The Zds proteins control entry into mitosis and target protein phosphatase 2A to the Cdc25 phosphatase

Sidonie Wicky‡, Hendri Tjandra‡, David Schieltz, John Yates III, and Douglas R. Kellogg

Abstract: The Wee1 kinase restrains entry into mitosis by phosphorylating and inhibiting cyclin-dependent kinase 1 (Cdk1). The Cdc25 phosphatase promotes entry into mitosis by removing Cdk1 inhibitory phosphorylation. Experiments in diverse systems have established that Wee1 and Cdc25 are regulated by protein phosphatase 2A (PP2A), but a full understanding of the function and regulation of PP2A in entry into mitosis has remained elusive. In budding yeast, entry into mitosis is controlled by a specific form of PP2A that is associated with the Cdc25 regulatory subunit (PP2ACdc25S). We show here that related proteins called Zds1 and Zds2 form a tight stoichiometric complex with PP2ACdc25S and target its activity to Cdc25 but not to Wee1. Conditional inactivation of the Zds proteins revealed that their function is required primarily at entry into mitosis. In addition, Zds1 undergoes cell cycle–dependent changes in phosphorylation. Together, these observations define a role for the Zds proteins in controlling specific functions of PP2ACdc25S and suggest that upstream signals that regulate PP2ACdc25S may play an important role in controlling entry into mitosis.

Introduction: Mitosis is initiated by synthesis of mitotic cyclins, which bind and activate cyclin-dependent kinase 1 (Cdk1) (Morgan, 2007). The Wee1 kinase inhibits Cdk1 via phosphorylation of a conserved tyrosine, which restrains entry into mitosis (Nurse, 1975; Nurse et al., 1976; Russell and Nurse, 1987). The Cdc25 phosphatase promotes entry into mitosis by removing the inhibitory phosphate (Russell and Nurse, 1986; Gautier et al., 1991; Kumagai and Dunphy, 1991). In both fission yeast and budding yeast, Wee1 mutants cause premature mitosis at a reduced cell size, whereas Cdc25 mutants cause delayed mitosis and increased cell size (Nurse, 1975; Fantes and Nurse, 1977; Russell and Nurse, 1986; Russell et al., 1989; Rupes et al., 2001; Jorgensen et al., 2002; Rupes et al., 2002; Harvey and Kellogg, 2003; Kellogg, 2003; Harvey et al., 2005; Pal et al., 2008; Rahal and Amon, 2008). These observations led to the hypothesis that Wee1 and Cdc25 mediate a cell size checkpoint that delays mitosis until a critical size has been reached. It also has been proposed that Wee1 and Cdc25 monitor the status of the actin cytoskeleton to link entry into mitosis to cellular morphogenesis (Lew and Reed, 1995; McMillan et al., 1998; Gachet et al., 2001; Lew, 2003). It has been difficult, however, to unambiguously define the cellular events that are monitored by Wee1 and Cdc25 because the upstream signals that control their activity are poorly understood. Elucidation of these signals is therefore an essential step toward understanding entry into mitosis.

Cdk1 is an important regulator of Wee1. The budding yeast Wee1 homologue is referred to as Swe1. Mitotic Cdk1 directly phosphorylates Swe1 on multiple Cdk1 consensus sites, which activates Swe1 to bind, phosphorylate, and inhibit Cdk1 (Harvey et al., 2005). Cdk1 also activates Swe1 in human cells, which suggests that this mechanism is conserved (Deibler and Kirschner, 2010). The initial activating phosphorylation of Swe1 by Cdk1 is followed by further phosphorylation events that lead to full hyperphosphorylation of Swe1.
Wee1 (Harvey et al., 2005). In yeast and vertebrates, it appears that mitotic Cdk1, when present at sufficiently high levels, can fully hyperphosphorylate and inactivate Wee1 family members (Tang et al., 1993; Mueller et al., 1995; Harvey et al., 2005). However, multiple kinases are required for full hyperphosphorylation of Wee1 family members in vivo, and their relative contributions are unclear (Wu and Russell, 1993; Mueller et al., 1995; Shulewitz et al., 1999; Sreenivasan and Kellogg, 1999; Asano et al., 2005). Phosphorylation of Xenopus Wee1 is further controlled by protein phosphatase 2A (PP2A), although the role played by PP2A is unknown (Tang et al., 1993).

Cdk1 is also an important regulator of Cdc25. In Xenopus, Cdk1 directly phosphorylates Cdc25, which stimulates a basal interphase control of Cdc25 via the Chk1 kinase and the PP1 phosphatase (Perry and Kellogg, 1999; Asano et al., 1999; Izumi and Maller, 1995). Cdc25-dependent inhibition of Wee1 and activation of Cdc25 are thought to constitute feedback loops that promote activation of Cdk1; however, the mechanisms that trigger the feedback loops are poorly understood. An oft proposed model is that the feedback loops are initiated by signals that stimulate Cdc25, and recent experimental evidence supports a model in which Cdc25 plays a crucial role in triggering full activation of Cdk1 (Deibler and Kirschner, 2010). However, the signals that control Cdc25 in this context are poorly understood. Phosphorylation of Xenopus Cdc25 is controlled by PP2A, yet the precise role of PP2A is unknown (Healy et al., 1991; Izumi et al., 1992; Kumagai and Dunphy, 1992; Clarke et al., 1993; Izumi and Maller, 1995; Zhao et al., 1997; Castilho et al., 2009; Mochida et al., 2009). It also appears that a kinase other than Cdk1 can phosphorylate Cdc25 in interphase, but the identity of the kinase is unknown (Kumagai and Dunphy, 1992; Izumi and Maller, 1995). In vertebrates and fission yeast, DNA damage checkpoint signals control Cdc25 via the Chk1 kinase and the PP1 phosphatase (Perry and Kornbluth, 2007; Wang et al., 2008; Reinhardt and Yaffe, 2009).

The budding yeast homologue of Cdc25, referred to as Mih1, is extensively hyperphosphorylated during interphase and undergoes dephosphorylation during entry into mitosis. The bulk of the hyperphosphorylation that occurs during interphase is dependent on a serine kinase 1, which is encoded by a pair of redundant genes called YCK1 and YCK2 (Robinson et al., 1992, 1993; Pal et al., 2008). Loss of Yck1/2 causes defects in regulation of Cdk1 inhibitory phosphorylation; however, it is unclear whether Yck1/2 play a positive or negative role in regulation of Mih1 because they may also regulate Swe1 (Pal et al., 2008). It is also unclear whether Yck1/2 phosphorylate Mih1 directly because thus far they have been found to phosphorylate Mih1 only weakly in vitro (Pal et al., 2008). Mih1 is phosphorylated by Cdk1 during mitosis and may therefore be subject to positive feedback, as proposed for vertebrate Cdc25 (Pal et al., 2008). The dephosphorylation of Mih1 that occurs as cells enter mitosis is dependent on PP2A (Pal et al., 2008). Inactivation of PP2A (Pal et al., 2008) causes increased Cdk1 inhibitory phosphorylation and a prolonged delay in entry into mitosis (Minshall et al., 1996; Yang et al., 2000; Pal et al., 2008). Similarly, inactivation of PP2A (Pal et al., 2008) catalytic subunits after cells have passed through the S phase causes delayed entry into mitosis and severe defects in activation of mitotic Cdk1 (Lin and Arndt, 1995). The phenotypes caused by cdc55Δ are largely rescued by swe1Δ or by a mutant version of Cdk1 that can not be inhibited by Swe1. These observations demonstrate that a key function of PP2A (Pal et al., 2008) is to regulate Cdk1 inhibitory phosphorylation (Lin and Arndt, 1995; Yang et al., 2000; Pal et al., 2008).

In summary, there has been significant progress toward elucidating the mechanisms that regulate Wee1 and Cdc25 family members, but we still lack a full understanding of the sequence of events that triggers removal of Cdk1 inhibitory phosphorylation and entry into mitosis. Key questions remain unanswered: How are the feedback loops that activate Cdc25 and inactivate Wee1 initiated? How is regulation of Wee1 and Cdc25 linked to upstream checkpoint signals? How are Cdc25 family members activated during entry into mitosis? What is the role of PP2A in entry into mitosis?

