cdc14. Because of its greater efficiency in this setting, we will use expression of Zds1 in most of the following experiments.

Cdc14 activation during early anaphase depends on Cdk-dependent phosphorylation of the Cdc14 inhibitor Net1 (Azzam et al., 2004). To analyze whether Cdc14 release by ectopic Zds1 expression involved Net1 phosphorylation, we analyzed the phosphorylation status of Net1 during the induction timecourse. At the time of Zds1 accumulation, slower migrating forms of Net1 became detectable by Western blotting, indicative of phosphorylation. To test whether indeed Net1 phosphorylation was responsible for Cdc14 release, we repeated the experiment in a strain carrying the *net1-6Cdk* allele (Azzam et al., 2004).
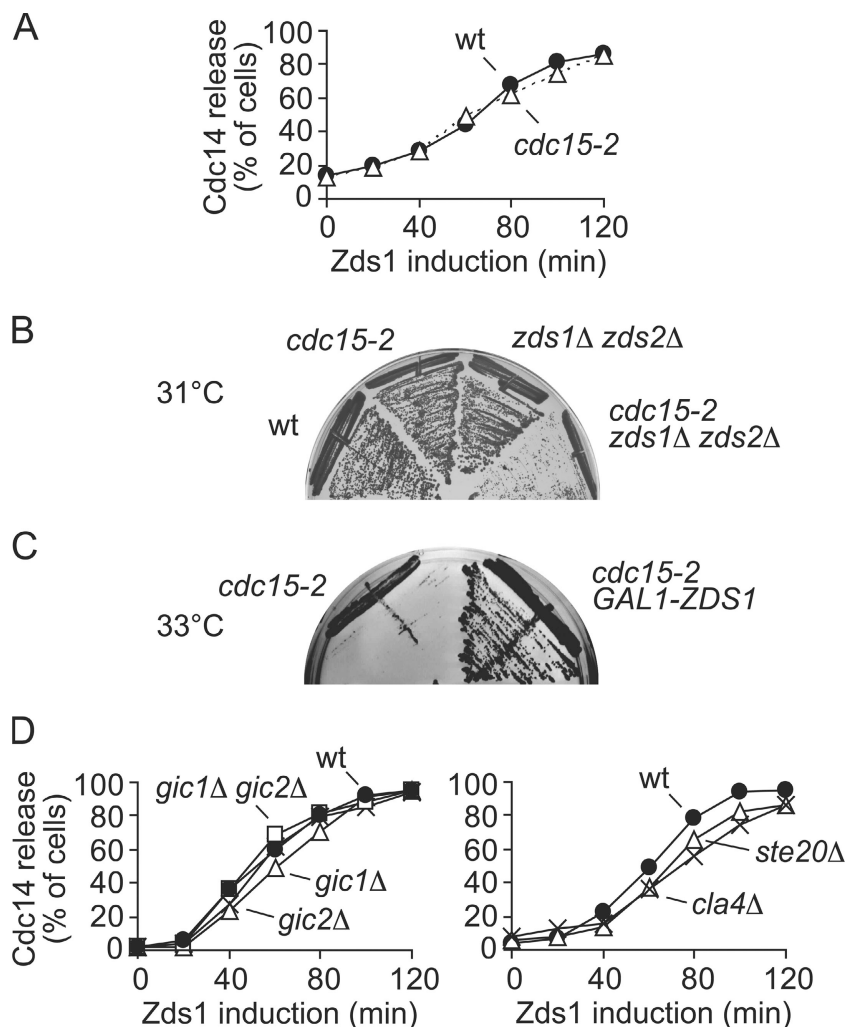
A

B

C

D

Figure 4. **Zds1 and the MEN.** (A) The essential MEN kinase Cdc15 is not required for Zds1-induced Cdc14 release. Strains Y3288 (*MATa MET-CDC20 GAL-Flag-ZDS1 CDC14-Pk₉*) and Y1429 (as Y3288, but *cdc15-2*) were arrested in metaphase by Cdc20 depletion and shifted to 37°C for 60 min before Zds1 induction. (B) *cdc15-2* and *zds1Δ zds2Δ* mutations display a synthetic growth defect. Strains Y699 (W303), Y3196 (*MATa cdc15-2*), Y3117 (*MATa zds1Δ zds2Δ*), and Y3428 (as Y3117, but *cdc15-2*) were streaked onto YPD plates and incubated at 31°C for 2 d. (C) Zds1 overexpression rescues growth of *cdc15-2* cells at restrictive temperature. Strains Y3196 and Y3191 (*MATa cdc15-2 GAL1-ZDS1*) were streaked onto YP plates containing raffinose and galactose, to induce Zds1 expression, at 33°C for 2 d. (D) Cdc42 effectors and PAK-kinases are not required for Zds1-induced Cdc14 release. Strains Y3288 (*MATa MET-CDC20 GAL-Flag-ZDS1 CDC14-Pk₉*), Y1409 (as Y3288, but *ste20Δ*), Y1410 (as Y3288, but *cla4Δ*), Y1412 (as Y3288, but *gic1Δ*), Y1411 (as Y3288, but *gic2Δ*), and Y1414 (as Y3288, but *gic1Δ gic2Δ*) were arrested in metaphase by Cdc20 depletion and Zds1 expression was induced. Cdc14 nucleolar release was monitored by immunofluorescence. Equal Zds1 expression levels were confirmed by Western blotting (not depicted).

