Spatial regulation of Cdc55–PP2A by Zds1/Zds2 controls mitotic entry and mitotic exit in budding yeast

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Budding yeast *CDC55* encodes a regulatory B subunit of the PP2A (protein phosphatase 2A), which plays important roles in mitotic entry and mitotic exit. The spatial and temporal regulation of PP2A is poorly understood, although recent studies demonstrated that the conserved proteins Zds1 and Zds2 stoichiometrically bind to Cdc55–PP2A and regulate it in a complex manner. Zds1/Zds2 promote Cdc55–PP2A function for mitotic entry, whereas Zds1/Zds2 inhibit Cdc55–PP2A function during mitotic exit. In this paper, we propose that Zds1/Zds2 primarily control Cdc55 localization. Cortical and cytoplasmic localization of Cdc55 requires Zds1/Zds2, and Cdc55 accumulates in the nucleus in the absence of Zds1/Zds2. By genetically manipulating the nucleocytoplasmic distribution of Cdc55, we showed that Cdc55 promotes mitotic entry when in the cytoplasm. On the other hand, nuclear Cdc55 prevents mitotic exit. Our analysis defines the long-sought molecular function for the zillion different screens family proteins and reveals the importance of the regulation of PP2A localization for proper mitotic progression.

Introduction

PP2A (protein phosphatase 2A) is a large family of heterotrimeric phosphatases that account for the majority of serine/threonine phosphatase activity and has important roles in mitotic progression in eukaryotic cells (Shi, 2009). The PP2A complex consists of a structural A subunit, a catalytic C subunit, and a regulatory B subunit, which binds to the AC heterodimer. These B subunits regulate both the substrate specificity and localization of the PP2A complexes.

Two B subunits, Cdc55 (B) and Rts1 (B'), have been identified in budding yeast. Cdc55 and Rts1 bind to the core PP2A subunits in a mutually exclusive manner, and *cdc55Δ* and *rts1Δ* exhibit distinct phenotypes, suggesting that they control different functions of PP2A (Shu et al., 1997; Zhao et al., 1997; Jiang, 2006). Cdc55 is important for mitosis, stress response, and polarized growth (Healy et al., 1991; Lin and Arndt, 1995; Minshull et al., 1996; Evans and Stark, 1997; Wang and Burke, 1997; Yang et al., 2000; Jiang, 2006; Queralt et al., 2006; Wang and Ng, 2006; Yellman and Burke, 2006; Chiroli et al., 2007). Cdc55 localizes to various sites, including the bud cortex, the bud neck, the vacuolar membrane, and in the nucleus, and recruits other PP2A subunits (Gentry and Hallberg, 2002). Furthermore, the dephosphorylation of Cdc55–PP2A substrates are cell cycle regulated (Queralt et al., 2006; Pal et al., 2008; Wicky et al., 2011). Thus, it is important to understand the spatiotemporal regulation of Cdc55. However, little is known about the mechanisms that control Cdc55 localization and/or activity.

Recent studies defined Zds1 (zillion different screens 1) and Zds2 proteins as regulators of Cdc55 (Yasutis et al., 2010; Wicky et al., 2011). Zds1 and Zds2 are paralogues and are widely conserved in fungi, including the fission yeast *Schizosaccharomyces pombe* (Yakura et al., 2006). As implied by their names, Zds1 and Zds2 have been identified as multicopy suppressors of mutants involved in divergent cellular processes, including cell cycle, transcription, and translation, cell polarity, and stress response (Bi and Pringle, 1996; Yu et al., 1996; Mizunuma et al., 1998; Schwer et al., 1998; Roy and Runge, 1999, 2000; Griffioen et al., 2001, 2003; Sekiya-Kawasaki et al., 2002; Hsu et al., 2004; Estruch et al., 2005; Zanelli and Valentini, 2005; Yokoyama et al., 2006). However, the molecular

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Abbreviations used in this paper: FEAR, early anaphase release of Cdc14; NES, nuclear export signal.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 193 No. 3 445–454
www.jcb.org/cgi/doi/10.1083/jcb.201101134

mechanism by which Zds1/Zds2 regulate such divergent processes remains a long-standing mystery.

Accumulating evidences suggest that Zds1/Zds2 directly regulate Cdc55–PP2A function. In large scale proteomics studies, both Zds1 and Zds2 were affinity purified with Cdc55–PP2A (Gavin et al., 2002; Ho et al., 2002; Krogan et al., 2006; Collins et al., 2007). A more recent study revealed that the Zds2 protein directly binds to Cdc55 in vitro via a highly conserved C-terminal region (Yasutis et al., 2010). Furthermore, Zds1 was shown to form a tight stoichiometric complex with Cdc55-containing PP2A (Queralt and Uhlmann, 2008; Wicky et al., 2011). Most importantly, association of Zds1 to PP2A is exclusively mediated via Cdc55 (Wicky et al., 2011). Thus, Zds1/2 is an attractive candidate that specifically controls Cdc55–PP2A and not Rts1–PP2A.

The best-characterized functions of Cdc55 and Zds1/Zds2 are their roles in mitotic entry (Wang and Burke, 1997; McMillan et al., 1999b; Yang et al., 2000; Pal et al., 2008; Yasutis et al., 2010; Wicky et al., 2011). Mitotic entry is driven by activation of the Cdk1 (Nurse, 1975). In budding yeast, Cdc28 (Cdk1) activation is prevented by the Swe1 kinase (Wee1 homologue), which phosphorylates Cdc28 on tyrosine 19 (Tyr-19; Booher et al., 1993; Lew and Reed, 1993), whereas Mih1 phosphatase (Cdc25 homologue) removes this inhibitory phosphorylation to promote mitotic entry (Russell et al., 1989). Because polarized growth and cell cycle are tightly coupled in budding yeast and activation of mitotic Cdc28 depolarizes cell polarity, mutants defective in mitotic entry often lead to prolonged apical bud growth (Lew and Reed, 1993). Both Cdc55 and Zds1/Zds2 are important for mitotic entry, as the *cdc55* deletion or *zds1Δ zds2Δ* double mutant leads to abnormally elongated cell morphology as a consequence of prolonged G2 delay (Healy et al., 1991; Bi and Pringle, 1996; Yu et al., 1996). In these mutants, Swe1 is stabilized (Yang et al., 2000), and Mih1 is hyperphosphorylated (Pal et al., 2008; Wicky et al., 2011). Furthermore, the hyperelongated morphology of *cdc55Δ* and *zds1Δ zds2Δ* is rescued either by deletion of *SWE1* (McMillan et al., 1999a; Yang et al., 2000), by overexpression of *MIH1* (McMillan et al., 1999a), or by introduction of an unphosphorylatable *CDC28Y19F* mutation (Wang and Burke, 1997; McMillan et al., 1999a; Wicky et al., 2011). Thus, Zds1/Zds2 are thought to function positively with Cdc55–PP2A to promote mitotic entry (Yasutis et al., 2010; Wicky et al., 2011).