To gain new insight into the upstream signals that control entry into mitosis via Wee1 and Cdc25 family members, we characterized the budding yeast Zds1 and Zds2 proteins. Previous work found that Zds1 and Zds2 are required for normal control of Cdk1 inhibitory phosphorylation, but the underlying mechanisms were unknown. Zds1 and Zds2 are redundant paralogues: Loss of either protein does not cause a severe phenotype, but cells lacking both are barely viable, show a prolonged delay at the G2/M transition, and become highly elongated (Bi and Pringle, 1996; Yu et al., 1996). These phenotypes are rescued by swe1Δ, which indicates that they are caused by increased Cdk1 inhibitory phosphorylation (Ma et al., 1996; McMillan et al., 1999). The elongated cell phenotype observed in mutants that cause increased Cdk1 inhibitory phosphorylation is due to the fact that mitotic Cdk1 is required for repression of polar bud growth (Surana et al., 1991; Fitch et al., 1992). Two general models could explain the role of Zds1/2 in controlling Cdk1 inhibitory phosphorylation. They may be required for execution of events that occur early in the cell cycle that are monitored by a Swe1-dependent checkpoint. Alternatively, Zds1/2 could play a more direct role in controlling the activity of Swe1 or Mih1, perhaps serving to relay checkpoint signals that determine when it is appropriate to enter mitosis. To help distinguish these models, we analyzed the function and regulation of Zds1/2 during the cell cycle, with the goal of defining how they control Cdk1 inhibitory phosphorylation.

RESULTS
Zds1 and Zds2 execute a function required for normal passage through mitosis
Phenotypes observed previously in zds1Δ zds2Δ cells suggested that Zds1/2 play a role in mitosis (Bi and Pringle, 1996; Yu et al., 1996; Queralt and Uhlmann, 2008). However, zds1Δ zds2Δ cells have severe cell-cycle defects, are difficult to synchronize, and rapidly accumulate suppressors. These factors have made it difficult to determine the primary and immediate effects caused by inactivation of Zds1/2. We therefore generated temperature-sensitive alleles of ZDS1 in a zds2Δ background. Seven mutants were isolated that showed a reduced growth rate and an elongated bud phenotype at 37°C. One of the mutants exhibited a growth defect at 34°C (zds1–1 zds2Δ), which allowed us to inactivate Zds1/2 without the transient nonspecific heat shock effects that can be caused by a shift to 37°C (Figure 1A). Observation of Zds1–1 zds2Δ cells shifted to 34°C revealed bud growth defects within 30 min, which indicated rapid inactivation of Zds1 (Figure 1B). The first defect that could be detected at 30 min was a slight elongation of large buds (arrows, Figure 1B). Prolonged incubation at the nonpermissive temperature caused all zds1–1 zds2Δ cells to grow highly elongated buds (Figure 1B). This kind of excessive polar growth can be caused by a failure to properly remove Cdk1 inhibitory phosphorylation, because activation of mitotic Cdk1 is required for repression of polar growth (Fitch et al., 1992; Booher et al., 1993; Lew and Reed, 1993; Ma et al., 1996; Sreenivasan and Kellogg, 1999; Longtine et al., 2000). To test whether inactivation of Zds1/2 causes increased Cdk1 inhibitory phosphorylation, we used a phospespecific antibody to assay Cdk1 inhibitory phosphorylation in synchronized zds1–1 zds2Δ and zds2Δ control cells. In the control cells, Cdk1 inhibitory phosphorylation peaked at 75 min and then began to decline, whereas in the mutant cells...
Cdk1 inhibitory phosphorylation increased throughout the time course (Figure 1C). We also found that the slow growth and elongated cell phenotypes caused by the zds1–1 mutant were rescued by swe1Δ, which demonstrated that these defects were due to misregulation of Swe1, Mih1, or both (data not shown). These observations are consistent with previous work that found that the slow growth and elongated cell phenotypes caused by zds1Δ zds2Δ are rescued by swe1Δ (McMillan et al., 1999).

To further characterize the function of Zds1/2 in cell-cycle progression, we analyzed the timing of mitotic spindle formation in synchronized zds1–1 zds2Δ cells that were released from a G1 arrest at the restrictive temperature. Short mitotic spindles are formed in early mitosis and require a low level of mitotic Cdk1 activity, whereas long spindles are generated during nuclear division (Fitch et al., 1992; Rahal and Amon, 2008). The timing of short spindle assembly was normal in zds1–1 zds2Δ cells; however, the cells underwent a prolonged delay at the short spindle stage (Figure 1D). Previous work found that activation of a Swe1-dependent checkpoint arrest causes cells to undergo a prolonged delay at the short spindle stage of early mitosis (Carroll et al., 1998; Sreenivasan and Kellogg, 1999; Theesfeld et al., 1999). Thus, this result is consistent with the idea that Zds1/2 are required for execution of events that are monitored by the Swe1-dependent checkpoint or that they are responsible for relaying signals that release inhibition of Cdk1 when checkpoint conditions have been satisfied.

To test whether Zds1/2 carry out functions earlier in the cell cycle, we assayed the timing of bud emergence and septin ring formation, both of which occur in the G1 phase. Inactivation of zds1–1 and deletions of genes that encode Cdk1 inhibitory phosphorylation (Sreenivasan and Kellogg, 1999; Longtine et al., 2000). Thus, this result is consistent with the idea that Zds1/2 are responsible for relaying signals that release inhibition of Cdk1 when checkpoint conditions have been satisfied.

To further define a role for Zds1/2 in control of Cdk1 inhibitory phosphorylation, we tested for genetic interactions between zds1Δ and deletions of genes that encode kinases known to be required for normal control of Cdk1 inhibitory phosphorylation, including CLA4, GIN4, and ELM1. Loss of any of these kinases causes a Swe1-dependent G2/M delay and growth of elongated buds (Sreenivasan and Kellogg, 1999; Longtine et al., 2000). We were unable to recover zds1Δ cla4Δ from crosses, which suggests that they are synthetically lethal. Moreover, zds1Δ gin4Δ and zds1Δ elm1Δ cells grew more poorly than either single deletion (data not shown).

Together, these results demonstrate that Zds1/2 execute functions required for normal regulation of Cdk1 inhibitory phosphorylation at G2/M.
Zds1 forms a tight stoichiometric complex with PP2ACdc55

Zds1/2 were found to associate with PP2A in coimmunoprecipitation assays (Gavin et al., 2002; Collins et al., 2007; Queralt and Uhmann, 2008; Yasutis et al., 2010). Canonical PP2A is a trimeric complex composed of a catalytic subunit, a scaffolding subunit, and a regulatory subunit (Janssens and Goris, 2001; Trinkle-Mulcahy and Lamond, 2006). In budding yeast, the catalytic subunit is encoded by a pair of redundant genes called PPH21 and PPH22, and the scaffolding subunit is encoded by the TPD3 gene. There are two main regulatory subunits called Cdc55 and Rts1 that associate with PP2A to form distinct complexes referred to as PP2ACdc55 and PP2ARts1, which perform different functions within the cell (Zhao et al., 1997; Gentry and Hallberg, 2002).

Although Zds1/2 were found to coprecipitate with components of PP2A Cdc55, it was unknown whether they interact with PP2A directly or via additional accessory proteins. It was also unknown whether they interact with multiple PP2A regulatory subunits. To learn more about the association of Zds1/2 with PP2A, we purified Zds1 complexes from yeast. The endogenous copy of Zds1 was tagged with three copies of the influenza hemagglutinin (HA) epitope and placed under the control of the GAL1 promoter. 3XHA-Zds1 complexes were purified by large-scale immunoaffinity chromatography using anti-HA antibodies and peptide elution. We found three proteins that appeared to bind stoichiometrically to Zds1 (Figure 2A). These proteins were identified by mass spectrometry as Cdc55, Tpd3, and Pph21/Pph22. The complex could be purified in the presence of 1 M KCl, which indicated a tight association.