In this strain, 6 Cdk consensus recognition sites within the regulatory Net1 N terminus have been made nonphosphorylatable by site-directed mutagenesis. After Zds1 expression in this strain, a Net1 mobility shift was no longer observed, and Cdc14 remained sequestered in the nucleolus (Fig. 3 B). This suggests that Net1 phosphorylation is required for Zds1-induced Cdc14 release.

The dependence of Zds1-induced Cdc14 release on 6 Cdk consensus sites in Net1 suggests that Zds1 expression facilitates Cdk-dependent Net1 phosphorylation. To directly address whether Cdk activity is required for Zds1-induced Cdc14 release, as opposed to another kinase that might recognize the Net1 Cdk consensus sites, we used cells carrying the ATP analogue (1NM-PP1)–sensitive Cdk allele *cdc28-as1* (Bishop et al., 2000). We again arrested cells in metaphase by Cdc20 depletion, then 1NM-PP1 was added to inhibit Cdk activity, and Zds1 expression was induced. Cells containing wild-type Cdc28, and *cdc28-as1* cells in the absence of inhibitor, released Cdc14 from the nucleolus as expected. Inhibition of Cdc28-as1 by 1NM-PP1 blocked Net1 phosphorylation and Cdc14 release (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200801054/DC1). This suggests that Zds1 expression causes Cdc14 release by promoting Cdk-dependent Net1 phosphorylation.

Cdc14 activation by Zds1 is independent of MEN and PAK kinases

To further address the mechanism of Zds1-induced Net1 phosphorylation and Cdc14 release, we analyzed the requirement of known components of the mitotic exit machinery. The MEN is essential to maintain Cdc14 activity during mitotic exit. We therefore tested whether Zds1 is still able to cause Cdc14 nucleolar release in the absence of MEN function. We expressed Zds1 in metaphase-arrested cells carrying a temperature-sensitive allele, *cdc15-2*, of the essential MEN kinase Cdc15. After ectopic Zds1 expression at the restrictive temperature, Cdc14 was released from the nucleolus in a manner indistinguishable between wild-type and *cdc15-2* mutant cells (Fig. 4 A). This suggests that Cdc14 activation by Zds1 is independent of MEN, and therefore that Zds1 acts in parallel or downstream of the MEN pathway.

To further dissect the genetic relationship between Zds1, Zds2, and MEN, we analyzed interactions between these factors in a synthetic growth defect assay. *cdc15-2* mutant cells grow well at temperatures up to 31°C (Fig. 4 B). Introduction of *zds1Δ* or *zds2Δ* individually into the *cdc15-2* background did not change this growth pattern (unpublished data). However, *cdc15-2* cells deleted for both paralogues (*cdc15-2 zds1Δ zds2Δ*) showed a synthetic growth defect at 31°C. This suggests
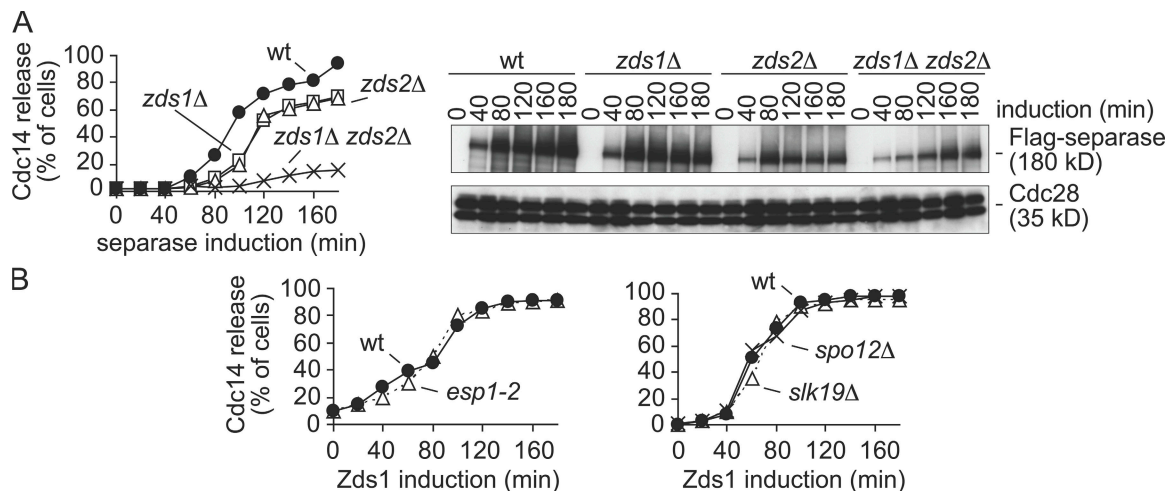
Figure 5. Zds1 is required for separase-induced Cdc14 early anaphase release. (A) Zds1 and Zds2 are required for separase-induced Cdc14 release. Strains Y1748 (*MATa GAL-ESP1-HA CDC14-Pk₉*), Y1790 (as Y1748, but *zds1Δ*), Y1959 (as Y1748, but *zds2Δ*), and Y2189 (as Y1790, but *zds1Δ zds2Δ*) were arrested in metaphase by nocodazole treatment and separase expression induced. Separase levels were analyzed by Western blotting, using Cdc28 as loading control. (B) Separase, Slk19, and Spo12 are not required for Zds1-induced Cdc14 release. Strains Y3288 (*MATa MET-CDC20 GAL-Flag-ZDS1 CDC14-Pk₉*) and Y1430 (as Y3288, but *esp1-2*) were arrested in metaphase by Cdc20 depletion at 23°C and shifted to 37°C for 60 min before Zds1 induction. Strains Y3288 (*MATa MET-CDC20 GAL-Flag-ZDS1 CDC14-Pk₉*), Y1521 (as Y3288, but *slk19Δ*), and Y1646 (as Y3288, but *spo12Δ*) were arrested in metaphase by Cdc20 depletion at 23°C and Zds1 expression was induced.