Although Zds1/Zds2 forms a tight complex with Cdc55 and function together with Cdc55–PP2A for mitotic entry, it is not always promoting Cdc55–PP2A functions. For example, Cdc55 is required for spindle assembly checkpoint (Minshull et al., 1996; Wang and Burke, 1997; Yellman and Burke, 2006), but Zds1/Zds2 are not required for the spindle assembly checkpoint (Wang and Burke, 1997). Furthermore, a recent paper demonstrated that Zds1/Zds2 is an inhibitor of Cdc55–PP2A during mitotic exit (Queralt and Uhlmann, 2008).

Cdc55–PP2A prevents mitotic exit by inhibition of a Cdc14 phosphatase (Queralt et al., 2006; Wang and Ng, 2006; Yellman and Burke, 2006). Cdc14 promotes mitotic exit by counteracting with Cdc28-dependent phosphorylation (Visintin et al., 1998). Cdc14 is kept inactive in the nucleolus by its inhibitor Net1/Cfi1 (Straight et al., 1999; Visintin et al., 1999). Phosphorylation of Net1/Cfi1 by Cdk1 results in Cdc14 release and activation during anaphase (Azzam et al., 2004). Cdc55 antagonizes this Net1/Cfi1 phosphorylation and prevents Cdc14 release, and Cdc55–PP2A activity toward Net1/Cfi1 is inhibited at the onset of anaphase (Queralt et al., 2006). Surprisingly, Zds1/Zds2 play opposite roles in mitotic exit. In contrast to Cdc55, Zds1/Zds2 are required for Net1/Cfi1 phosphorylation and optimal Cdc14 release from the nucleolus, and *zds1Δ zds2Δ* shows a severe defect in mitotic exit (Queralt and Uhlmann, 2008). Because overexpression of Zds1 partially inhibits Cdc55–PP2A catalytic activity toward Net1 and because the mitotic exit defect of *zds1Δ zds2Δ* is fully rescued by deletion of *CDC55*, it is proposed that Zds1/Zds2 are inhibitors of Cdc55–PP2A activity (Queralt and Uhlmann, 2008).

Here, we propose that Zds1/Zds2 primarily control Cdc55 localization. Cortical and cytoplasmic localization of Cdc55 requires Zds1/Zds2 because Cdc55 accumulates in the nucleus in the absence of Zds1/Zds2. By genetically manipulating nucleocytoplasmic distribution of Cdc55, we show that Zds1/Zds2 act as positive regulators for cytoplasmic Cdc55–PP2A but as negative regulators for nuclear Cdc55–PP2A functions. Our analysis reveals the importance of the regulation of PP2A localization for proper mitotic progression.

Results and discussion

Zds1 and Zds2 are required for cytoplasmic localization of Cdc55

We first examined subcellular localization of Zds1, Zds2, and Cdc55. To ensure that the proteins were expressed at the native level, we added a GFP sequence to the genes encoding Cdc55, Zds1, and Zds2 at their endogenous locus. These GFP fusion constructs were fully functional.

As it has been previously reported with exogenously expressed GST-Zds1, GST-Zds2 (Bi and Pringle, 1996), and Zds2-i-9×myc (Yasutis et al., 2010), both Zds1-GFP and Zds2-GFP localized to the bud cortex of small to medium budded cells (Fig. 1 A and Fig. S1). We also observed bud neck localization of Zds1-GFP and Zds2-GFP in late mitotic cells (Fig. 1 A and Fig. S1), which is consistent with the previous observation of GST-Zds1 localization (Bi and Pringle, 1996). Similarly, Cdc55-GFP localized to the bud cortex of small to medium budded cells and to the bud neck in late mitotic cells that is consistent with a previous study using GFP-Cdc55 (Fig. 1 B; Gentry and Hallberg, 2002). Zds1, Zds2, and Cdc55 also exist in the cytoplasm and are excluded from the vacuole (Fig. 1, A and B).

Interestingly, Zds1 and Zds2 were excluded from the nucleus, as judged by DAPI staining of the nucleus throughout the cell cycle (Fig. 1 A and Fig. S1). Nuclear exclusion of Zds1-GFP was also observed when it was highly overexpressed under a strong *GAL1* promoter. Bi and Pringle (1996) also reported that GST-Zds1 and GST-Zds2 are exclusively cytoplasmic and that nuclear localization was hardly detected. These observations suggest that Zds1 and Zds2 most likely function at the bud cortex and in the cytoplasm but not in the nucleus. In contrast, Cdc55-GFP was localized also in the