The same protein complex also could be purified by using Zds1–3XHA expressed from its own promoter or by using Cdc55–3XHA expressed from its own promoter (data not shown). Tpd3 and Pph21/Pph22 failed to copurify with Zds1 in cdc55Δ cells: This result indicated that Cdc55 mediates binding of Zds1 to PP2A (Figure 2A). Note that Zds1–3XHA showed a reduced electrophoretic mobility when isolated from cdc55Δ cells. This issue is addressed later in this article. Observation of cdc55Δ cells, in which Zds1/2 fail to interact with PP2A, revealed that they exhibit a morphological phenotype similar to the one observed in zds1Δzds2Δ cells (Figure 2B). Together, these observations suggest that Zds1/2 mediate the functions of PP2ACdc55 via binding to the Cdc55 subunit.

Zds1/2 target dephosphorylation of Mih1 by PP2ACdc55

We next sought to identify targets of the PP2ACdc55–Zds1/2 complex. Swe1 and Mih1 were good candidates because genetic analysis demonstrated that Zds1/2 regulate Cdk1 inhibitory phosphorylation (Figure 1; McMillan et al., 1999). Moreover, previous work demonstrated that Mih1 and Swe1 undergo dramatic cell cycle–dependent changes in phosphorylation that may be regulated by PP2ACdc55 (Sreenivasan and Kellogg, 1999; Harvey et al., 2005; Pal et al., 2008). We therefore used Western blotting to assay phosphorylation of Mih1 and Swe1 in synchronized zds1Δ zds2Δ cells that carry a mutant allele of CDK1 that can not be inhibited by Swe1 (cdk1-Y19F). The cdk1-Y19F allele largely rescued the severe morphological defects and mitotic delay caused by zds1Δ zds2Δ, which allowed the cells to be synchronized (Figure S1). The cdk1-Y19F allele also removed the concern that effects of zds1Δ zds2Δ on regulation of Swe1 or Mih1 could be caused indirectly by inhibition of Cdk1, which is possible because Cdk1 regulates both Swe1 and Mih1 (Harvey et al., 2005; Pal et al., 2008). Thus, using cdk1-Y19F allowed us to focus on the role of Zds1/2 in signals that act upstream of Mih1 and Swe1. We assayed accumulation of the mitotic cyclin Clb2 in the same samples, which provided a molecular marker for mitotic progression (Figure 3C). Western blot signals in each strain were normalized to a loading control, so protein levels could be compared between strains.

In cdk1-Y19F control cells, Mih1 underwent dephosphorylation as cells entered mitosis, as previously observed in wild-type cells (Pal et al., 2008) (Figure 3A). Strikingly, in cdk1-Y19F zds1Δ zds2Δ cells, Mih1 failed to undergo full dephosphorylation as cells entered mitosis. An identical effect was previously observed in cdc55Δa cells (Pal et al., 2008).

The effects of zds1Δ zds2Δ on Swe1 phosphorylation were more subtle. In the cdk1-Y19F control cells, Swe1 initially accumulated in a partially hyperphosphorylated form (Figure 3B, band marked with one asterisk) and then accumulated in a fully hyperphosphorylated form (band marked with two asterisks), as previously reported in wild-type and cdk1-Y19F cells (Harvey et al., 2005). In cdk1-Y19F zds1Δ zds2Δ cells, the Swe1 protein accumulated with timing that was similar to that of the control cells, but the initial phosphorylation of Swe1 was slightly delayed. In addition, a smaller amount of Swe1 reached full hyperphosphorylation, and Swe1 accumulated in the partially phosphorylated form (band marked with an asterisk in Figure 3B). These results were unexpected because, in independent experiments, we discovered that PP2ACdc55 opposes phosphorylation of Swe1 by Cdk1/Cdc1 (S. Harvey and D. Kellogg, unpublished data). Specifically, we found that the initial partial hyperphosphorylation of Swe1 occurred prematurely in cdk1-Y19F cdc55Δ cells. Different experiments suggest a model in which the opposing activity of PP2ACdc55 creates a threshold that limits the initial activating phosphorylation of Swe1 by Cdk1, thereby allowing a low level of Cdk1 activity to escape Swe1 inhibition to initiate early mitotic events (S. Harvey and D. Kellogg, unpublished data). If Zds1/2 mediate the functions of PP2ACdc55, one would expect to see premature hyperphosphorylation of Swe1 in cdk1-Y19F zds1Δ zds2Δ cells.
A model that could explain the different phenotypes caused by cdc55Δ and zds1Δ zds2Δ is that PP2A<sup>Cdc55</sup> is active against Swe1 in the absence of Zds1/2 but strictly requires Zds1/2 for dephosphorylation of Mih1. To test this model, we deleted the CDC55 gene in the cdc1-k19F zds1Δ zds2Δ cells. This deletion caused premature phosphorylation of Swe1, loss of hypophosphorylated forms of Swe1, and accumulation of Swe1 in a partially phosphorylated form, as we observed in cdc1-k19F cdc55Δ cells (Figure 1B) (S. Harvey and D. Kellogg, unpublished data). The failure of Mih1 to undergo dephosphorylation in zds1Δ zds2Δ cells was not affected by additional deletion of CDC55.

In summary, the data in Figure 3, A and B, demonstrate that Zds1/2 are strictly required for the PP2A<sup>Cdc55</sup>-dependent dephosphorylation of Mih1, but are not required for the PP2A<sup>Cdc55</sup>-dependent dephosphorylation of Swe1. Thus, Zds1/2 activate PP2A<sup>Cdc55</sup>-dependent dephosphorylation of Mih1. In contrast, previous work reached the conclusion that Zds1/2 inhibit PP2A<sup>Cdc55</sup>-dependent dephosphorylation of Net1, which suggests that they can play both positive and negative roles in the regulation of PP2A<sup>Cdc55</sup> (Queralt and Uhlmann, 2008). This issue is addressed further in the Discussion.

Inactivation of Zds1/2 also caused subtle effects on the accumulation of Clb2 protein. Clb2 accumulation was delayed in zds1Δ zds2Δ cdc1-k19F cells compared to cdc1-k19F control cells (Figure 3C; peak Clb2 levels are marked with an asterisk), and overall levels of Clb2 appeared to be reduced. The delay in reaching peak levels was rescued by cdc55Δ. The causes of these effects are unknown. Several high-throughput screens identified physical interactions between Zds1/2 and proteins involved in transcription, so the effects on Clb2 accumulation could be due to a transcriptional role for Zds1/2. In addition, previous studies suggest that the effect of inactivating Zds1/2 on the timing of peak Clb2 levels may be due to a role for Zds1/2 in mitotic exit. In these studies, it was found that PP2A<sup>Cdc55</sup> inhibits mitotic exit by opposing phosphorylation of Net1, which sequesters the Cdc14 phosphatase in the nucleolus (Queralt and Uhlmann, 2008). Phosphorylation of Net1 triggers release of Cdc14, which then promotes mitotic exit. Zds1/2 are thought to promote mitotic exit by inhibiting PP2A<sup>Cdc55</sup>-dependent dephosphorylation of Net1 (Queralt and Uhlmann, 2008). Thus, PP2A<sup>Cdc55</sup> should be hyperactive against Net1 in zds1Δ zds2Δ cdc1-k19F cells.

The hyperactivity should cause a delay in mitotic exit and a corresponding delay in destruction of Clb2 that should be eliminated by cdc55Δ, which is consistent with the observed effects of inactivating Zds1/2 on Clb2 levels (Figure 3C). Control of mitotic exit by PP2A<sup>Cdc55</sup> is independent of Cdk1 inhibitory phosphorylation (Queralt et al., 2006).

**The phosphorylation state of Zds1 is controlled by opposing kinase and phosphatase activities**

The discovery that Zds1/2 bind tightly to PP2A<sup>Cdc55</sup> and are required for dephosphorylation of Mih1 suggested that they may play a role in relaying upstream signals that control Mih1 during entry into mitosis. We therefore carried out experiments to identify mechanisms that could regulate Zds1. We noticed that Zds1 purified from cdc55Δ cells appeared to migrate with a slower electrophoretic mobility, which suggested hyperphosphorylation (Figure 2A). To confirm this, we used Western blotting to assay the electrophoretic mobility of Zds1 in crude extracts of wild-type and cdc55Δ cells. To improve resolution, electrophoresis was carried out for a longer period of time, which revealed a quantitative shift in Zds1 mobility in cdc55Δ cells (Figure 4A). Treatment of Zds1 isolated from cdc55Δ cells with phosphatase caused Zds1 to shift to a lower apparent molecular weight (Figure 4B). These observations demonstrate that Zds1 undergoes hyperphosphorylation when it is dissociated from PP2A<sup>Cdc55</sup>. They also suggest that phosphorylation of Zds1 is normally opposed by PP2A<sup>Cdc55</sup>. To further test this idea, we assayed Zds1 phosphorylation in cells that were dependent on a temperature-sensitive allele of one of the redundant PP2A catalytic subunits (pph22Δ-172 pph21Δ pph3Δ) (Figure 4C) (Stark, 1996). This strain showed reduced growth...
Zds1 protein remained hyperphosphorylated after G1, in contrast to wild-type Zds1, which underwent partial dephosphorylation (Figure 5, A–C). Thus, zds1−1 is phosphorylated in late G1 similar to wild type but shows phosphorylation defects at other stages of the cell cycle, which may be the cause of the mutant phenotype.