that Zds1 and Zds2 are unlikely to be downstream effectors of MEN, and are likely to act in a parallel pathway. Consistent with this possibility, *GAL1*-promoter driven overexpression of Zds1 rescued the growth of *cdc15-2* cells at their restrictive temperature of 33°C (Fig. 4 C).

A large-scale two-hybrid screen of cell polarity proteins has reported an interaction of Zds1 and Zds2 with two Cdc42 GTPase effector proteins, Gic1 and Gic2 (Drees et al., 2001). Gic1 and Gic2, as well as two additional Cdc42 effectors, the p21-activated kinase (PAK) kinases Cla4 and Ste20, have been shown to take part in mitotic exit regulation (Höfken and Schiebel, 2002, 2004; Chiroli et al., 2003; Seshan and Amon, 2005). The mechanism by which these Cdc42 effectors promote mitotic exit is not yet understood. The reported genetic interactions with components of the mitotic exit machinery are consistent with the possibility that these proteins act in parallel to MEN, and therefore possibly in conjunction with Zds1 and Zds2. We analyzed whether Gic1, Gic2, Cla4, or Ste20 were involved in Zds1-induced Cdc14 activation. We constructed *gic1Δ*, *gic2Δ*, *gic1Δ gic2Δ*, *ste20Δ*, and *cla4Δ* strains that we arrested in metaphase by Cdc20 depletion. Then, ectopic Zds1 expression was induced. Cdc14 nucleolar release occurred with similar kinetics in all these mutant strains as compared with the wild-type control (Fig. 4 D). This suggests that Zds1 promotes Cdc14 release independently of the Cdc42 effectors Gic1 and Gic2 and the PAK kinases Ste20 and Cla4. These observations leave open the possibility that Zds1, or Zds2, act downstream of Gic1, Gic2, Ste20, or Cla4 to mediate their effect on mitotic exit.

Zds1 is required for separase-induced Cdc14 activation

The findings that Zds1 and Zds2 are required for Cdc14 nucleolar release in early anaphase, and that they act independently of MEN, prompted us to further analyze the relationship of these proteins with separase, which is essential for Cdc14 activation at this time. Cdc14 nucleolar release triggered by ectopic separase expression depends on Slk19 and Spo12 (Sullivan and Uhlmann, 2003), and we wondered whether it also depended on Zds1 or Zds2. To analyze this, we used the microtubule poison nocodazole to arrest wild-type, *zds1Δ*, *zds2Δ*, and *zds1Δ zds2Δ*, cells in metaphase and induced ectopic separase expression under control of the *GAL1* promoter by galactose addition to the growth medium. Separase accumulated in all strains, but *zds1Δ* and *zds2Δ* cells released Cdc14 from the nucleolus with a delay compared with wild-type cells (Fig. 5 A). Cdc14 nucleolar release was almost completely abolished in *zds1Δ zds2Δ* cells. This indicates that Zds1 and Zds2 are required for separase-induced Cdc14 activation.