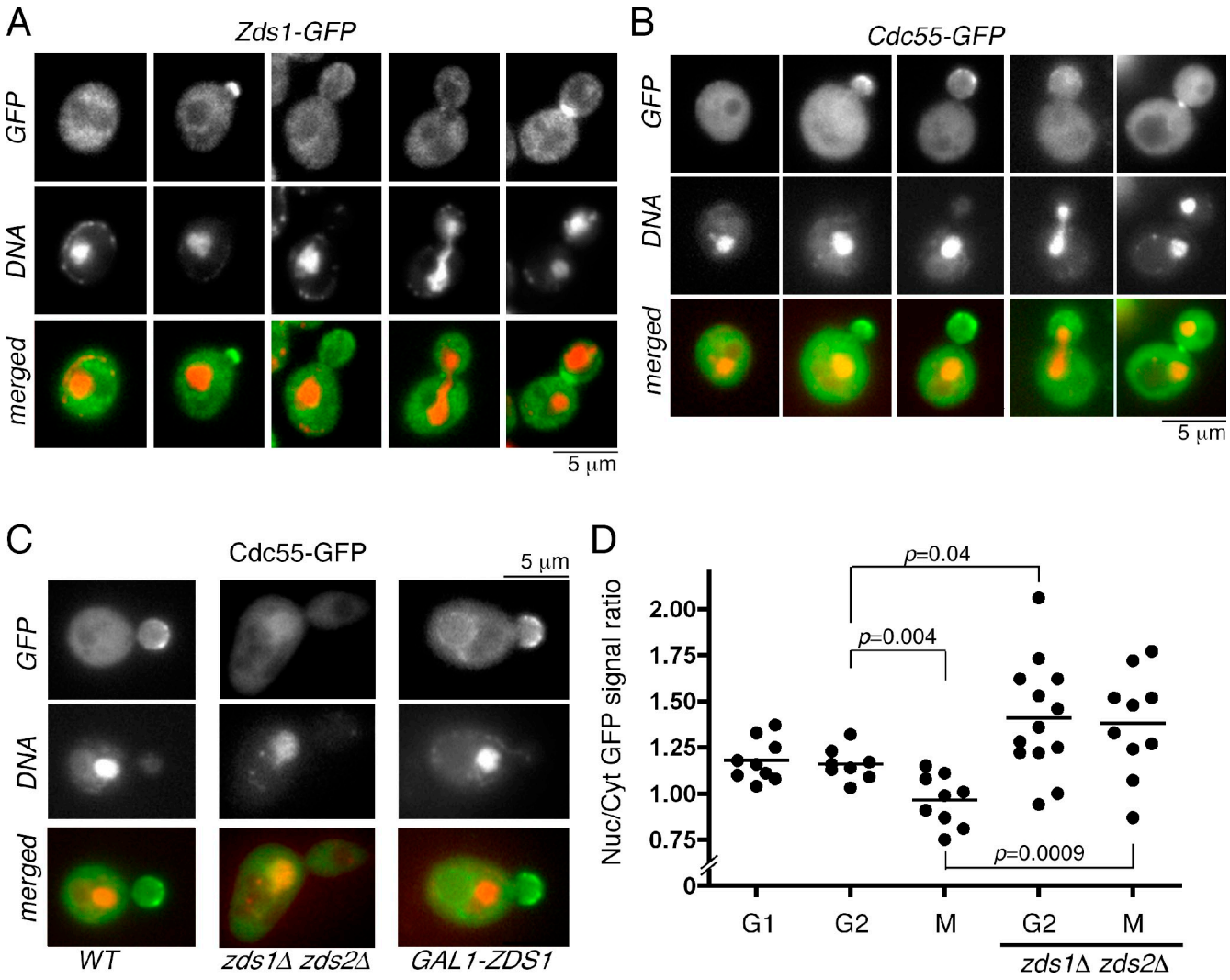

Figure 1. **Localization of Zds1 and Cdc55.** (A) Cell cycle localization of endogenous Zds1-GFP. Cells were fixed with formaldehyde and stained with DAPI. (B) Cell cycle localization of endogenous Cdc55-GFP. In live cells, DNA was stained with Hoechst 33258. (C) Nuclear localization of Cdc55-GFP is affected in *zds1Δ zds2Δ* cells and in the cells overexpressing Zds1 (*GAL1-ZDS1*). (D) Nuclear accumulation of Cdc55-GFP is prevented by Zds1 and Zds2. The ratio of mean fluorescence intensity of nuclear (Nuc) and cytoplasmic (Cyt) Cdc55-GFP in each individual cell was plotted. Horizontal lines indicate means. *n* = 8–13 for each category. G1, unbudded cells; G2, budded with a single nucleus; M, large budded cells with dividing or divided nuclei. P-value was calculated by Student's unpaired *t* test.

nucleus (Fig. 1 B). Nuclear localization of Cdc55-GFP was observed throughout the cell cycle, which is consistent with the previous study using GFP-Cdc55 (Gentry and Hallberg, 2002) or 3HA-Cdc55 (Queralt et al., 2006) and with the fact that several Cdc55–PP2A targets, such as Esp1 and Net1, are localized in the nucleus or the nucleolus (Queralt et al., 2006; Clift et al., 2009).

To gain further insight into Zds1 and Cdc55 localization, we examined the interdependence of their localization. Localization of Zds1-GFP to the bud cortex and to the bud neck was severely impaired in *cdc55Δ* but still remained excluded from the nucleus (Fig. S2). We also examined the localization of Zds1ΔC800-GFP, which lacks the C-terminal Cdc55-binding domain (Yasutis et al., 2010). Zds1ΔC800-GFP localization to the bud cortex and at the bud neck was also defective but still excluded from nucleus (Fig. S2). Similarly, Cdc55-GFP failed to localize to the bud cortex and at the bud neck in the *zds1Δ zds2Δ* cells (Fig. 1 C). Thus, cortical and bud neck localization of Zds1 and Cdc55 is interdependent.

Interestingly, Cdc55-GFP accumulated more in the nucleus in the *zds1Δ zds2Δ* cells (Fig. 1 C). In contrast, overexpression of *ZDS1* from a strong *GAL1* promoter resulted in enhancement of cortical, cytoplasmic, and vacuolar membrane localization of Cdc55-GFP and a reduction of the GFP signal from the nucleus (Fig. 1 C).

Quantification of mean GFP fluorescence intensity in the nucleus and in the cytoplasm further confirmed that Zds1/Zds2 affect the nuclear to cytoplasmic ratio of Cdc55-GFP. In wild-type cells, Cdc55-GFP was more concentrated in the nucleus for cells both in G1 and G2 than cells in mitosis (Fig. 1 D). Reduction of nuclear Cdc55-GFP signal in mitosis was dependent on Zds1/Zds2 (Fig. 1 D). Nuclear Cdc55-GFP signal was significantly increased not only in mitotic cells but also in G2 cells for the *zds1Δ zds2Δ* strain (Fig. 1 D), suggesting that Zds1/Zds2 prevent nuclear accumulation of Cdc55 throughout the cell cycle.

