Zds1 regulates PP2A^{Cdc55} activity and Cdc14 activation during mitotic exit through its Zds_C motif

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Summary

At anaphase onset, highly active mitotic cyclin-dependent kinase (Cdk) is inactivated to promote exit from mitosis and completion of cytokinesis. The budding yeast Cdc14p phosphatase is a key mitotic regulator that counteracts cyclin-dependent kinase (Cdk) activity during mitotic exit. Separase, together with Zds1p, promotes the downregulation of the protein phosphatase 2A in conjunction with its Cdc55p regulatory subunit (PP2A^{Cdc55}) in early anaphase, enabling accumulation of phosphorylated forms of Net1p and release of Cdc14p from the nucleolus. Here we show that the C-terminal domain of Zds1p, called the Zds_C motif, is required for Zds1-induced release of Cdc14p, and the N-terminal domain of the protein might be involved in regulating this activity. More interestingly, Zds1p physically interacts with Cdc55p, and regulates its localization through the Zds_C motif. Nevertheless, expression of the Zds_C motif at endogenous levels cannot induce timely release of Cdc14p from the nucleolus, despite the proper (nucleolar) localization of Cdc55p. Our results suggest that the activity of PP2A^{Cdc55} cannot be modulated solely through regulation of its localization, and that an additional regulatory step is probably required. These results suggest that Zds1p recruits PP2A^{Cdc55} to the nucleolus and induces its inactivation by an unknown mechanism.

Key words: Cell cycle, Cdc14 phosphatase, Mitotic exit, PP2A phosphatase, Zds1, *Saccharomyces cerevisiae*

Introduction

Mitotic exit involves an intricately ordered series of events, from the splitting of sister chromatids at anaphase onset to completion of cell division by cytokinesis. Cells enter mitosis when cyclin-B–Cdk (Clb2p–Cdc28p in budding yeast) reaches peak kinase activity (reviewed by Morgan, 2007). An essential step during mitotic exit is the activation of the mitotic Cdk-counteracting phosphatase Cdc14p in budding yeast, *Saccharomyces cerevisiae* (reviewed by Mocciaro and Schiebel, 2010; Stegmeier and Amon, 2004). Cdc14p contributes to the downregulation of Cdk activity by dephosphorylating mitotic Cdk substrates.

Cdc14p is kept inactive in the nucleolus by binding to its nucleolar inhibitor Net1p (also called Cfi1p) (Shou et al., 1999; Visintin et al., 1999) for most of the cell cycle. During anaphase, Cdk-dependent phosphorylation of Net1p releases active Cdc14p. After release from the nucleolus, Cdc14p initially appears throughout the nucleus, and shortly thereafter, spreads throughout the cytoplasm. Phosphorylated Net1p has less affinity for, and cannot inhibit, Cdc14p (Azzam et al., 2004; Shou and Deshaies, 2002; Yoshida and Toh-e, 2002). There are two essential, complementary regulatory pathways for Cdc14p activation: the Cdc fourteen early anaphase release (FEAR) pathway (reviewed by Queralt and Uhlmann, 2008a; Rock and Amon, 2009) and a G-protein-coupled kinase signaling cascade called the mitotic exit network (MEN). During early anaphase, the main FEAR component, separase, initiates Net1p phosphorylation and Cdc14p release in conjunction with the proteins Slk19p, Spo12p and Fob1p (Azzam et al., 2004; Queralt et al., 2006; Stegmeier et al., 2002; Sullivan and Uhlmann, 2003; Tomson et al., 2009). At anaphase onset, the release of separase from its inhibitor securin triggers proteolytic cleavage of cohesin, and at this point, separase exhibits a second non-proteolytic activity to release Cdc14p from the nucleolus (Sullivan and Uhlmann, 2003). Initial Cdc14p release is promoted by Cdk-dependent phosphorylation of Net1p (Azzam et al., 2004), which is counteracted in metaphase by type 2A protein phosphatase in conjunction with its Cdc55p regulatory subunit (PP2A^{Cdc55}) (Queralt et al., 2006; Yellman and Burke, 2006). Separase-dependent downregulation of PP2A^{Cdc55} at anaphase onset enables Cdk-dependent Net1p phosphorylation as well as release of Cdc14p from the nucleolus. At this point, the MEN is activated by declining Cdk activity and by released Cdc14p (Jaspersen et al., 1998; Lee et al., 2001). MEN is a GTPase-driven signaling cascade that is associated with the spindle-pole body (SPB) (Monje-Casas and Amon, 2009; Pereira et al., 2000; Valerio-Santiago and Monje-Casas, 2011). It includes the Ras-like GTPase Tem1p, and its downstream kinases Cdc15p and Mob1p–Dbf2p. The MEN keeps Cdc14p active during the later stages of anaphase, when mitotic Cdk activity declines (Jaspersen and Morgan, 2000; Mohl et al., 2009; Queralt et al., 2006; Stegmeier et al., 2002). Kinases in the MEN, including Polo kinase, help sustain Net1p phosphorylation at this time (Lee et al., 2001; Liang et al., 2009; Shou and Deshaies, 2002; Visintin et al.,