These experiments also revealed that zds1−1 zds2Δ cells undergo a prolonged arrest with high levels of Cln2 and Clb2 at the restrictive temperature (Figure 5B). This finding supports a role for Zds1 in controlling activation of Cdk1/Clb2, because previous work demonstrated that Cdk1/Clb2 represses Cln2 transcription by phosphorylating the transcription factor that promotes G1 cyclin transcription (Amon et al., 1993). Because Cdk1/Cln2 promotes polar growth, the failure to repress CLN2 transcription is the likely cause of the elongated cell phenotype observed in the zds1−1 zds2Δ mutant.

at the permissive temperature, which indicated decreased PP2A function. We found that Zds1 was hyperphosphorylated at both the permissive and restrictive temperatures, which further confirmed that phosphorylation of Zds1 is opposed by PP2A Cdc55. We showed that Zds1 is also phosphorylated in wild-type cells by immunoprecipitating Zds1 in the presence of absence of phosphatase inhibitors. In the absence of phosphatase inhibitors, Zds1 migrated more rapidly, which demonstrated that Zds1 became dephosphorylated during the immunoprecipitation, most likely by associated PP2A Cdc55 (Figure 4D).

Together, these results suggest that the phosphorylation state of Zds1 is determined by the opposing activities of PP2ACdc55 and at least one kinase. Dissociation of Zds1 from PP2ACdc55 shifts the balance entirely to the kinase, which causes Zds1 to undergo quantitative hyperphosphorylation.

Zds1 undergoes cell cycle–dependent phosphorylation

We next tested whether Zds1 undergoes cell cycle–dependent phosphorylation. To do this, we used Western blotting to follow the behavior of the Zds1 protein during the cell cycle in cells synchronized in G1 by centrifugal elutriation. To provide molecular markers for cell–cycle progression, we also monitored the behavior of the G1 cyclin Cln2 and the mitotic cyclin Clb2. Zds1 appeared as two bands in early G1 and shifted to a form with reduced electrophoretic mobility in late G1 phase (Figure 5A). The reduced mobility correlated with the appearance of Cln2. We observed the same result regardless of whether cells were synchronized by centrifugal elutriation (Figure 5A) or by α-factor arrest and release (Figure 5B).

We carried out a similar analysis in zds1−1 zds2Δ cells and zds2Δ control cells synchronized in G1 by mating pheromone arrest and release. This analysis revealed that the temperature-sensitive zds1−1 protein also showed reduced electrophoretic mobility that was strongly correlated with Cln2 levels (Figure 5B). The mobility shift was larger than that of the wild-type protein because the zds1−1 protein was hypophosphorylated in early G1 (Figure 5C). Treatment of the zds1−1 protein with phosphatase confirmed that it was full length, because it ran as a single band of the same molecular weight as that of dephosphorylated wild-type Zds1 (Figure 5D). The zds1−1

FIGURE 4: Zds1 undergoes phosphorylation and is regulated by PP2ACdc55. (A) Cell extracts of rapidly growing wild-type and cdc55Δ cells were analyzed by Western blotting using an anti-Zds1 antibody. (B) Zds1 was immunoprecipitated from cdc55Δ cells using anti-Zds1 antibodies bound to protein G beads. At the end of the purification, the beads were split in half and λ–phosphatase was added to one half. After a 30-min incubation at 30°C, the immunoprecipitates were boiled and analyzed by SDS–PAGE and Western blotting. (C) Asynchronous pph21Δ pph3Δ pph22−172 and pph21Δ pph3Δ PPH22 cells were shifted to the restrictive temperature (37°C) for the indicated times. Zds1 phosphorylation was analyzed by Western blotting. (D) Zds1 was immunoprecipitated from rapidly growing wild-type cells using anti-Zds1 antibodies bound to protein G beads in the presence or absence of phosphatase inhibitors. Immunoprecipitates were analyzed by SDS–PAGE and Western blotting.
Mih1 but not for PP2A<sub>Cdc55</sub>-dependent dephosphorylation of Swe1, which demonstrates that they target PP2A<sub>Cdc55</sub> to Mih1. Zds1 undergoes complex cell cycle–dependent changes in phosphorylation, which suggests that Zds1/2 may be targets of upstream signals that control entry into mitosis. Together, these observations suggest a model in which Zds1/2 control Cdk1 inhibitory phosphorylation by relaying upstream signals that control entry into mitosis to Mih1 via PP2A<sub>Cdc55</sub>. In this model, inactivation of Zds1/2 causes a mitotic arrest because the signal to remove Cdk1 inhibitory phosphorylation is not properly relayed. Although we favor this model, we cannot rule out the possibility that inactivation of Zds1/2 triggers a checkpoint arrest by causing defects in early cell-cycle events that could not be detected by our assays. It is also possible that Zds1/2 regulate entry into mitosis via additional mechanisms that are independent of PP2A<sub>Cdc55</sub> and Mih1.

Our understanding of the function of Zds1/2 is currently limited because we do not yet have comprehensive data on the functional consequences of dephosphorylation of Mih1 by PP2A<sub>Cdc55</sub>-Zds1/2. To rigorously determine the functional significance of Mih1 dephosphorylation it will be necessary to assay the activity of differently phosphorylated forms of Mih1 and to map and mutate the relevant
entry into mitosis, even though PP2A Cdc55 retains activity against complete failure in Mih1 dephosphorylation and severe defects in entry into mitosis that are rescued by increased Cdk1 inhibitory phosphorylation, and severe defects in Swe1. The facts that Zds1/2 play a specific role in targeting PP2A Cdc55 against Swe1. Because PP2A Cdc55 opposes phosphorylation of Mih1 by Cdk1, Zds1/2 inhibit the activity of PP2A Cdc55 against Swe1. Previous work reached the conclusion that Zds1/2 inhibit the activity of PP2A Cdc55 in the absence of mitotic Cdc1 activity (Kumagai and Dunphy, 1992; Izumi and Maller, 1995). Although the identity of the kinase is unknown, this observation suggests that the potential for similar checkpoint signaling may exist in vertebrate cells and that the checkpoint is turned off in Xenopus oocytes.

Previous work reached the conclusion that Zds1/2 inhibit the activity of PP2A Cdc55 against Net1 (Queralt and Uhlmann, 2008). Interestingly, the behavior of Swe1 in cells that lack Zds1/2 is also consistent with an inhibitory role for Zds1/2. In this case, the delayed phosphorylation of Swe1 and accumulation of Swe1 in an intermediate phosphorylation form could be explained by a role for Zds1/2 in inhibiting the activity of PP2A Cdc55 against Swe1. Because PP2A Cdc55 opposes phosphorylation of Mih1 by Cdk1, Zds1/2 inhibit the activity of PP2A Cdc55 against Swe1. Previous work reached the conclusion that Zds1/2 inhibit the activity of PP2A Cdc55 in the absence of mitotic Cdc1 activity (Kumagai and Dunphy, 1992; Izumi and Maller, 1995). Although the identity of the kinase is unknown, this observation suggests that the potential for similar checkpoint signaling may exist in vertebrate cells and that the checkpoint is turned off in Xenopus oocytes.