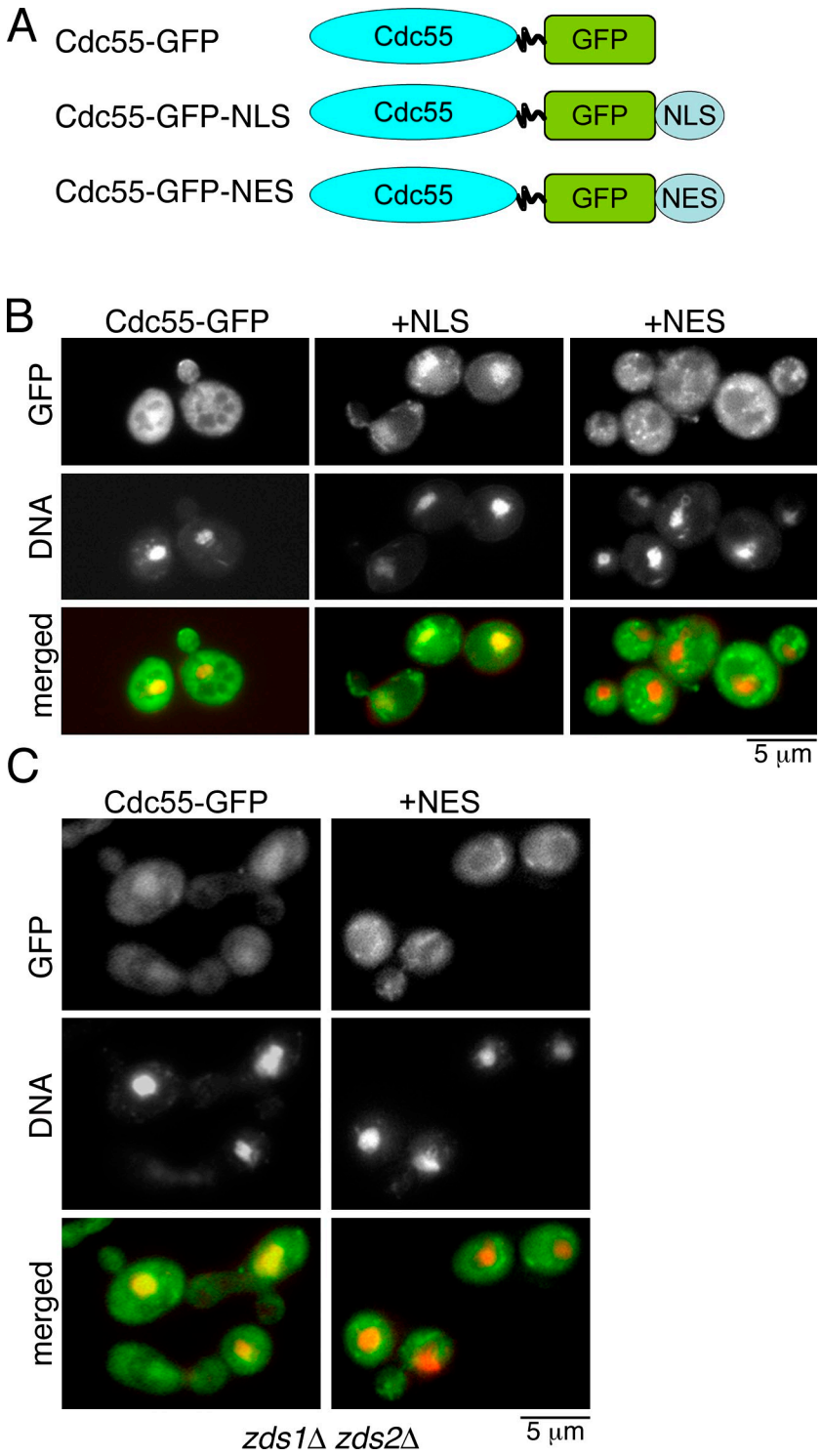

Figure 2. **Generation of nuclear and cytoplasmic Cdc55 mutants.** (A) Schematic representation of Cdc55 mutants. (B) Localization of Cdc55-NLS and Cdc55-NES in living cells. (C) Localization of Cdc55 and Cdc55-NES in *zds1Δ zds2Δ* cells. For all images in B and C, the DNA was stained with Hoechst 33258.

These observations prompted us to test whether Zds1/Zds2 promote mitotic progression by preventing nuclear accumulation of Cdc55.

CDC55 mutants specifically targeted to the cytoplasm or the nucleus

To test whether the nucleocytoplasmic distribution of Cdc55 is important for proper mitotic progression, we first generated two novel mutants of *CDC55* (Fig. 2 A). By adding a strong nuclear export signal (NES; AAALALKLAGLNI; Hodel et al., 2006) to Cdc55-GFP, we were able to make a *cdc55-NES* mutant, which is constitutively cytoplasmic (Fig. 2 B) even in the absence of both Zds1/Zds2 (Fig. 2 C). We also made a *cdc55-NLS* mutant by the addition of a strong NLS of the SV40 antigen (AAAPKKKRKVG; Hodel et al., 2006), which is constitutively nuclear (Fig. 2 B). With these unique *cdc55* mutants, we are now able to test our hypothesis that nucleocytoplasmic distribution of Cdc55 under the control of Zds1 is important for mitotic progression.

Cytoplasmic Cdc55 and Zds1/Zds2 promote mitotic entry

In our strain background (BY4741), all the *cdc55Δ*, *zds1Δ*, *zds2Δ*, or *zds1Δ zds2Δ* strains were viable. However, *cdc55Δ* and *zds1Δ zds2Δ* strains exhibited severe growth defects at a lowered temperature (Fig. 3 A) as it has been previously described in the other genetic background (Bi and Pringle, 1996; Minshull et al., 1996). The cold-sensitive growth of these mutants likely reflects defective mitotic entry, as the cold-sensitive growth defect of *cdc55* is rescued by a *CDC28Y19F* mutation (Wang and Burke, 1997). Consistent with their positive roles in mitotic entry, both *cdc55Δ* and *zds1Δ zds2Δ* exhibited abnormally elongated bud morphology at all the temperatures tested (Fig. 3 B and Fig. S3 B). In agreement with a similar domain analysis on Zds2 (Yasutis et al., 2010), we also found that a conserved Cdc55-binding region of Zds1 was important for Zds1 functions. In a *zds2Δ* background, Zds1 mutants lacking the Cdc55-binding domain (*zds1ΔC800* and *zds1ΔC400*) exhibited growth defects at a low temperature and a hyperelongated morphology similar to *zds1Δ* (Fig. S3 C). Furthermore, overexpression of the Cdc55-binding domain of Zds1 (aa 801–913) was sufficient to rescue the cold sensitivity and elongated morphology of *zds1Δ zds2Δ* cells (Fig. S3 D). Thus, Zds1 function in mitotic entry is likely mediated via Cdc55 binding.

cdc55-NES mutant cells did not display abnormal morphology. However, *cdc55-NLS* mutant cells displayed a highly elongated morphology like *cdc55Δ* or *zds1Δ zds2Δ* mutants (Fig. 3 B). To confirm that the elongated morphology is caused by a delay in mitotic entry, we deleted *SWE1* from these strains. Consistent with the idea that Zds1/Zds2 and Cdc55 promote mitotic entry via prevention of the inhibitory phosphorylation of Cdc28 by Swe1 (Fig. 3 C), deletion of *SWE1* was sufficient to suppress the elongated morphology of *cdc55Δ*, *zds1Δ zds2Δ*, and *cdc55-NLS* cells (Fig. 3 B).

Next, we confirmed that the elongated morphology of *cdc55Δ* and *zds1Δ zds2Δ* mutants was indeed caused by abnormal phosphorylation of Cdc28 at Tyr-19 (Minshull et al., 1996; Yang et al., 2000; Pal et al., 2008). We synchronized the cells in G2/M by nocodazole (a microtubule-destabilizing drug) treatment for 3 h and monitored phosphorylation status of Cdc28 by Western blotting using a phosphospecific antibody specific to Cdc28-Y19. As expected, the levels of Cdc28-Y19 phosphorylation were high in the cells lacking the Cdc28 phosphatase *mih1Δ* and were absent in the *swe1Δ* cells (Fig. 3 D). Mutants that showed the elongated morphology (*cdc55Δ*, *zds1Δ zds2Δ*, and *cdc55-NLS*) have significantly elevated levels of Cdc28-Y19 phosphorylation, suggesting a prolonged delay in G2, and this phosphorylation was fully eliminated by deleting *SWE1* in these strains (Fig. 3 D). These morphological and biochemical data are consistent with the idea that both Cdc55 and Zds1/Zds2 function together to inactivate Swe1 for mitotic entry.