We next investigated whether reciprocally also Zds1 depended on separase in its ability to activate Cdc14. Zds1 expression in metaphase-arrested wild-type or separase mutant *esp1-2* cells led to Cdc14 nucleolar release with indistinguishable kinetics (Fig. 5 B). This suggests that although separase depends on Zds1 in its ability to promote Cdc14 activation, Zds1 can act independently of separase. Cdc55 levels are reduced in *esp1-2* cells (Queralt et al., 2006), and we cannot exclude that this could have facilitated Zds1-induced Cdc14 activation and masked a possible separase requirement. Together, these findings are consistent with a role of Zds1, and probably Zds2, in mitotic exit in concert with or downstream of separase.

Separase-dependent Cdc14 nucleolar release in early anaphase requires the two proteins Slk19 and Spo12, but their mechanism of action is poorly understood. We therefore analyzed a possible dependence of Zds1 on Slk19 or Spo12 in its ability to induce Cdc14 nucleolar release. After Zds1 expression in metaphase-arrested wild-type, *slk19Δ*, and *spo12Δ* cells, Cdc14 was released from the nucleolus with similar kinetics in
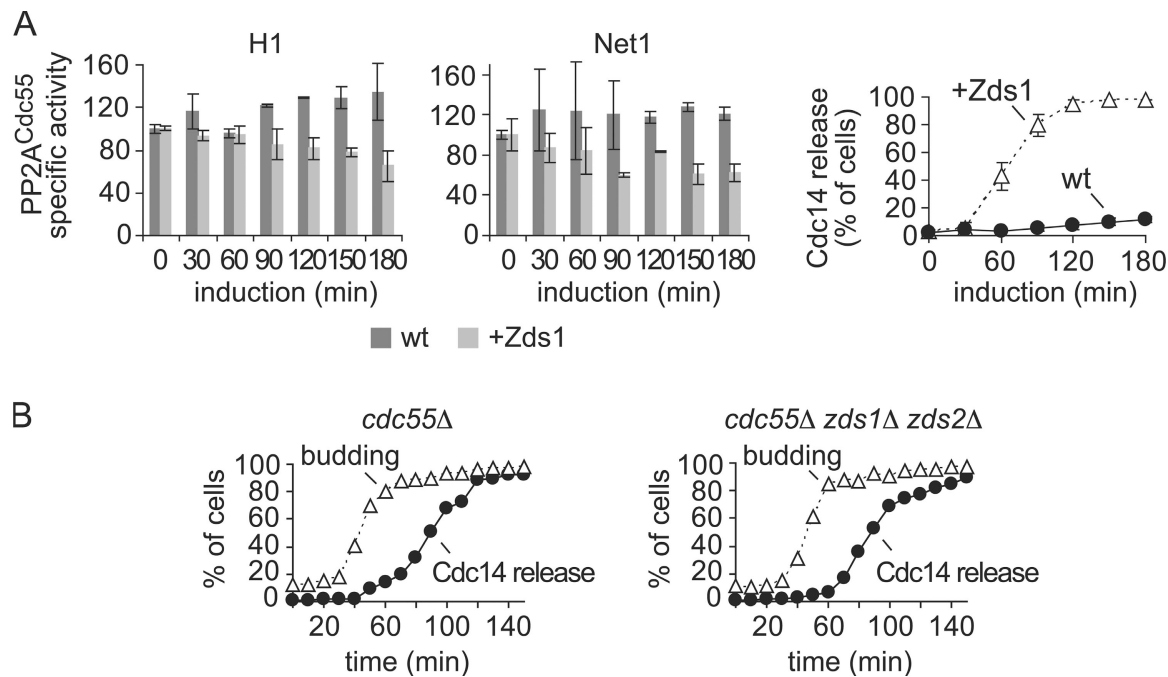
Figure 6. **Zds1 promotes PP2A^Cdc55 down-regulation.** (A) Zds1 causes down-regulation of PP2A^Cdc55 phosphatase activity. Strains Y2627 (*MATa MET-CDC20 HA₃-CDC55 CDC14-myc₁₈*) and Y1015 (as Y2627, but *GAL-Flag-ZDS1 CDC14-Pk₉*) were arrested in metaphase by Cdc20 depletion and galactose added to induce Zds1 expression. The specific phosphatase activity of immunopurified Cdc55 was measured as described in Materials and methods. Mean and standard deviation of the specific phosphatase activity relative to metaphase in three experiments are shown. Cdc14 nucleolar release in these experiments was analyzed in parallel. (B) Zds1 and Zds2 are dispensable for early Cdc14 nucleolar release in the absence of Cdc55. Strains Y2831 (*MATa cdc55Δ CDC14-Pk₉*) and Y3419 (as 2831, but *zds1Δ zds2Δ*) were synchronized in G1 by pheromone α-factor treatment and released to progress through the cell cycle into nocodazole-imposed metaphase arrest. The budding index and Cdc14 nucleolar release were determined.

all strains (Fig. 5 B). This shows that Zds1 can promote Cdc14 release independently of Slk19 and Spo12. Therefore, Slk19 and Spo12 are likely to either function as mediators between separase and Zds1, or act in a pathway that promotes Cdc14 release in parallel to separase.