Phosphorylation sites, which has not yet been possible. Several observations suggest, however, that Yck1/2-dependent phosphorylation inhibits Mih1 activity and that dephosphorylation leads to increased Mih1 activity. First, Yck1/2-dependent hyperphosphorylation of Mih1 occurs during interphase, when Mih1 activity should be suppressed, and PP2A Cdc55 Zds1/2 dephosphorylates Mih1 during entry into mitosis, when Mih1 activity must be increased (Pal et al., 2008). Second, cdc55Δ causes a failure in Mih1 dephosphorylation, increased Cdk1 inhibitory phosphorylation, and severe defects in entry into mitosis that are rescued by swe1Δ (Minshull et al., 1996; Yang et al., 2000; Pal et al., 2008). Finally, zds1Δ zds2Δ causes a complete failure in Mih1 dephosphorylation and severe defects in entry into mitosis, even though PP2A Cdc55 retains activity against Swe1. The facts that Zds1/2 play a specific role in targeting PP2A Cdc55 to Mih1 and that loss of Zds1/2 causes severe defects in entry into mitosis strengthen the evidence that removal of Yck1/2-dependent Mih1 phosphorylation promotes entry into mitosis. Inactivation of Yck1/2 causes a failure in Mih1 hyperphosphorylation but does not cause premature entry into mitosis, as would be predicted if Yck1/2 inhibited Mih1 (Pal et al., 2008). Preliminary experiments suggest, however, that Yck1/2 may also be required for inactivation of Swe1, which complicates interpretation of the phenotype caused by loss of Yck1/2 (S. Anastasia and D. Kellogg, unpublished data).

The hyperphosphorylation of Mih1 early in interphase contrasts with Xenopus Cdc25, which is not hyperphosphorylated during interphase. A possible explanation is that hyperphosphorylation of Mih1 early in the cell cycle reflects an added layer of checkpoint control that does not operate in Xenopus egg extracts. In budding yeast, Wee1 and Cdc25 respond to checkpoint signals that coordinate entry into mitosis with bud growth or morphogenesis, whereas in early Xenopus embryos, cell division occurs without accompanying growth or morphogenesis. Hyperphosphorylation of Mih1 early in the cell cycle may therefore inhibit Mih1 until bud growth or morphogenesis is complete. In this model, Zds1/2 would play a critical role in delaying checkpoint signals that trigger dephosphorylation of Mih1 when checkpoint conditions have been satisfied. Interestingly, inhibition of PP2A in interphase Xenopus extracts leads to extensive and quantitative hyperphosphorylation of Cdc25 in the absence of mitotic Cdc1 activity (Kumagai and Dunphy, 1992; Izumi and Maller, 1995). Although the identity of the kinase is unknown, this observation suggests that the potential for similar checkpoint signaling may exist in vertebrate cells and that the checkpoint is turned off in Xenopus oocytes.

Previous work reached the conclusion that Zds1/2 inhibit the activity of PP2A Cdc55 against Net1 (Queralt and Uhlmann, 2008). Interestingly, the behavior of Swe1 in cells that lack Zds1/2 is also consistent with an inhibitory role for Zds1/2. In this case, the delayed phosphorylation of Swe1 and accumulation of Swe1 in an intermediate phosphorylation form could be explained by a role for Zds1/2 in inhibiting the activity of PP2A Cdc55 against Swe1. Because PP2A Cdc55 opposes phosphorylation of Mih1 by Cdk1, Zds1/2 inhibit the activity of PP2A Cdc55 against Swe1. Previous work reached the conclusion that Zds1/2 inhibit the activity of PP2A Cdc55 in the absence of mitotic Cdc1 activity (Kumagai and Dunphy, 1992; Izumi and Maller, 1995). Although the identity of the kinase is unknown, this observation suggests that the potential for similar checkpoint signaling may exist in vertebrate cells and that the checkpoint is turned off in Xenopus oocytes.

Previous work reached the conclusion that Zds1/2 inhibit the activity of PP2A Cdc55 against Net1 (Queralt and Uhlmann, 2008). Interestingly, the behavior of Swe1 in cells that lack Zds1/2 is also consistent with an inhibitory role for Zds1/2. In this case, the delayed phosphorylation of Swe1 and accumulation of Swe1 in an intermediate phosphorylation form could be explained by a role for Zds1/2 in inhibiting the activity of PP2A Cdc55 against Swe1. Because PP2A Cdc55 opposes phosphorylation of Mih1 by Cdk1, Zds1/2 inhibit the activity of PP2A Cdc55 against Swe1. Previous work reached the conclusion that Zds1/2 inhibit the activity of PP2A Cdc55 in the absence of mitotic Cdc1 activity (Kumagai and Dunphy, 1992; Izumi and Maller, 1995). Although the identity of the kinase is unknown, this observation suggests that the potential for similar checkpoint signaling may exist in vertebrate cells and that the checkpoint is turned off in Xenopus oocytes.

Previous work reached the conclusion that Zds1/2 inhibit the activity of PP2A Cdc55 against Net1 (Queralt and Uhlmann, 2008). Interestingly, the behavior of Swe1 in cells that lack Zds1/2 is also consistent with an inhibitory role for Zds1/2. In this case, the delayed phosphorylation of Swe1 and accumulation of Swe1 in an intermediate phosphorylation form could be explained by a role for Zds1/2 in inhibiting the activity of PP2A Cdc55 against Swe1. Because PP2A Cdc55 opposes phosphorylation of Mih1 by Cdk1, Zds1/2 inhibit the activity of PP2A Cdc55 against Swe1. Previous work reached the conclusion that Zds1/2 inhibit the activity of PP2A Cdc55 in the absence of mitotic Cdc1 activity (Kumagai and Dunphy, 1992; Izumi and Maller, 1995). Although the identity of the kinase is unknown, this observation suggests that the potential for similar checkpoint signaling may exist in vertebrate cells and that the checkpoint is turned off in Xenopus oocytes.

Previous work reached the conclusion that Zds1/2 inhibit the activity of PP2A Cdc55 against Net1 (Queralt and Uhlmann, 2008). Interestingly, the behavior of Swe1 in cells that lack Zds1/2 is also consistent with an inhibitory role for Zds1/2. In this case, the delayed phosphorylation of Swe1 and accumulation of Swe1 in an intermediate phosphorylation form could be explained by a role for Zds1/2 in inhibiting the activity of PP2A Cdc55 against Swe1. Because PP2A Cdc55 opposes phosphorylation of Mih1 by Cdk1, Zds1/2 inhibit the activity of PP2A Cdc55 against Swe1. Previous work reached the conclusion that Zds1/2 inhibit the activity of PP2A Cdc55 in the absence of mitotic Cdc1 activity (Kumagai and Dunphy, 1992; Izumi and Maller, 1995). Although the identity of the kinase is unknown, this observation suggests that the potential for similar checkpoint signaling may exist in vertebrate cells and that the checkpoint is turned off in Xenopus oocytes.
1984; Ferrell, 1996). If the kinase and phosphatase operate at or near their maximal velocities, the target protein can exhibit large switchlike changes in its phosphorylation state in response to small changes in enzyme velocity. This behavior is referred to as “zero-order ultrasensitivity.” The fact that PP2Aβδ5 is associated with Zds1/2 suggests that it is acting at its maximal velocity, which fulfills one requirement for a zero-order ultrasensitivity. Thus the PP2Aβδ5, Zds1/2 complex may contribute to switchlike activation of Cdk1 during entry into mitosis.

The discovery that Zds1 undergoes cell cycle–dependent changes in phosphorylation raises the possibility that regulation of PP2Aβδ5 plays an important role in the mechanisms that trigger entry into mitosis. An interesting model is that phosphorylation of Zds1/2 by Cdk1/Cln2 early in the cell cycle inhibits their ability to target dephosphorylation of Mih1. Because Cdk1/Cln2 drives bud growth, this would ensure that entry into mitosis does not occur while bud growth is ongoing (McCusker et al., 2007). The fact that multiple signals feed into Zds1/2 suggests that they could be an integration point for checkpoint signals that report on the status of cell size or morphogenesis. Zds1 is localized to the growing bud, so it is well positioned to relay these kinds of signals (Bi and Pringle, 1996). An important goal for future work will be to fully define the signals that control Zds1/2, as this will likely lead to a better understanding of the checkpoint signals that control entry into mitosis.