The observation that the *cdc55-NLS* (nuclear), but not *cdc55-NES* (cytoplasmic), mutant was defective in mitotic entry suggests that the cytoplasmic localization of Cdc55 is important for mitotic entry. To test this hypothesis, we examined whether *cdc55-NES* can bypass the requirement of Zds1/Zds2 not only for cytoplasmic localization of Cdc55 (Fig. 2 C) but also for mitotic entry. Indeed, both the abnormally elongated morphology (Fig. 3 B) and elevated phosphorylation of Cdc28-Y19 in *zds1Δ zds2Δ* cells (Fig. 3 D) were almost fully rescued by the *cdc55-NES* mutation. Furthermore, the cold-sensitive growth defect of *zds1Δ zds2Δ* was also rescued by the *cdc55-NES* (Fig. 3 A). Thus, the cytoplasmic localization of Cdc55 mediated by Zds1/Zds2 is required and sufficient for normal mitotic entry. The finding that the *cdc55-NES* mutation bypassed the requirement of Zds1/Zds2 for mitotic entry suggests that the most important function of Zds1/Zds2 in mitotic entry is to promote Cdc55 export from the nucleus or to maintain Cdc55 in the cytoplasm.

Nuclear Cdc55 interferes with mitotic exit

It is known that Cdc55–PP2A is an inhibitor of mitotic exit (Queralt et al., 2006; Wang and Ng, 2006; Yellman and Burke, 2006). Cdc55 inhibits Cdc14 release from the nucleolus, a key step for mitotic exit, by preventing Net1/Cfi1 phosphorylation. In contrast, Zds1/Zds2 were recently shown to be activators of mitotic exit (Queralt and Uhlmann, 2008). Because Zds1/Zds2 and Cdc55 physically interact and the mitotic exit defect of the *zds1Δ zds2Δ* cells was suppressed by deletion of *CDC55*, it has been proposed that Zds1/Zds2 are inhibitors of Cdc55–PP2A activity (Fig. 4 A).

To examine the role of Cdc55 and Zds1/Zds2 proteins in mitotic exit, we first tested for genetic interactions using *LTE1* deletion strains. Lte1 is a nonessential component of the mitotic exit network and shows synthetic lethality with genes involved in the early anaphase release of Cdc14 (FEAR) pathway (Stegmeier et al., 2002). Consistent with the requirement of Zds1/Zds2 in the FEAR pathway, we found a synthetic lethality between *lte1Δ* and *zds1Δ zds2Δ* (Fig. 4 B). We hypothesized that this synthetic lethality is derived from the nuclear accumulation of Cdc55. Consistent with our hypothesis, *cdc55-NLS* showed synthetic lethality with *lte1Δ* (Fig. 4 B). In contrast, *cdc55-NES* showed no synthetic growth defects with *lte1Δ* (Fig. 4 B).

To further confirm that nuclear Cdc55 is preventing the FEAR pathway, we analyzed the timing of Cdc14 release from the nucleolus using spindle length as an internal marker for mitotic progression. Wild-type, *cdc55Δ*, and *cdc55-NES* cells partially released Cdc14 in early anaphase (spindle length of 3–7 µm) and fully released Cdc14 in late mitosis (spindle length >7 µm; Fig. 4 C). In contrast, Cdc14 release in early anaphase was significantly impaired in *zds1Δ zds2Δ* and *cdc55-NLS* cells (Fig. 4 C). Furthermore, Cdc14 release was partially impaired in the later stages of mitosis in *zds1Δ zds2Δ* and *cdc55-NLS* cells (Fig. 4 C). These results suggest that nuclear Cdc55 interferes with mitotic exit by inhibiting the release of Cdc14 from the nucleolus. In support of this hypothesis, artificial exclusion of Cdc55 from the nucleus by the *cdc55-NES* mutation fully rescued Cdc14 release defects of the *zds1Δ zds2Δ* mutant (Fig. 4 C). Thus, exclusion of Cdc55 from the nucleus is the key function of Zds1/Zds2 to promote mitotic exit.

Zds1/Zds2 promote mitotic entry via Cdc55 function in the cytoplasm

In this study, we show that Zds1/Zds2 proteins promote cytoplasmic functions of Cdc55–PP2A (Fig. 5). In the absence of