Zds1 regulates PP2A^Cdc55 activity

The above results suggest that Zds1 and Zds2 act together with separase to promote Cdc14 nucleolar release in early anaphase. Furthermore, ectopic Zds1 expression induces Cdk-dependent Net1 phosphorylation. Together with the physical interaction of Zds1 and Zds2 with PP2A^Cdc55, this opens the possibility that the two paralogues are involved in separase-dependent down-regulation of PP2A^Cdc55 phosphatase activity. To test this possibility, we measured the specific in vitro phosphatase activity of PP2A^Cdc55 isolated from cells before and after ectopic Zds1 expression. The PP2A^Cdc55 phosphatase-specific activity decreased to about half of its initial value in response to Zds1 induction (Fig. 6 A). This suggests that Zds1 has the ability to cause inhibition of PP2A^Cdc55 activity, opening the possibility that it is directly involved in PP2A^Cdc55 down-regulation in early anaphase. Unfortunately, we were unable to measure PP2A^Cdc55 activity during anaphase in the absence of Zds1 and Zds2. Cells lacking both paralogues did not tolerate *GAL1* or *MET3* promoter-driven *CDC20* expression, which would be required to provide the necessary synchrony for PP2A^Cdc55 analysis during anaphase progression.

If the role of Zds1 in mitotic exit is indeed down-regulation of PP2A^Cdc55 activity, then deletion of Cdc55 should make Zds1

and Zds2 dispensable for timely Cdc14 release. In the absence of Cdc55, nucleolar release of Cdc14 occurs prematurely when cells enter mitosis. The increasing Cdk activity at this time phosphorylates Net1 without being counteracted by PP2A^Cdc55 activity, and thus without having to await PP2A^Cdc55 down-regulation at anaphase onset (Queralt et al., 2006). We therefore analyzed whether Cdc14 nucleolar release during mitotic entry in *cdc55Δ* cells depended on Zds1 and Zds2. We synchronized *cdc55Δ* cells in G1 by pheromone α-factor treatment, and released them to progress through S phase and into nocodazole-imposed rearrest in mitosis. As expected, Cdc14 was released from the nucleolus as cells entered mitosis. The timing of Cdc14 activation during the cell cycle was indistinguishable in a parallel culture of *cdc55Δ* cells lacking Zds1 and Zds2 (Fig. 6 B). Therefore, Zds1 and Zds2 no longer influence Cdc14 release from the nucleolus in the absence of Cdc55. This is consistent with the possibility that PP2A^Cdc55 acts as the downstream target of Zds1 and Zds2, and that the two paralogues act in its inhibition.

Mutual interactions between separase, Zds1, and Cdc55

To begin to understand the mechanism by which Zds1 contributes to separase-mediated PP2A^Cdc55 down-regulation, we studied the interactions between these three components. We have above, and previously (Queralt et al., 2006), described interactions between Zds1 and PP2A^Cdc55, as well as between separase and PP2A^Cdc55. Zds1 might therefore interact with PP2A^Cdc55 directly, or the interaction might be mediated by separase. To address this,

we performed coimmunoprecipitation analysis between Zds1 and Cdc55 in protein extracts obtained from both wild-type and separase mutant *esp1-2* strains in which separase had been inactivated at the restrictive temperature for the *esp1-2* allele. The interaction between Zds1 and Cdc55 remained unaffected by separase inactivation (Fig. 7 A), suggesting that Zds1 and Cdc55 interact with each other independently of separase. Because of the strong phenotype of the *esp1-2* allele in mitotic exit we consider it less likely, but cannot exclude, that separase mediates an interaction between Zds1 and PP2A^{Cdc55} that is maintained despite separase inactivation by the *esp1-2* mutation.

Next, we asked whether the interaction between separase and Cdc55 is mediated by Zds1. We compared coimmunoprecipitates of Cdc55 with separase in protein extracts obtained from wild-type and *zds1Δ zds2Δ* strains. The interaction of Cdc55 with separase appeared relatively weaker than that of Cdc55 with Zds1, but it remained unaffected in the *zds1Δ zds2Δ* strain (Fig. 7 B). Therefore separase interacts with Cdc55 independently of Zds1 and Zds2.