**MATERIALS AND METHODS**

Yeast strains and culture conditions

The strains used in this study are listed in Table 1. All yeast strains are the YPH250 background (his3–11,15, leu2–3,112, trp1–1, ura3–52, ade2–1, can1–100) except YDH6 and YDH8, which are in the YPH250ΔΔΔΔ background (trp1–1Δ, his3–200Δ, leu2–1Δ, lys2–801amber ade2–1Δ ura3–52) and DK608, which was created by sporulating a ZDS2ΔΔΔΔ diploid from the BY4743 background (his3Δ1, his1Δ, leu2Δ0/leu2α0, lys2Δ0/lys2α0, met15α0/MET17, ura3Δ0/ura3Δ0). Unless otherwise indicated, all strains were grown in yeast extract–peptone–dextrose (YPD) medium supplemented with 40 mg/l adenine. Experiments using the cdk1–1α1 strain (JAU05) were carried out in YPD lacking supplemental adenine. DK589 was generated by transformation of DK186 with the pGAL-CLN2 plasmid (a gift of Ray Deshaies, California Institute of Technology, Pasadena, CA). DK599 was generated by sporulation of a diploid generated by crossing HT138 with ADR473. HT125, HT126, HT127, and HT128 were generated by sporulating the diploid DY4036. HT133 was generated by sporulating the diploid DY4037. HT138 was generated by integrating a PCR product containing the GAL1 promoter and a 3XHA tag upstream of ZDS1 into SW34, SW24, ADR510, and SW117 by integrating pDK31B (CLN2–3XHA, ADE2, LEU2) into SW34, SW24, ADR510, and SW117 at the CLN2 locus.

Generation of ZDS1 temperature-sensitive alleles

To generate zds1-ts alleles, the Schizosaccharomyces pombe his5+ gene was integrated downstream of ZDS1 in strain HT127 using a standard PCR-based approach to create strain SW24 (oligos: GGGAAGATGGAACAAACACAAACTCTTCTATAACATC-GATCCCCCGGGTTAATTAA and GGCAATTTTCTTCGCCCCGGG-CAGAAAGCTCTTCTTTATCGAATTGCGTCTTTAAC; vector: pFA6α-His3MX6) (Longtine et al., 1998). The ZDS1 gene and the downstream his5+ gene were then amplified under mutagenic conditions (oligos: CACCAACAGGTGTCCGTC and CGCCTG- GCCGTCCTTGG), and the PCR product was transformed into HT127. Colonies were screened at the restrictive temperature for slow growth and rough colony morphology, which are characteristic of a loss of ZDS1/2 function. As a secondary screen, cells were examined to determine whether they have elongated buds at 37°C, which is a further indication of a loss of ZDS1/2 function. All of the zds1-ts mutants could be rescued by a CEN plasmid that carries wild-type ZDS1 (pSM1), which was constructed by cloning ZDS1 into Ycpplac33 (oligos: CGCGGATCCCTCTGAGGAACCT-CTGGTGTTGAGGGCAGCCTGGCCG; vector: pCEN2-His3MX6) (Longtine and Pringle, 1996). All of the ZDS1/2 alleles were complemented by introducing ZDS1 by Cdk1/Cln2 early in the cell cycle inhibits their ability to target dephosphorylation of Mih1. Because Cdk1/Cln2 drives bud growth, this would ensure that entry into mitosis does not occur while bud growth is ongoing (McCusker et al., 2007). The fact that multiple signals feed into Zds1/2 suggests that they could be an integration point for checkpoint signals that report on the status of cell size or morphogenesis. Zds1 is localized to the growing bud, so it is well positioned to relay these kinds of signals (Bi and Pringle, 1996). An important goal for future work will be to fully define the signals that control Zds1/2, as this will likely lead to a better understanding of the checkpoint signals that control entry into mitosis.
beta-mercaptoethanol) supplemented with 50 mM NaF, 50 mM beta-glycerophosphate, and 2 mM phenylmethylsulfonyl fluoride (PMSF). Bead beating was carried out with a Multibeater-8 (BioSpec Products, Bartlesville, OK) at top speed for 100 s. lysates were incubated in a boiling water bath for 5 min and centrifuged for 5 min in a tabletop microfuge at top speed. Then, 20 μl was loaded on a gel. For Figure 1, D–F, 1-ml samples were collected for each time point and immediately fixed with 4% formaldehyde. At the end of the time course, cells were washed in 1X phosphate-buffered saline (PBS) and either analyzed directly under the microscope for bud emergence (Figure 1E) or processed for immunofluorescence (Figure 1, and either analyzed directly under the microscope for bud emergence (Figure 1E) or processed for immunofluorescence (Figure 1, D and F). For Figure 6A, cells were released from α-factor arrest into fresh YPD at 30°C and split into four tubes. At 50 min, 25 μM 1NM-PP1 (a gift from C. Zang and K. Shokat, University of California, San Francisco) was added to one tube, and an equivalent amount of dimethyl sulfoxide (DMSO) was added to a second tube. Samples (1 ml) were collected at 0, 2.5, and 5 min (1NM-PP1) or at 5 min (DMSO) after treatment. The same procedure was used with the two remaining tubes at 90 min after release from α-factor arrest. For Figure 5A, small, unbudded cells were isolated by centrifugal elution from logarithmically growing cells in a Beckman Coulter J6-M1 centrifuge and a JE-5.0 rotor at 4°C as previously described (Schwob and Nasmyth, 1993). The time-course experiment was carried out at 30°C, collecting 1-ml samples at the indicated time points. To test whether PP2A acts on Zds1, log-phase cultures of temperature-sensitive alleles of ppp22–12::URA3 ppp21–1::LYS2 (S. pombe) were shifted to the restrictive temperature (37°C), and 1.6-ml samples were collected at the indicated time points (Figure 4C).

**Immunoaffinity purifications and mass spectrometry**

Immunoaffinity purifications of 3XHA-Zds1 complexes (Figure 3A) were carried out in the presence of 0.5 M KCl using the same protocol as that used for purification of 3XHA-Mih1, with some modifications (Pal et al., 2008). Cells containing a 3XHA-Zds1 under the