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Journal of Cell Science

2008; Yoshida and Toh-e, 2002). The MEN is inhibited by high Cdk levels and activated by Cdc14p, at the level of Cdc15p kinase (Jaspersen and Morgan, 2000; König et al., 2010; Stegmeier et al., 2002).

Our previous work revealed that separase cooperates with Zds (zillion different screens) 1 and 2 to activate Cdc14p phosphatase in early anaphase (Queralt and Uhlmann, 2008b). Ectopic Zds1p expression is sufficient to trigger PP2A^{Cdc55} downregulation, Cdk-dependent phosphorylation of Net1p and release of Cdc14p from the nucleolus. Furthermore, Zds1p physically interacts with separase, and separase-induced Cdc14p activation requires the presence of Zds1p and Zds2p. These observations indicate that separase acts through Zds1p and Zds2p to cause PP2A^{Cdc55} downregulation at anaphase onset. No sequence motifs have been detected in the primary amino acid sequences of Zds1p and Zds2p that could yield any hints as to their activity. Orthologs of Zds1p and Zds2p can be found in most ascomycetes and in some Basidiomycota, with the strongest similarity between them confined to a C-terminal motif: the Zds_C motif (Pfam Zds_C PF08632) (Finn et al., 2008). It was recently reported that the Zds proteins control mitotic entry as putative regulators of the kinase Swe1p (Wee1 in higher eukaryotes) (Yasutis et al., 2010) and guide PP2A to Mih1p phosphatase (Wicky et al., 2011). These observations define a role for the Zds proteins as controllers of specific functions of PP2A^{Cdc55}. Their ability to regulate PP2A^{Cdc55} suggests that they act as common PP2A modulators.

Here we demonstrate that the Zds C-terminal region of Zds1p is required to regulate release of Cdc14p from the nucleolus, which occurs through regulation of PP2A^{Cdc55} activity. Ectopic expression of the C-terminal Zds_C motif in metaphase-arrested cells releases Cdc14p from the nucleolus. However, ectopic expression of the N-terminal region of Zds1p does not promote Cdc14 activation. Interestingly, a truncated Zds1 protein, comprising the Zds1 N-terminal region (resides 1–460) fused to the Zds_C motif, induces timely nucleolar Cdc14p release at endogenous levels. Nevertheless, when expressed at endogenous levels, the Zds1p N-terminal and C-terminal domains are both necessary for timely release of Cdc14p from the nucleolus in early anaphase. Here, we demonstrate that Zds1p physically interacts with, and regulates the localization of, Cdc55p through the Zds_C motif. These findings imply that Zds1p acts as a regulator of PP2A by regulating PP2A^{Cdc55} localization to the nucleolus by their physical interaction through its Zds_C motif. However, when expressed at endogenous levels, the Zds_C motif alone does not induce timely release of Cdc14, despite proper localization of Cdc55p in the nucleolus. This fact suggests that the activity of PP2A^{Cdc55} cannot be modulated solely through its localization, and that an additional regulatory step is probably required. These results suggest that Zds1p recruits PP2A^{Cdc55} to the nucleolus and induces its inactivation by an unknown mechanism.