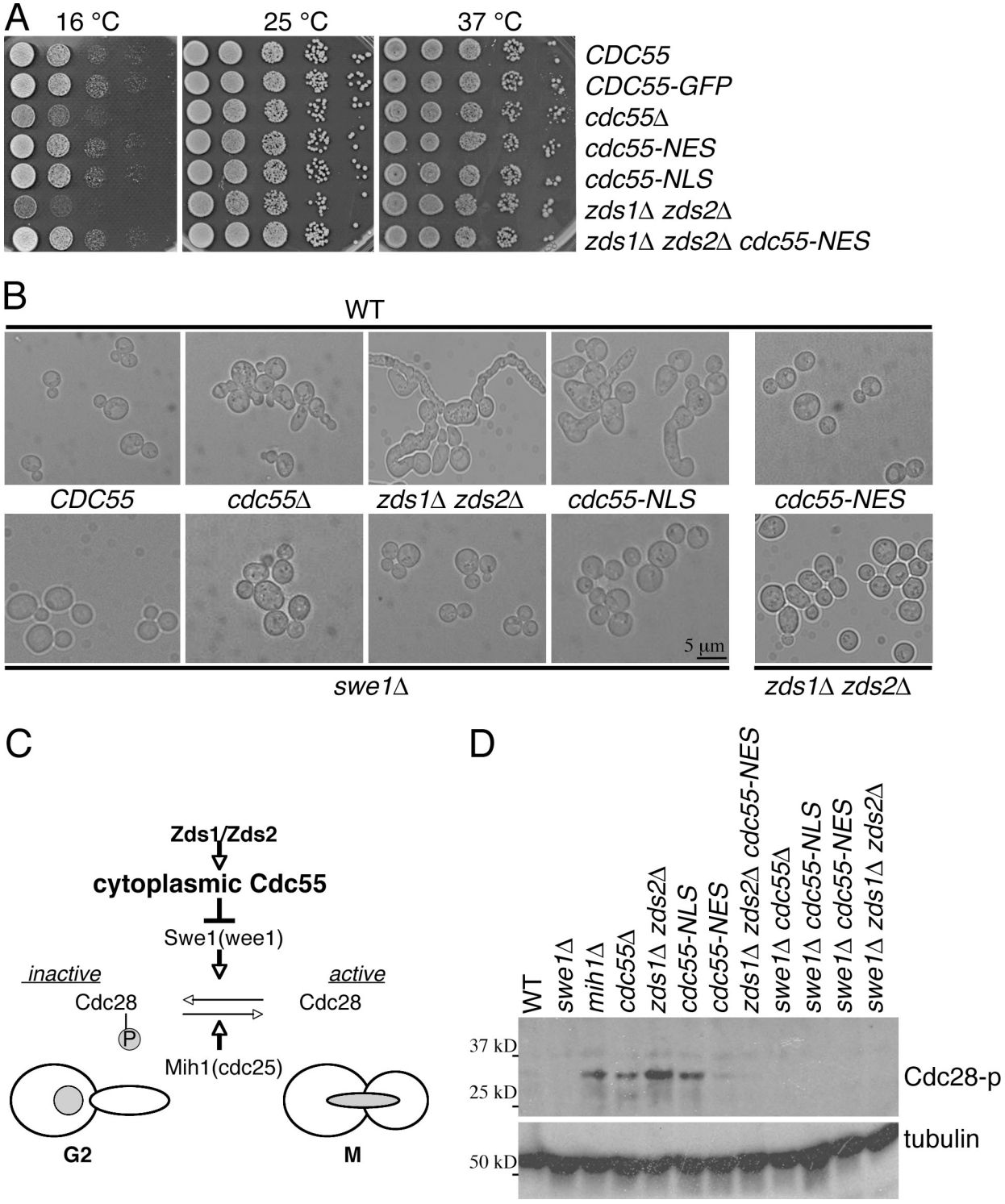

Figure 3. **Zds1/Zds2 and cytoplasmic Cdc55 promote mitotic entry.** (A) Serial dilutions of indicated strains spotted on YPD (yeast peptone dextrose) at different temperatures. (B) Representative images of cells with the indicated genotypes in the presence (top row) or absence (bottom row) of *SWE1* except for *cdc55-NES zds1Δ zds2Δ*. Cells were grown in YPD media at 24°C. (C) Our model of mitotic entry regulation by Zds1/Zds2 and cytoplasmic Cdc55. (D) Cytoplasmic Cdc55 is required for dephosphorylation of Cdc28-Y19. Cells were treated with 15 µg/ml nocodazole for 3 h to prevent mitotic progression before cell lysate extraction for Western blotting. Antiphospho–Cdc2-Y15 and antitubulin (loading control) antibodies were used for detection. P, phosphorylation. WT, wild type.

Zds1/Zds2, Cdc55 accumulates in the nucleus, resulting in defective mitotic entry. Reduction of cytoplasmic Cdc55 is likely the reason for the G2 delay because the *cdc55-NLS* mutant, whose gene product is dominantly nuclear, phenocopies *zds1Δ zds2Δ*. The G2 delay is not caused by the nuclear accumulation of Cdc55 because the elongated bud morphology of *cdc55-NLS* was rescued by an extra copy of *CDC55* (Fig. S2). In contrast, the *cdc55-NES* mutant, whose gene product is dominantly cytoplasmic, is