The interactions of separase with Cdc55, and of Cdc55 with Zds1, suggested that we should also be able to detect an interaction of separase with Zds1, and that this interaction may be Cdc55 dependent. Consistent with this expectation, we detected separase in Zds1 immunoprecipitates by Western blotting. In the absence of Cdc55, this interaction was maintained, albeit with somewhat reduced intensity (Fig. 7 C). This suggests that separase makes contact with Zds1 independently of Cdc55, but that the presence of Cdc55 may stabilize this interaction. Together, these interaction studies open the possibility that separase, Zds1, and PP2A^{Cdc55} are found together in a protein complex formed by pairwise interactions between all three components. Molecular size analysis of separase in protein extracts from both budding and fission yeast has shown separase to exist as part of large protein complexes (Funabiki et al., 1996; and unpublished data). These results suggest that PP2A^{Cdc55}, Zds1, and likely also Zds2, form part of these complexes.

Discussion

Mitotic exit encompasses an ordered series of events, leading from chromosome segregation during anaphase to completion of cell division by cytokinesis. The budding yeast Cdc14 phosphatase is a key regulator of these events. Cdc14 is kept inactive in the nucleolus for much of the cell cycle, but is released from there during mitotic exit to contribute to Cdk down-regulation and dephosphorylation of numerous Cdk substrates. Initial models for Cdc14 activation in anaphase suggested that anaphase spindle elongation brings the spindle pole–bound MEN GTPase Tem1 in contact with its activator Lte1 at the bud cortex (Bardin et al., 2000; Pereira et al., 2000). In this way Cdc14 would be activated, and mitotic exit events initiated, once anaphase is complete. Meanwhile, it has become clear that Cdc14 is already activated at the onset of anaphase, and that release of separase from its inhibitor securin plays a crucial role in Cdc14 activation (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003; Queralt et al., 2006). The early activated Cdc14 makes crucial contributions to successful anaphase progression. It leads to relocation of Fin1 and
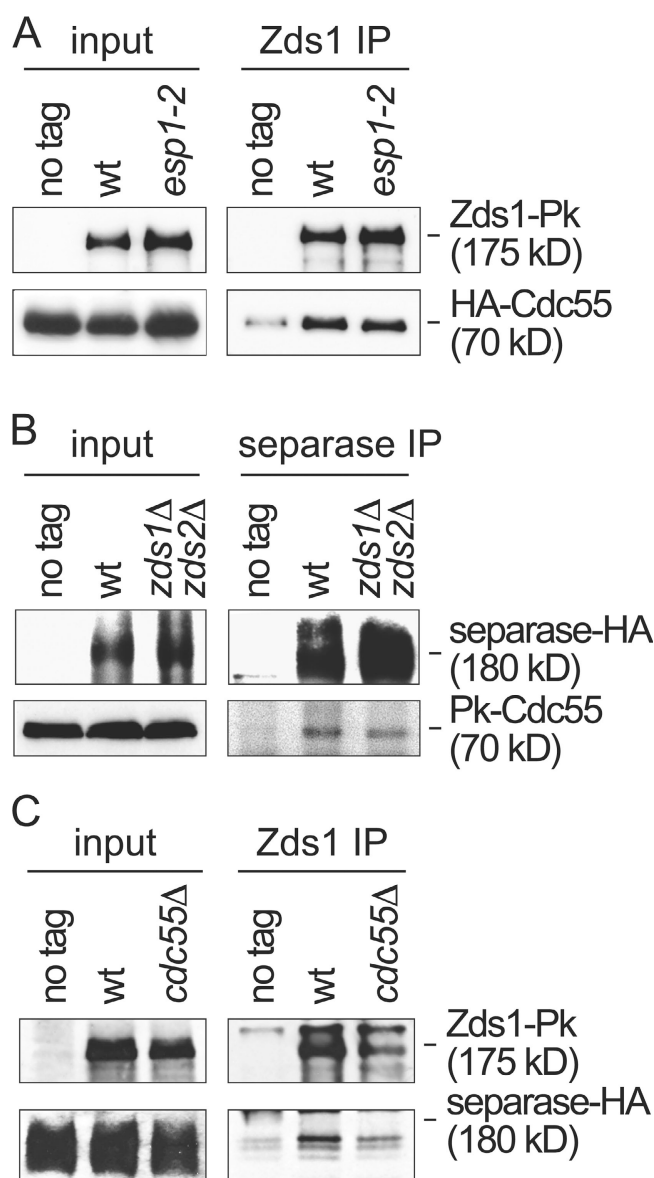
Figure 7. **Interactions between separase, Zds1, and Cdc55.** (A) Zds1 and Cdc55 interact independently of separase. Co-immunoprecipitation between Zds1 and Cdc55 was analyzed in protein extracts from strains Y585 (*MATa Zds1-Pk₆ HA₃-CDC55*) and Y586 (as 585, but *esp1-2*) that were grown at 33°C, a restrictive temperature for the separase *esp1-2* allele, for 1 h before extract preparation. Protein extracts from strain Y584 (*MATa HA₃-CDC55*) lacking a Pk epitope on Zds1 served as a control. (B) Separase and Cdc55 interact independently of Zds1 and Zds2. (B) As (A), but Cdc55 co-immuoprecipitation with separase from extracts of strains Y587 (*MATa ESP1-HA₆*), Y3434 (as 587, but *Pk₃-CDC55*), and Y3427 (as 3434, but *zds1Δ zds2Δ*) grown at 25°C was analyzed. (C) An interaction of separase and Zds1. Co-immunoprecipitation of separase with Zds1 was analyzed in extracts from strains Y587 (*MATa ESP1-HA₆*), Y588 (as 587, but *Zds1-Pk₆*), and Y589 (as 588, but *cdc55Δ*).