Results

The Zds_C motif is required for phosphorylation of Net1p and for release of Cdc14p from the nucleolus

We have previously demonstrated that ectopic Zds1p expression in metaphase downregulates PP2A^{Cdc55} and releases Cdc14p in a Net1p phosphorylation-dependent reaction (Queralt and Uhlmann, 2008b). However, the mechanism by which Zds1p impinges on PP2A^{Cdc55} activity, and how this mechanism is controlled by separase, are not known. In budding yeast the two sequence paralogs Zds1p and Zds2p share 37.5% sequence identity, concentrated in five regions of homology (Bi and Pringle, 1996). However, no sequence motifs have been detected in their primary amino acid sequence that could yield any hints as to their activity. Orthologs of Zds1p and Zds2p can be found in most ascomycetes and in some Basidiomycota, with the strongest similarity confined to the Zds_C motif. To elucidate the molecular mechanism by which Zds1p downregulates PP2A^{Cdc55} activity, we employed a functional protein domain approach to identify the regions within Zds1p that are required for mitotic exit. We prepared strains expressing truncated versions of Zds1p lacking different regions of homology between Zds1p and Zds2p (Fig. 1, the black boxes indicate the homology regions). First, we deleted the homology regions located in the C-terminal half of the proteins, to generate two truncated Zds1p proteins: one lacking the Zds_C motif (hereafter referred to as *zds1Δ804–916*) and one lacking two regions of homology in the C-terminal (*zds1Δ461–916*). We arrested cells in metaphase (by depletion of Cdc20p), and induced expression of the different truncated Zds1p proteins under control of the galactose-inducible promoter *GAL1* (Fig. 1A). As we previously described (Queralt and Uhlmann, 2008b) (supplementary material Fig. S1), ectopic expression of full-length Zds1p leads to accumulation of the phosphorylated forms of Net1p and to release of Cdc14p from the nucleolus. By contrast, after induction of *zds1Δ804–916* and *zds1Δ461–916*, Net1p mobility shift is no longer observed and Cdc14p remains in the nucleolus. This suggests that the C-terminal region of Zds1p containing the Zds_C motif is needed to promote Net1p phosphorylation and release of Cdc14p from the nucleolus.

To further study the domain within the Zds1p protein involved in the downregulation of PP2A^{Cdc55}, we constructed a new truncated Zds1p protein (referred to as *zds1Δ1–72*) lacking the N-terminal region (which contains the first region of homology between Zds1p and Zds2p). To analyze whether the N-terminal region of Zds1p is also important for promoting Cdc14p release, we induced ectopic expression of *zds1Δ1–72* in metaphase-arrested cells (Fig. 1B). Zds1p protein expression levels were equivalent to those of the Zds1p full-length protein, after galactose induction. Expression of either full-length Zds1p or *zds1Δ1–72* led to release of Cdc14p from the nucleolus with similar kinetics, although in the case of the former, Cdc14p was released at higher levels at later time points. Consistent with this discrepancy, ectopic expression of full-length Zds1p was also more effective at promoting Net1p phosphorylation. Thus, we concluded that the N-terminal region of Zds1p helps regulate Net1p phosphorylation and release of Cdc14p from the nucleolus, but is not essential for the Zds1p activity during mitotic exit.

The Zds_C motif induces partial release of Cdc14p from the nucleolus

The aforementioned results suggest that the C-terminal region of Zds1p, which contains the Zds_C motif, is required for promotion of Net1p phosphorylation and release of Cdc14p from the nucleolus. We next sought to determine if ectopic expression of the Zds_C motif alone is able to activate Cdc14p. Thus, we prepared strains expressing either the Zds_C-terminal region containing the Zds_C motif (*zds1Δ1–802*), or the Zds_C motif alone (*zds1Δ1–840_Δ894–916*). We induced ectopic expression of *zds1Δ1–802* or *zds1Δ1–840_Δ894–916* in metaphase-arrested cells (Fig. 2). In cells containing full-length Zds1p, release of Cdc14p from the nucleolus occurred as expected. Interestingly, induction of *zds1Δ1–802* or *zds1Δ1–840_Δ894–916* led to Cdc14p