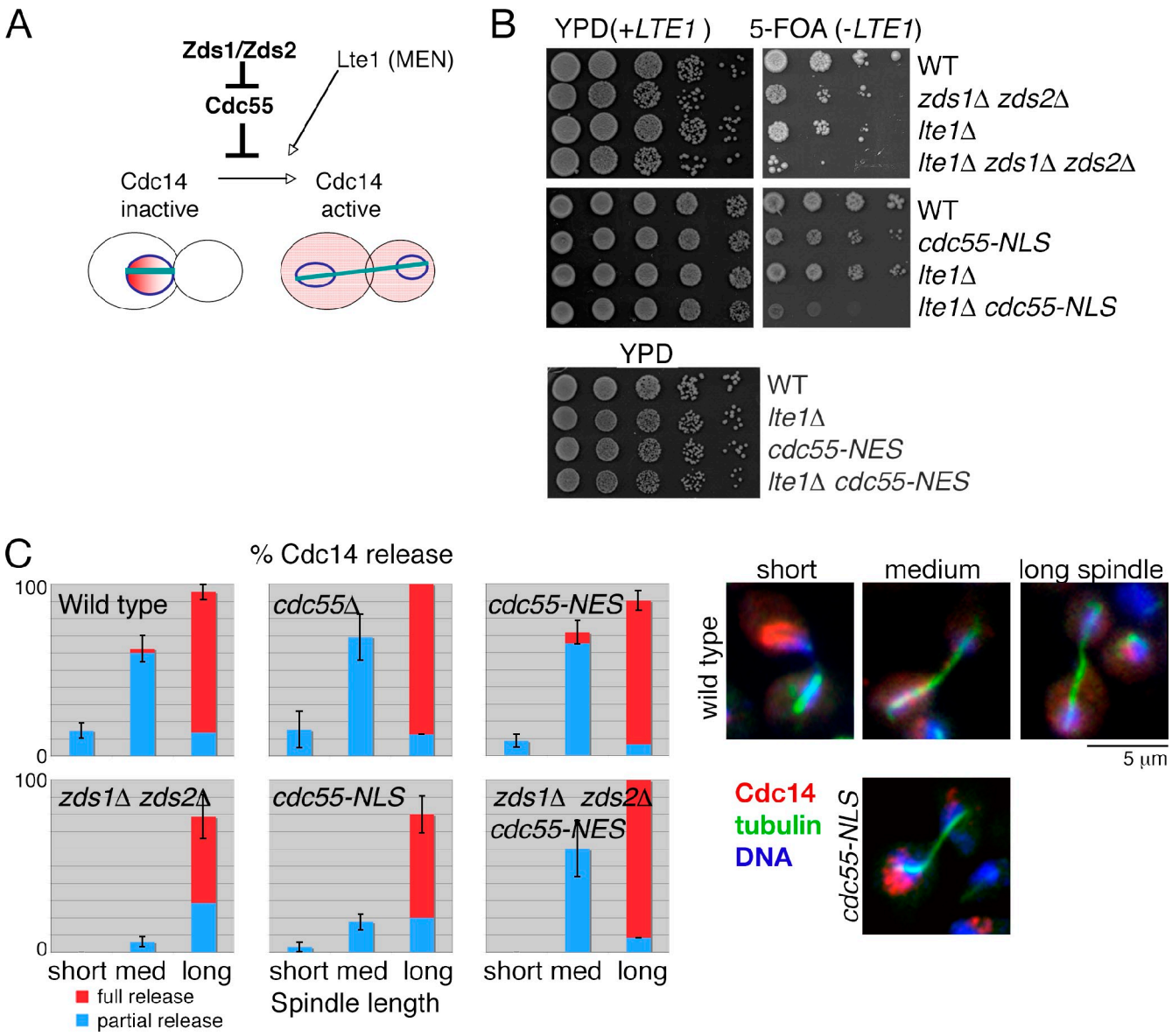

Figure 4. **Nuclear Cdc55 prevents mitotic exit by inhibiting Cdc14 release from the nucleolus.** (A) Model of Cdc14 release controlled by Cdc55 and Zds1/Zds2 proteins. (B) Nuclear Cdc55 is toxic to *lte1Δ* mutant cells. Serial dilutions of the indicated strains containing a complementing *LTE1* plasmid (with a *URA3* marker) were grown on YPD plates or plates containing 5-fluoroorotic acid (5-FOA) to counter the select *LTE1* plasmid at 30°C for 3 d (top). *cdc55-NES* and *lte1Δ* are not synthetically lethal. Serial dilutions of the indicated strains were spotted on YPD at 24°C (bottom). (C) Quantification of Cdc14 release during anaphase. Mitotic cells were categorized into short spindle (0–3 µm), medium (med) spindle (3–7 µm), and long spindle (>7 µm). In each category, Cdc14 release status was classified as partial release or full release. Error bars are SEM. *n* = 133 (wild type), 33 (*cdc55*), 122 (*cdc55-NES*), 123 (*cdc55-NLS*), 107 (*zds1 zds2*), and 65 (*zds1 zds2 cdc55-NES*). Examples of wild-type Cdc14 localization during anaphase are on the top right, and an example of a midanaphase *cdc55-NLS* cell is shown on the bottom right. Cdc14 and tubulin were visualized by indirect immunofluorescence microscopy using the anti-Cdc14 antibody and α-tubulin antibody. DNA was stained with DAPI. MEN, mitotic exit network.

fully competent in mitotic entry and can even rescue the mitotic entry defects of *zds1Δ zds2Δ* cells. Our data suggest that the primary function of Zds1/Zds2 during the cell cycle is to keep Cdc55–PP2A in the cytoplasm because not only the mitotic entry defect but also the cold-sensitive growth defect of the *zds1Δ zds2Δ* cells was rescued by the *cdc55-NES*. This bypass effect also suggests that Zds1/Zds2 is not required for the catalytic activity or substrate specificity of Cdc55–PP2A in the cytoplasm.

Our genetic data suggest that the critical target of Cdc55 in mitotic entry is Swe1 because mitotic entry defects of *cdc55Δ*, *zds1Δ zds2Δ*, and *cdc55-NLS* are almost fully rescued by deletion of *SWE1*. Dephosphorylation of Mih1 is regulated by Cdc55 (Pal et al., 2008; Wicky et al., 2011). Therefore, Mih1 is also a target of Zds1/Zds2–Cdc55–PP2A. However, *mih1Δ* cells have a relatively milder defect in mitotic entry (Russell et al., 1989; Pal et al., 2008) compared with the *cdc55Δ* or the *zds1Δ zds2Δ*, suggesting that Mih1 is not the only target. It is important to mention that both Swe1 and Mih1 are regulated by nucleocytoplasmic transport, and a *swe1* mutant defective in nuclear export is a more potent inhibitor of mitotic entry (Keaton et al., 2008). Thus, we speculate that Swe1 inactivation by Zds1/Zds2–Cdc55–PP2A takes place in the cytoplasm, which is also

A

	Cdc55 localization	Mitotic entry	Mitotic exit
WT	cytoplasm/nucleus	+	+
cdc55Δ	-	-	+
zds1Δ zds2Δ	nucleus	-	-
cdc55-NLS	nucleus	-	-
cdc55-NES	cytoplasm	+	+
zds1Δ zds2Δ +cdc55-NES	cytoplasm	+	+

B

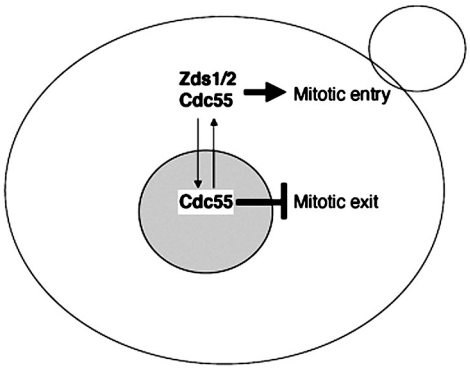

Figure 5. **Summary and model of Zds1/Zds2–Cdc55–PP2A regulation of mitosis.** (A) Summary table of Cdc55 localization and mitotic entry and exit defects. (B) A model of the regulation of mitosis by Cdc55 and Zds1/Zds2 proteins. When bound to Zds1/Zds2, Cdc55 functions in the cytoplasm to promote mitotic entry by activating the Cdk. Exclusion of Cdc55 from the nucleus by Zds1/Zds2 proteins is also required for mitotic exit by preventing Cdc14 release. In the absence of Zds1/Zds2, Cdc55 remains in the nucleus, leading to a delay in mitotic entry and mitotic exit. See Results and discussion for more details. WT, wild type.

consistent with the fact that Swe1-inactivating kinases are also in the cytoplasm (McMillan et al., 1999a; Shulewitz et al., 1999; Asano et al., 2005).

The role and significance of the bud tip and the bud neck localization of Zds1/Zds2 and Cdc55 remain an area of interest for future research. Zds1/Zds2 interact with cell polarity proteins of the Cdc42- and the Rho1-signaling pathways both physically and genetically (Bi and Pringle, 1996; Drees et al., 2001; Sekiya-Kawasaki et al., 2002), we speculate that Zds1/Zds2 also have direct roles in cell polarity beyond controlling Cdk1 activity. Alternatively, cell polarity factors might influence cell cycle progression via Zds1/Zds2–Cdc55 regulation.

Zds1/Zds2 promote mitotic exit by nuclear exclusion of Cdc55

It was proposed that Zds1/Zds2, together with the separase Esp1, inhibit Cdc55–PP2A catalytic activity to promote Cdc14 release in anaphase (Queralt and Uhlmann, 2008). Given the fact that Esp1, Cdc55, and its substrate Net1 are all in the nucleus and/or nucleolus, the Zds1/Zds2-mediated inhibition of Cdc55–PP2A must occur within the nucleus. Our genetic data are generally consistent with Queralt and Uhlmann (2008), and we do not exclude the possibility that Zds1/Zds2 directly inhibit Cdc55–PP2A activity specific to Net1, but the following evidence supports our hypothesis that Zds1/Zds2 promotes mitotic exit primarily by excluding Cdc55 from the nucleus (Fig. 5).