### Table 1: Strains used in this study.

| Strain   | Genotype                          | Reference or source            |
|----------|-----------------------------------|--------------------------------|
| ADR473   | MATa, cdc55::LEU2                 | Adam Rudner                    |
| ADR489   | MATa, cdc55::HIS3                 | Adam Rudner                    |
| ADR510   | MATa, cdc1-T1-Y19F-HA::URA3       | Adam Rudner                    |
| AS1      | MATa, bar1α ELM::TRP1             | Altman and Kellogg (1997)      |
| DK17     | MATa                              | Altman and Kellogg (1997)      |
| DK186    | MATa, bar1α                       | This study                     |
| DK303    | MATa, bar1α CL2B-3XHA::TRP1       | This study                     |
| DK589    | MATa, bar1α (pGal-CLN2(CEN/URA3)) | This study                     |
| DK599    | MATa, bar1α GAL2-3XHA-ZDS1::his5+ (S. pombe) | This study                     |
| DK608    | MATa, Zds2::kanMX6                | This study                     |
| DEY213   | MATa, bar1α PHH22::URA3 pph3Δ1::LYS2 pph2Δ1::HIS3 | Evans and Stark (1997)         |
| DEY214   | MATa, bar1α pph22–12::URA3 pph3Δ1::LYS2 pph2Δ1::HIS3 | Evans and Stark (1997)         |
| DMY305   | MATa, bar1α GAL1-3XHA-CLN2::TRP1  | McCusker et al. (2007)         |
| DY4036   | MAT α/α, Zds1::LEU2/ZDS1 zds2::TRP1/ZDS2 | David Stillman                |
| DY4037   | MAT α/α, Zds1::URA3/ZDS1 zds2::HIS3/ZDS2 | David Stillman                |
| JAU05    | MATα, bar1α cdk1::cdk1-as1        | Bishop et al. (2000)           |
| HT125    | MATa, Zds1::LEU2                  | This study                     |
| HT126    | MATa, Zds1::LEU2                  | This study                     |
| HT127    | MATa, Zds2::TRP1                  | This study                     |
| HT128    | MATa, Zds2::TRP1                  | This study                     |
| HT133    | MATa, Zds1::URA3                  | This study                     |
| HT138    | MATa, bar1α GAL1–3XHA-ZDS1::his5+ (S. pombe) | This study                     |
| HT153    | MATa, ZDS1::URA3 gin4::LEU2       | This study                     |
| HT16     | MATa, ZDS1::LEU2 elm1::TRP1       | This study                     |
| KA61     | MATa, bar1α chn1::TRP1 chn2::LEU2 | This study                     |
| RA5      | MATa, bar1α gin4::LEU2            | This study                     |
| RA19     | MATa, bar1α gin4::LEU2            | This study                     |
| SW24     | MATa, Zds2::TRP1 ZDS1::his5+ (S. pombe) | This study                     |
| SW34     | MATa, Zds2::TRP1 zds1–1::his5+ (S. pombe) | This study                     |
| SW49     | MATa, Zds1::LEU2 cdc55::HIS3      | This study                     |
| SW52     | MATa, ZDS1::URA2/ZDS1::LEU2/ZDS2::kanMX6 CDC55::cdc55::HIS3 | This study                     |
| SW61     | MATa, cdc55::HIS3                 | This study                     |
| SW62     | MATa, Zds1::LEU2 zds2::kanMX6     | This study                     |
| SW77     | MATa, Zds2::TRP1 zds1–1::his5+ (S. pombe) CLN2-3XHA-ADE2 | This study                     |
| SW80     | MATa, Zds2::TRP1 ZDS1::his5+ (S. pombe) CLN2–3XHA-ADE2 | This study                     |
| SW116    | MATa, Zds2::TRP1 cdc28-Y19F-HA::URA3 | This study                     |
| SW117    | MATa, Zds1::LEU2 zds2::TRP1 cdc28–19F-HA::URA3 | This study                     |
| SW127    | MATa, cdc1-Y19F-HA::URA3 CLN2-3XHA::ADE2 | This study                     |
| SW129    | MATa, Zds1::LEU2 zds2::TRP1 cdc55::kanMX6 cdk1-Y19F-HA::URA3 | This study                     |
| SW131    | MATa, Zds1::LEU2 Zds2::TRP1 cdk1-Y19F-HA::URA3 CLN2–3XHA::ADE2 | This study                     |
control of the GAL1 promoter (HT138, DK599) and control cells (DK186) were grown overnight at 30°C in yeast extract peptone medium containing 2% glycerol and 2% ethanol to OD600 = 0.8. Galactose was then added to 2% and the cells were incubated for 3 h at 30°C to induce 3XHA-Zds1 synthesis. Preparation of cell extracts, immunopurification beads, and incubation of extract with the anti-HA antibody beads was carried out exactly as described, except that the extract buffer contained 0.5 M KCl rather than 1 M KCl (Pal et al., 2008). At the end of the incubation, the beads were washed two times with 15 ml of ice-cold extract buffer without PMSF. The beads were then transferred to a 1.5-ml Biospin column (Bio-Rad, Hercules, CA) and washed five times with 1 ml of extract buffer without PMSF and two times with 1 ml of extract buffer without PMSF or Tween 20. To elute the column, 250 μl of elution buffer containing HA dipeptide at 0.5 μg/ml was added to the column. After a 30-min incubation at room temperature, another aliquot was added and the flow-through fraction was collected. This process was repeated for a total of four fractions. The fractions were combined and flash frozen in liquid nitrogen. For mass spectrometry, 600 μl of eluate was precipitated in the presence of 10% trichloroacetic acid for 10 min on ice, followed by centrifugation for 5 min in a tabletop microfuge at 13,000 rpm. The pellet was resuspended in 50 μl of protein sample buffer, boiled for 5 min, and centrifuged for 5 min, then 25 μl was loaded on a 9% SDS-polyacrylamide gel. Protein bands were stained with Coomassie blue, then were excised and analyzed by mass spectrometry. Gel bands were digested with trypsin as previously described (Wilm et al., 1996), and proteins were identified with a nano-LC ion source coupled to a Finnigan LCQ tandem mass spectrometer (Gatin et al., 1998). The tandem mass spectra were searched against the yeast open reading frame database obtained from Stanford University using the SEQUEST algorithm (Eng et al., 1994). Each tandem mass spectrum that had a high scoring match was manually inspected to ensure that the match was correct.

The protocol used to purify 3XHA-Cln2/Cdk1 for kinase assays was the same as the one used for 3XHA-Zds1, with the following modifications: Cell expressing 3XHA-Cln2 under the control of the GAL1 promoter (DMY305) were grown at 30°C to OD600 = 0.8 and induced as described earlier in text for 3XHA-Zds1. Cells (10 g) and 20 ml of extract buffer were used to prepare the extract. The extract buffer used was the same as the one used for 3XHA-Zds1 purification, except that it also contained 100 mM b-glycerophosphate, 50 mM NaF, 1 mM vanadate, and 1 mM dithiothreitol (DTT). The elution buffer was the same as the one used for 3XHA-Zds1 elution. To purify Clb2–3XHA/Cdk1, 1.5 l of cells (DK303) was grown in YPD at 30°C to OD600 = 0.8. Cells were pelleted and resuspended in 1.6 l of YPD containing benomyl at 20 μg/l. After a 3-h incubation at room temperature, cells were washed with 50 mM HEPES-KOH, pH 7.6, and frozen in liquid nitrogen. Cells (6 g) and 13 ml of extract buffer were used to prepare the extract. The same protocol used to purify 3XHA-Zds1 was then followed. The purification of dephosphorylated 3XHA-Zds1 was carried out as described earlier in the text for 3XHA-Zds1 with the following modifications: At the end of the 3-h incubation, the beads were washed twice with 15 ml of extract buffer and once with 15 ml of lambda-phosphatase buffer (New England Biolabs, Ipswich, MA; 50 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, 1 mM MnCl2). The beads were transferred to a 1.5-ml conical tube, and the supernatant was aspirated. Phosphatase buffer (100 μl) and 20 μl of lambda-phosphatase (New England Biolabs; 400,000 U/ml) were added. The beads were incubated for 2 h at 30°C. Phosphatase buffer (100 μl) was added, and the 1.5-ml tube containing the beads was placed on a rotator and incubated overnight at 4°C. The beads were transferred into a 15-ml conical tube, washed two times with extract buffer, transferred onto a 1.5-ml Bio-Spin column (Bio-Rad), and washed and eluted as described earlier in the text for 3XHA-Zds1.

In vitro kinase assays
In vitro kinase assays using purified dephosphorylated 3XHA-Zds1, 3XHA-Cln2/Cdk1, and Clb2–3XHA/Cdk1 were carried out as follows: 25-μl reactions containing 10 μl of eluate of dephosphorylated 3XHA-Zds1 and 10 μl of eluate of either 3XHA-Cln2/Cdk1 or Clb2–3XHA-Cdk1 were prepared in kinase assay buffer (50 mM HEPES-KOH, pH 7.6, 2 mM MgCl2, 0.05% Tween-20, 10% glycerol, 1 mM DTT, all final concentrations) in the presence or absence of 1 mM ATP. Reactions were incubated at 30°C for 30 min and were terminated by freezing in liquid nitrogen. Then, 4 μl of 4X protein sample buffer was added, and samples were incubated in a boiling water bath for 3 min and resolved on a 9% SDS-polyacrylamide gel. To assess relative levels of each cyclin, 2 μl of 3XHA-Cln2/Cdk1 eluate and 2 μl of Clb2–3XHA/Cdk1 eluate were diluted in sample buffer and loaded onto a 10% SDS-polyacrylamide gel. Gels were analyzed by Western blotting using anti-Zds1 and anti-HA antibodies. The activity of Clb2–3XHA/Cdk1 was confirmed by performing the in vitro kinase assay with dephosphorylated Mih1 as substrate instead of Zds1 (see Pal et al., 2008).

ACKNOWLEDGMENTS
S.W. was supported by a fellowship from the Swiss National Foundation. This work was supported by the Pew Biomedical Scholars Program and by Grant GM06962 from the National Institutes of Health. We thank members of the Kellogg lab for helpful discussions and critical reading of the manuscript, and we thank Michael Polymenis for supporting S.W. during this work.