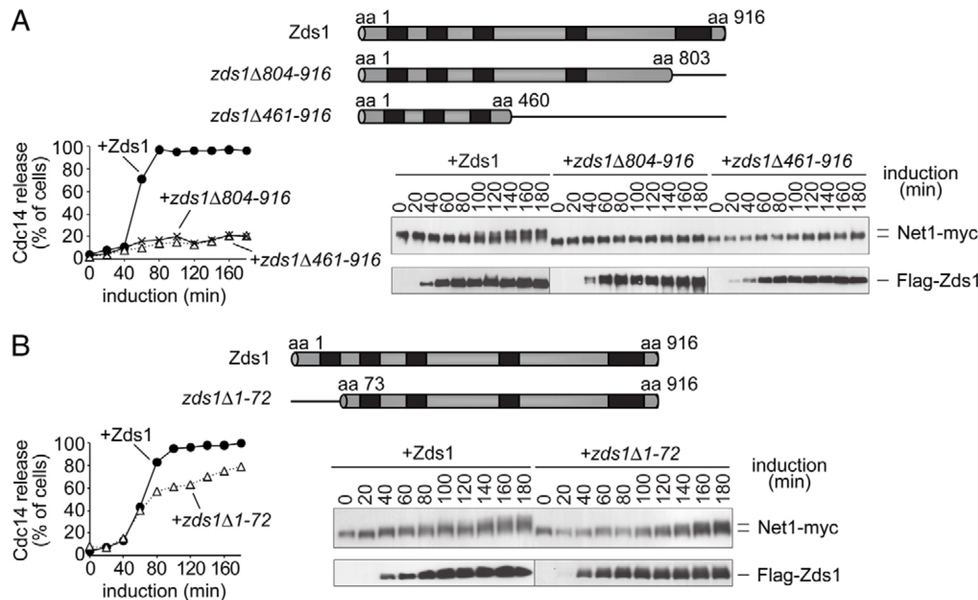

Fig. 1. The C-terminal region of Zds1p is necessary for inducing phosphorylation of Net1p and release of Cdc14p from the nucleolus. (**A**) Ectopic expression of truncated Zds1p proteins lacking the C-terminal domain of the protein fails to promote release of Cdc14p from the nucleolus. Strains Y496 (*MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ NET1-myc$_9$*), Y497 (as Y496, but *GAL1-Flag$_3$-zds1Δ804–916*) and Y498 (as Y496, but *GAL1-Flag$_3$-zds1Δ461–916*) were arrested in metaphase by Cdc20p depletion, and then Zds1p expression was induced in them. Release of Cdc14p from the nucleolus was monitored by immunofluorescence. Zds1p expression and Net1p phosphorylation were analyzed by western blotting. Samples for determination of Net1p phosphorylation status were run in the same protein gel. Western blots used to study the Zds1p protein levels were from different gels, because each truncated Zds1 protein is best visualized in a gel of a different acrylamide percentage. (**B**) The N-terminal region of Zds1p is not required for Zds1p-induced Net1p phosphorylation and Cdc14p release. As in A, but using strains Y496 (*MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ NET1-myc$_9$*) and Y501 (as Y496, but *GAL1-Flag$_3$-zds1Δ1–72*).

release from the nucleolus in 40% of cells. At the time of Zds1 accumulation, after induction of either *zds1Δ1–802* or *zds1Δ1–840_Δ894–916*, no slower-migrating forms of Net1p were detected by western blotting. This can be explained by the different experimental approaches employed: protein extracts give information on a mixed population of cells and sometimes the Net1-phosphorylated forms are below the threshold of detection by western blotting, whereas in situ immunofluorescence entails counting of single cells. We concluded that ectopic expression of the Zds_C motif in metaphase-arrested cells is able to cause release of Cdc14p from the nucleolus. As a control, we maintained the cultures without Zds1p induction, and did not observe either release of Cdc14p from the nucleolus or Net1p phosphorylation (supplementary material Fig. S2). Because of difficulties we encountered cloning the truncated versions of Zds1p, and because the C-terminal fragments *zds1Δ1–802* and *zds1Δ1–840_Δ894–916* yielded the same results, we chose to use the fragment *zds1Δ1–802* as the Zds_C motif for the majority of subsequent experiments.