First, the majority of Zds1/Zds2 is in the cytoplasm. Given that Zds1 forms a stoichiometric complex with Cdc55–PP2A, it is unlikely that a minor fraction of Zds1/Zds2 in the nucleus is sufficient for inhibiting Cdc55–PP2A in the nucleus. Second, nuclear-accumulated Cdc55-NLS dominantly inhibits mitotic exit even in the presence of Zds1/Zds2, suggesting that nuclear Cdc55–PP2A activity is not effectively inhibited by Zds1/Zds2. Third, the nuclear Cdc55 signal is significantly reduced during mitosis in a Zds1/Zds2-dependent manner. It is important to mention that reduction of Cdc55 intensity from the nucleus was underestimated in our assay because it was impossible to exclude contaminating strong cytoplasmic signals from other focal planes with our standard fluorescent microscope. Our genetic data are also consistent with our hypothesis that nuclear exclusion of Cdc55 is the key function of Zds1/Zds2 because *zds1Δ zds2Δ* defects in Cdc14 release are fully rescued by the *cdc55-NES* mutation.

All these lines of evidence suggest that either cytoplasmic Cdc55 or nuclear exclusion of Cdc55 is important for mitotic exit. We favor the latter hypothesis because deletion of *CDC55* is sufficient for rescuing the mitotic exit defects of *zds1Δ zds2Δ* (Queralt and Uhlmann, 2008), and overexpression of *CDC55* prevents mitotic exit and is toxic to mitotic exit network mutants (Wang and Ng, 2006).

Evolutional conservation of PP2A regulation

Recent studies revealed that the PP2A–B55 (a counterpart of PP2A–Cdc55) complex is the major regulator of mitotic entry and exit in animal cells (Mochida et al., 2009; Schmitz et al., 2010). In the *Xenopus laevis* egg and in HeLa cells, PP2A–B55 activity is directly inhibited by Arpp19 and α-endosulfine during mitosis (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). Budding yeast Igo1/Igo2 share sequence homology to Arpp19 and α-endosulfine (Dulubova et al., 2001; Talarek et al., 2010), suggesting a possible conservation of PP2A inhibition during mitosis.

On the other hand, Zds1 is highly conserved in fungi, including pathogenic *Candida albicans* and *Cryptococcus neoformans*, but no obvious homologue was found in higher eukaryotes. Considering the fact that a key function of Zds1/Zds2 is to regulate the nucleocytoplasmic distribution of Cdc55–PP2A, it is possible that Zds1/Zds2 has uniquely evolved in fungi, which undergo "closed mitosis," but is not required in higher eukaryotes, which undergo "open mitosis." Alternatively, it is also possible that functional counterparts to the zillion different screens protein family exist in higher eukaryotes but are not easily recognizable by their primary sequences, such as the case for Cdk inhibitors.

Materials and methods

Yeast genetics

All yeast strains used in this study were isogenic or congenic to BY4741 (*MATa leu2Δ0 his3Δ1 met15Δ0 ura3Δ0*, obtained from Thermo Fisher Scientific). Standard yeast genetics was used to generate the strains. Yeast strains are listed in Table S1. PY3295 and SY strains were gifts from D. Pellman (Dana-Farber Cancer Institute, Boston, MA). D. Lew (Duke University, Durham, NC) provided a *SWE1* gene knockout plasmid. Gene deletions or modifications were performed with PCR-mediated one-step gene replacement using pFA6a vectors provided by J. Pringle (Stanford University, Stanford, CA; Longtine et al., 1998) and confirmed by PCR. For tagging *CDC55*, *ZDS1*, and *ZDS2* with GFP, a flexible amino acid linker (GGSGGS) was introduced between the target protein and GFP. For generation of the *cdc55-NES* and *cdc55-NLS* mutants, the NES sequence

(AAALALKLAGLNI) and SV40 NLS sequence (AAAPKKKRKVG) were directly introduced after the GFP sequence of *CDC55-GFP* by PCR-mediated one-step gene replacement (Longtine et al., 1998).

Biochemistry

Protein extracts were prepared as previously described (von der Haar, 2007). In brief, cells were treated with the solution containing 0.1 N NaOH, 0.05 M EDTA, 2% SDS, and 2% β-mercaptoethanol and immediately boiled for 10 min. The samples were neutralized with acetic acid, and SDS sample buffer (Boston Bioproducts) was added. Rabbit antiphospho–Cdc2-Y15 antibody (Cell Signaling Technology) and mouse anti-GFP antibody (Millipore) were purchased from the commercial source. Rabbit anti-Rho1 antibody was custom made. Rabbit antitubulin antibody is a gift from B. Goode (Brandeis University, Waltham, MA). HRP-conjugated secondary antibodies were obtained from Millipore, and proteins were detected by an enhanced chemiluminescence system (ECL Plus; GE Healthcare).

Fluorescence microscopy

Fluorescence images were acquired with a fluorescence microscope (Eclipse E600; Nikon) equipped with a charge-coupled device camera (DC350F; Andor) with 100×, NA of 1.45, or 60×, NA 1.4, oil objectives. The images were captured and analyzed with NIS-Elements software (Nikon), and the figures were processed and assembled in Photoshop (Adobe).

For indirect immunofluorescence methods, we fixed the cells with 4% formaldehyde for 10 min at room temperature, and the cell walls were digested with Zymolyase 20T (Zymo Research). The mitotic spindle was immunostained with the YOL1/34 rat monoclonal antibody (AbD Serotec) followed by an FITC-conjugated anti–rat antibody (Jackson ImmunoResearch Laboratories, Inc.). Cdc14 was immunostained with sc-12045 polyclonal antibody (Santa Cruz Biotechnology, Inc.) followed by a CY3-conjugated anti–goat antibody (Rockland Immunochemicals, Inc.). DAPI was used to visualize the nucleus in fixed cells. For live-cell imaging, all strains were grown in synthetic media at room temperature. We used Hoechst 33258 (AnaSpec) at a 2-µM concentration in PBS to stain the nucleus. For quantifying the nucleocytoplasmic ratio of Cdc55-GFP, mean nuclear fluorescence intensity and mean cytoplasmic fluorescence intensity were calculated with NIS-Elements software.

Online supplemental material

Fig. S1 shows that Zds2, like Zds1, is excluded from the nucleus. Fig. S2 shows that cortical and bud neck localization of Zds1 and Cdc55 are interdependent. Fig. S3 shows that the conserved Cdc55-binding region of Zds1 is important for Zds1 function in mitotic entry. Table S1 contains the list of yeast strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201101134/DC1.

We thank David Pellman for the discussion and support. We thank David Pellman, Daniel Lew, John Pringle, and Bruce Goode for the gift of reagents and Erin Jonasson for her critical reading of the manuscript and for discussion.

This paper was supported by a Massachusetts Life Sciences Center grant and by a start-up fund of Brandeis University.

Submitted: 26 January 2011
Accepted: 31 March 2011

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