the aurora B kinase complex to the spindle midzone, stabilizes the dynamic behavior of mitotic spindle microtubules, and is required for condensation and resolution of the late-segregating rDNA locus (Pereira and Schiebel, 2003; D'Amours et al., 2004; Sullivan et al., 2004; Higuchi and Uhlmann, 2005; Woodbury and Morgan, 2007). It thus appears that the "mitotic exit" machinery orchestrates both, progression through faithful anaphase,

then followed by mitotic exit and cytokinesis. How the ordering of anaphase and subsequent mitotic exit events is achieved by a common set of regulators remains an important open question.

In this study, we address the question of how separase causes Cdc14 activation at anaphase onset. We have previously shown that separase-dependent PP2A^{Cdc55} down-regulation at anaphase onset allows Cdk-dependent phosphorylation of the nucleolar Cdc14 inhibitor Net1, thus promoting Cdc14 release (Queralt et al., 2006). The mechanistic basis for separase-dependent PP2A^{Cdc55} down-regulation remained to be elucidated. Separase and PP2A^{Cdc55} interact, and this interaction might be enhanced, or it may change its quality, as securin is degraded at anaphase onset. A model by which separase binding to PP2A^{Cdc55} directly inhibits its phosphatase activity nevertheless appears unlikely, as PP2A^{Cdc55} is present in a large stoichiometric excess over separase. Separase would thus only be able to regulate a small fraction of PP2A^{Cdc55} directly, while overall PP2A^{Cdc55} activity is reduced by approximately half during anaphase. This suggests that a catalytic step must be involved downstream of separase, and therefore that additional factors are likely required to convey separase-dependent down-regulation of the phosphatase. The observation of a direct interaction of Zds1 and Zds2 with PP2A^{Cdc55} prompted us to analyze the contribution of these two proteins to mitotic exit regulation.

Our analysis revealed that Zds1 and Zds2 are required for timely Cdc14 activation during anaphase. Ectopic Zds1 expression in turn is sufficient to trigger PP2A^{Cdc55} down-regulation, Cdk-dependent Net1 phosphorylation, and Cdc14 nucleolar release. Furthermore, we found that Zds1 physically interacts with separase. These observations open the possibility that separase acts through Zds1 and Zds2 to cause PP2A^{Cdc55} down-regulation at anaphase onset. This notion is further supported by the similarity in requirements for Cdc14 activation by ectopic expression of either separase or Zds1. In both cases, Cdc14 nucleolar release depends on Cdk-dependent Net1 phosphorylation. Consistent with this requirement, ectopic expression of Zds1, like that of separase, leads to Cdc14 activation in mitotic cells, but not cells arrested in G1 or S phase of the cell cycle (Sullivan and Uhlmann, 2003; and unpublished data). The mechanism by which Zds1 and Zds2 impinge on PP2A^{Cdc55} activity, and how this mechanism is in turn controlled by separase, remain important questions for future studies. It is noteworthy that, like separase, Zds1 and Zds2 are relatively low abundant proteins (Ghaemmaghami et al., 2003). Therefore, it does not appear likely that Zds1 and Zds2 act as direct inhibitory components of the PP2A^{Cdc55} complex. Instead, they might be involved in regulating the status of posttranslational modifications or in controlling conformational changes in the PP2A^{Cdc55} holoenzyme that impinge on its activity (Hombauer et al., 2007).

Zds1 and Zds2 have been found as high copy modulators of a large variety of cellular processes. These include cell cycle regulation, cell polarity establishment, and chromatin silencing, among others. These processes are known, or are conceivable, to be influenced by PP2A (Evans and Stark, 1997). The pleiotropic effect of Zds1 and Zds2 on cell physiology may therefore stem from the multitude of processes that are influenced by PP2A. If this possibility holds true, Zds1 and Zds2 might emerge as PP2A regulators

not only during mitotic exit, but in a variety of cellular settings. Recently, fission yeast zds1 has been identified and characterised (Yakura et al., 2006). Like its budding yeast orthologue, it has been found to contribute to a variety of cellular processes, including sexual differentiation, cell wall integrity, and cell morphology. This spectrum of activities again overlaps with the physiological roles described for PP2A in this organism (Kinoshita et al., 1996). It will now be of interest to elucidate the mechanism by which separase, together with Zds1 and Zds2, regulate PP2A^{Cdc55} activity to promote progression through anaphase and out of mitosis. This may serve as a paradigm for PP2A regulation by Zds1 and Zds2 also in other contexts. Finally, a contribution of mammalian separase to Cdk down-regulation and progression through meiotic cell division has been documented (Gorr et al., 2006; Kudo et al., 2006). Although some of the downstream effectors in mammalian cells may differ from those in budding yeast, an understanding of the nonproteolytic function of separase during mitotic exit in the yeast model may yield insight that will also help to elucidate the function of human separase in cell cycle progression.