The Zds1p domain, comprising residues 74–460, is required for complete release of Cdc14p from the nucleolus

On the basis of the aforementioned results we hypothesized that the role of the N-terminal region of Zds1p is either to regulate the activity of the protein or to target protein–protein interactions. Thus, to analyze the contribution of the N-terminal region of Zds1p, we constructed new truncated versions of Zds1p containing the first N-terminal homology domain between Zds1p and Zds2p. First, we expressed only residues 1–73 of the Zds1p protein (*zds1Δ74–916*; Fig. 3A). We induced ectopic expression of *zds1Δ74–916* in metaphase-arrested cells. In the cells expressing full-length Zds1p, slower migrating forms of Net1p accumulated and Cdc14p was released

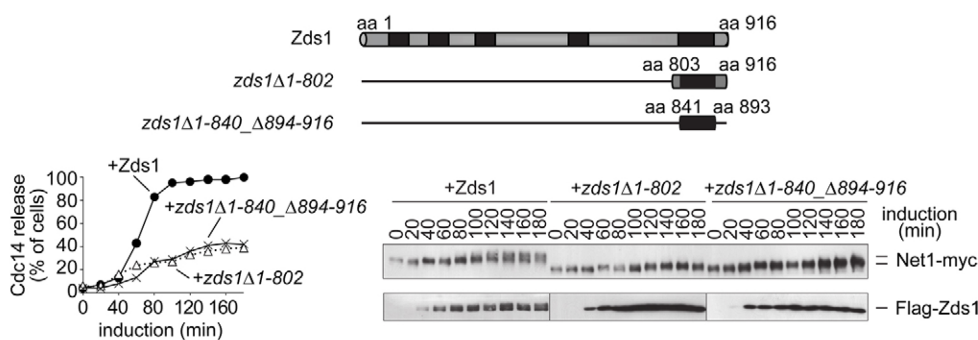

Fig. 2. The Zds1p Zds_C motif induced partial release of Cdc14p from the nucleolus. Strains Y496 (*MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ NET1-myc$_9$*), Y623 (as Y496, but *GAL1-Flag$_3$-zds1Δ1–802*) and Y651 (as Y496, but *GAL1-Flag$_3$-zds1Δ1–840_Δ894–916*) were arrested in metaphase by Cdc20p depletion, and then treated with galactose to induce Zds1p expression.

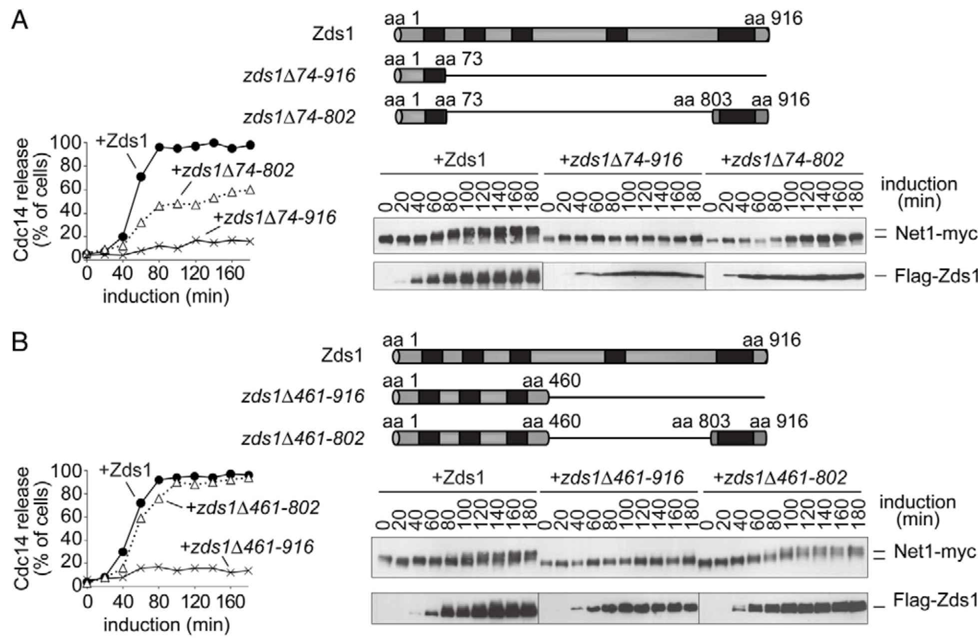

Fig. 3. The Zds1p N-terminal domain is required for full release of Cdc14p from the nucleolus. (**A**) Strains Y496 (*MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ NET1-myc$_9$*), Y691 (as Y496, but *GAL1-Flag$_3$-zds1Δ74–916*) and Y673 (as Y496, but *GAL1-Flag$_3$-zds1Δ74–802*) were arrested in metaphase by Cdc20p depletion, and then Zds1p expression was induced in them. (**B**) Strains Y496 (*MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ NET1-myc$_9$*), Y498 (as Y496, but *GAL1-Flag$_3$-zds1Δ461–916*) and Y533 (as Y496, but *GAL1-Flag$_3$-zds1Δ461–802*) were arrested in metaphase by Cdc20p depletion, and then treated with galactose to induce Zds1p expression.

from the nucleolus, as expected. By contrast, after induction of *zds1Δ74–916*, Net1p mobility shift was no longer observed and Cdc14p remained in the nucleolus. This result further confirmed that the N-terminal region of Zds1p is not sufficient to induce either phosphorylation of Net1p or release of Cdc14p from the nucleolus.