REFERENCES
Altmann R, Kellogg DR (1997). Control of mitotic events by Nap1 and the Gin4 kinase. J Cell Biol 138, 119–130.
Amon A, Tyers M, Futcher B, Nasmyth K (1993). Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. Cell 74, 993–1007.
Anderson CW, Baum PR, Gesteland RF (1993). Processing of adenovirus 2-induced proteins. J Virol 12, 241–252.
Asano S, Park JE, Sakchaisri K, Yu LR, Song S, Supavilai P, Veenstra TD, Lee KS (2005). Concerted mechanism of SWe1/SWe1 regulation by multiple kinases in budding yeast. EMBO J 24, 2194–2204.
Bi E, Pringle JR (1994). ZDS1 and ZDS2, genes whose products may regulate Cdc42p in Saccharomyces cerevisiae. Mol Cell Biol 16, 5264–5275.
Bishop AC et al. (2000). A chemical switch for inhibitor-sensitive alleles of the p34cdc2 protein kinase. Nature 407, 395–401.
Booher RN, Deshaies RJ, Kirschner MW (1993). Properties of Saccharomyces cerevisiae wee1 and its differential regulation of p34cdc2 in response to G1 and G2 cyclins. EMBO J 12, 3417–3426.
Carroll C, Altman R, Schieltz D, Yates J, Kellogg DR (1998). The septicins are required for the mitosis-specific activation of the Gin4 kinase. J Cell Biol 143, 709–717.
Castilho PV, Williams BC, Mochida S, Zhao Y, Goldberg ML (2009). The M phase kinase Greatwall (Gwl) promotes inactivation of PP2A/B55(delta), a phosphatase directed against CDK phosphosites. Mol Biol Cell 20, 4777–4789.
Clarke PR, Hofmann I, Draela G, Karsenti E (1993). Dephosphorylation of cdk2-C by a type-2A protein phosphatase: specific regulation during the cell cycle in Xenopus egg extracts. Mol Biol Cell 4, 397–411.
Collins SR, Kemmeren P, Zhao XC, Bi E, Pringle JR (1996). ZDS1 and ZDS2, genes whose products may regulate Cdc42p in Saccharomyces cerevisiae. Mol Cell Biol 16, 5264–5275.
Debelsier RW, Kirschner MW (2010). Quantitative reconstitution of mitotic CDK1 activation in somatic cell extracts. Mol Cell 37, 753–767.
Egelhofer TA, Villen J, McCusker D, Gygi SP, Kellogg DR (2008). The septins function in G1 pathways that influence the pattern of cell growth in budding yeast. PLoS ONE 3, e2002.

Eng JK, McCormack AL, Yates JR 3rd (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J Am Soc Mass Spectrom 5, 976–989.

Evans DRH, Stark MJR (1997). Mutations in the Saccharomyces cerevisiae type 2A protein phosphatase catalytic subunit reveal roles in cell wall integrity, actin cytoskeleton organization and mitosis. Genetics 145, 227–241.

Fantas P, Nurse P (1977). Control of cell size in fission yeast by a growth regulated module size control over nuclear division. Exp Cell Res 107, 377–386.

Ferre J, Jr (1996). Tripping the switch: a flash. How a protein kinase cascade can convert graded inputs into switch-like outputs. Trends Biochem Sci 21, 460–466.

Fitch I, Dahmann C, Surana U, Amon A, Nasmyth K, Goetsch L, Byers B, Fitcher B (1992). Characterization of four S. pombe cyclin genes of the budding yeast Saccharomyces cerevisiae. Mol Biol Cell 3, 805–818.

Gachet Y, Tourrier M, Millar JB, Hyams JS (2001). A MAP kinase-dependent actin checkpoint ensures proper spindle orientation in fission yeast. Nature 412, 352–355.

Galil GL, Kleiman GR, Hays LG, Link AJ, Yates JR Jr (1998). Protein identification at the low femtomole level from silver-stained gels using a new filterless electrospray interface for liquid chromatography-microspray and nanospray mass spectrometry. Anal Biochem 263, 93–101.

Gautier J, Salomon MJ, Bohrer RN, Bazar JF, Kirschner MW (1991). cdc25 is a specific tyrosine kinase that directly activates p34cdc2. Cell 67, 197–211.

Gavin AC et al. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415, 141–147.

Gentry MS, Hallberg RL (2002). Localization of Saccharomyces cerevisiae Zds proteins control entry into mitosis: budding yeast wee1 delays entry into mitosis and is a specific cdc25 phosphatase inhibitor. Mol Biol Cell 13, 3477–3492.

Goldbeter A, Koshland DE, Jr (1981). An amplified sensitivity arising from covalent modification in biological systems. Proc Natl Acad Sci USA 78, 6840–6844.

Goldbeter A, Koshland DE, Jr (1984). Ultrasensitivity in biochemical systems controlled by covalent modification. Interplay between zero-order and multistep effects. J Biol Chem 259, 14441–14447.

Harvey SL, Charlet A, Haas W, Gygi SP, Kellogg DR (2005). Cdc1-dependent regulation of the mitotic inhibitor Weel1. Cell 122, 407–420.

Harvey SL, Kellogg DR (2003). Conservation of mechanisms controlling entry into mitosis: budding yeast wee1 delays entry into mitosis and is required for cell size control. Curr Biol 13, 264–275.

Healy AM, Zdzieszewski S, Stapleton AE, Goebl M, DePaoli-Roach AA, Pringle JR (1991). Cdc5 phosphatase activity. Mol Cell Biol 11, 5767–5780.

Hoffman I, Clarke PR, Marcote MJ, Karsten E, Draetta G (1993). Phosphorylation and activation of human cdc25C by cdc2-cyclin B and its involvement in the self amplification of MPF at mitosis. EMBO J 12, 53–63.

Izumi T, Maller JL (1993). Elimination of cdc2 phosphate sites in the cdc25 phosphatase blocks initiation of M-phase. Mol Biol Cell 4, 1337–1350.

Izumi T, Maller JL (1995). Phosphorylation and activation of the Xenopus Cdc25 phosphatase in the absence of Cdc2 and Cdc2 kinase activity. Mol Biol Cell 6, 215–216.

Izumi T, Walker DH, Maller JL (1992). Periodic changes in the phosphorylation of the Xenopus Cdc25 phosphatase regulate its activity. Mol Biol Cell 3, 923–939.

Janssens V, Goris J (2001). Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J 353, 417–439.

Jorgensen P, Nishikawa J, Tyers M (2002). Systematic identification of pathways that couple cell growth and division in yeast. Science 297, 395–400.

Kellogg DR (2003). Wee1-dependent mechanisms required for coordination of cell growth and cell division. J Cell Sci 116, 4833–4890.

Kellogg DR, Alberts BM (1992). Purification of a multiprotein complex containing centrosomal proteins from the Drosophila embryo by chromato-
Wang Z, Kar S, Carr BI (2008). Cdc25A protein phosphatase: a therapeutic target for liver cancer therapies. Anticancer Agents Med Chem 8, 863–871.

Wilm M, Shevchenko T, Houthaeve T, Breit S, Schweigerer L, Fotsis T, Mann M (1996). Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature 379, 466–499.

Wu L, Russell P (1993). Nim1 kinase promotes mitosis by inactivating Wee1 tyrosine kinase. Nature 363, 738–741.

Yang H, Jiang W, Gentry M, Hallberg RL (2000). Loss of a protein phosphatase 2A regulatory subunit (Cdc55p) elicits improper regulation of Swe1p degradation. Mol Cell Biol 20, 8143–8156.

Yasutis K, Vignali M, Ryder M, Tameire F, Dighe SA, Fields S, Kozminski KG (2010). Zds2p regulates Swe1p-dependent polarized cell growth in Saccharomyces cerevisiae via a novel Cdc55p interaction domain. Mol Biol Cell 21, (in press).

Yu Y, Jiang YW, Wellinger RJ, Carlson K, Roberts JM, Stillman DJ (1996). Mutations in the homologous ZDS1 and ZDS2 genes affect cell cycle progression. Mol Cell Biol 16, 5254–5263.

Zhao Y, Boguslawski G, Zitomer RS, DePaoli-Roach AA (1997). Saccharomyces cerevisiae homologs of mammalian B and B′ subunits of protein phosphatase 2A direct the enzyme to distinct cellular functions. J Biol Chem 272, 8256–8262.