Materials and methods

Yeast strains, plasmids, and cell cycle synchronization procedures
All strains were derivatives of W303. Epitope tagging of endogenous genes and gene deletions were performed by gene targeting using PCR products. For ectopic expression of Zds1, a *GAL1-ZDS1* fragment from YEp352-GAL1-ZDS1 (Bi and Pringle, 1996) was introduced into the YIplac204 vector. Three tandem Flag epitopes were inserted in front of the open reading frame, and plasmids were integrated into the yeast genome after linearization within the *TRP1* marker gene. YIplac204-GAL1-ZDS2 was created by inserting *GAL1-3Flag* into YIplac204, and adding a fragment containing the *ZDS2* open reading frame originating from plasmid M2655 (Yu et al., 1996). For N-terminal tagging of endogenous *CDC55*, 500 base pairs of the *CDC55* promoter, and in a second reaction the first 178 base pairs of the *CDC55* open reading frame, were amplified by PCR using genomic DNA as the template. The fragments were introduced into plasmid pRS306 and three tandem HA epitopes inserted between promoter and open reading frame. The plasmid was integrated at the *CDC55* locus after linearization with Msc I. Ectopic expression of separase in cells arrested in metaphase by Cdc20 depletion was as described previously (Uhlmann et al., 2000). Ectopic expression of Zds1 and Zds2 followed the same protocol. Cell synchronization using α-factor, and metaphase arrest by Cdc20 depletion and release into synchronous anaphase by Cdc20 re-induction, were described previously (Uhlmann et al., 1999).

Immunoprecipitation and PP2A^{Cdc55} phosphatase assay
Protein extracts were prepared after spheroplast lysis as described previously (Uhlmann et al., 1999). For immunoprecipitation, the clarified extracts were precleared with protein A sepharose beads (GE Healthcare), incubated with antibody, and immunocomplexes adsorbed to magnetic protein A dynabeads (Invitrogen). Beads were washed in extraction buffer and boiled with SDS-PAGE loading buffer. For the phosphatase assays, ^{32}P-phosphorylated histone H1 and Net1(1–600) were prepared as substrates as described previously (Queralt et al., 2006). PP2A^{Cdc55} was immunopurified from cell extracts via N-terminally HA epitope-tagged Cdc55 as above, and the dynabeads were washed with phosphatase buffer (50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 1 mg/ml BSA [Sigma-Aldrich], and 0.1% β-mercaptoethanol). Reaction mix (1 μg ^{32}P-phospho-histone H1, or 0.5 μg ^{32}P-phospho-Net1[1–600], in 50 μl phosphatase buffer) was added to the beads, followed by incubation at 30°C for 20 min. Reactions were terminated on ice, protein was precipitated 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
Antibodies used for immunoprecipitation and Western blotting were α-Pk clone SV5-Pk1 (AbD Serotec), α-HA clone 12CA5 (Roche), α-myc clone

9E10, α-FLAG clone M2 (Sigma-Aldrich), α-Tpd3 (a gift from E. Ogris; Hombauer et al., 2007), anti-α-tubulin clone YOL1/34 (AbD Serotec), and α-PSTAIRE serum recognizing Cdc28 sc-53 (Santa Cruz Biotechnology, Inc.). Antibodies used for indirect immunofluorescence were α-Pk clone SV5-Pk1 and anti-α-tubulin clone YOL1/34. Secondary antibodies were Cy3 labeled goat α-mouse antibody (GE Healthcare) to visualize Pk-epitope tagged Cdc14, and affinity-purified fluorescein-conjugated donkey α-rat antibody (Millipore) to visualize tubulin. Stained cells were mounted in Fluoroguard (Bio-Rad Laboratories), containing 0.1 μg/ml DAPI. Cells were imaged at room temperature using an Axioplan 2 imaging microscope (Carl Zeiss, Inc.) equipped with a 100× (NA 1.45) Plan-Neofluar objective and an ORCA-ER camera (Hamamatsu Photonics). Anaphase spindle length measurements were conducted with the quantitative analysis tools of the Volocity software (Improvision), which was used for image acquisition. Brightness and contrast of the images were adjusted using Photoshop (Adobe).

Online supplemental material
Fig. S1 shows that Zds1-induced Cdc14 release depends on Cdk activity. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801054/DC1.

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