Secondly, we prepared a new construct (*zds1Δ74–802*) containing the first homology domain between Zds1p and Zds2p fused to the Zds_C motif. We induced ectopic expression of *zds1Δ74–802* in metaphase-arrested cells. Net1p phosphorylation was not detected, but in 50% of the cells, Cdc14p was released from the nucleolus: thus, *zds1Δ74–802* behaved similarly to the Zds_C motif alone (compare Fig. 3A with Fig. 2). This result indicated that the region comprising the first 73 residues of Zds1p, which encompasses one region of homology between Zds1p and Zds2p, does not contribute to Cdc14p activation.

Lastly, to further study whether the N-terminal region of Zds1p helps regulate Cdc14p release, we induced ectopic expression of a second fusion construct (*zds1Δ461–802*) between the N-terminal and the C-terminal regions of Zds1p: it contained the three N-terminal homology regions in Zds1p and Zds2p, plus the Zds_C motif (Fig. 3B). As previously observed, after induction of the truncated Zds1p protein *zds1Δ461–916* (Fig. 1A), Net1p mobility shift was no longer observed and Cdc14p remained in the nucleolus. This indicated that the N-terminal domain of Zds1p cannot promote release of Cdc14p from the nucleolus. Interestingly, when we ectopically expressed the fusion construct *zds1Δ461–802*, Net1p phosphorylation and release of Cdc14p from the nucleolus each occurred with similar kinetics to those of the full-length protein. These results indicated that the fusion construct is able to induce both Net1p phosphorylation and release of Cdc14p from the nucleolus. Together, these findings are consistent with the premise that the N-terminal region of Zds1p helps regulate Zds1p activity during mitotic exit.

We used a recently developed quantitative single-cell assay to quantify nucleolar localization of Cdc14p (Lu and Cross, 2009; Lu and Cross, 2010), determining the value from the ratio between the coefficients of variation (CV) of the Cdc14p–eGFP signal and of the Net1p–mCherry signal: when Cdc14p remains in the nucleolus, this ratio is high (values close to 1), and when the Cdc14p–eGFP signal is redistributed to the whole cell, this ratio is low (values close to 0.5). We repeated the above experiments for all the truncated Zds1p proteins in strains containing Cdc14p–eGFP and Net1p–mCherry. We arrested cells in metaphase by depletion of Cdc20p and then induced ectopic expression of the different truncated Zds1p proteins (Fig. 4). As shown previously, expression of the full-length Zds1p induced release of Cdc14p from the nucleolus. The ratio CV Cdc14:CV Net1 was equal to 0.35, indicating a high level of release of Cdc14p from the nucleolus. By contrast, after induction of the N-terminal-truncated Zds1p proteins *zds1Δ74–916* and *zds1Δ461–916*, the ratios values were close to 1, indicating that Cdc14p remained in the nucleolus. However, ectopic expression of either the Zds_C motif (*zds1Δ1–802*) or the Zds_C motif fused to the smaller N-terminal region of Zds1p (*zds1Δ74–802*) gave a ratio of roughly 0.62, which is consistent with partial release of Cdc14p. Finally, induction of the larger fusion construct *zds1Δ461–802*, which contains the three N-terminal homology regions in Zds1p and Zds2p, plus the Zds_C motif, gave a ratio of approximately 0.4, which is similar to the value obtained with the full-length protein. These quantifications paralleled the results that we obtained in the immunofluorescence assays (Figs 1–3), thereby reinforcing our earlier conclusions.

The Zds1p N-terminal and C-terminal domains are both necessary for timely release of Cdc14p from the nucleolus in early anaphase

To further characterize the functional Zds1p domains involved in timely release of Cdc14p from the nucleolus, we studied the truncated Zds1p proteins expressed at endogenous levels. We constructed a centromeric plasmid containing the different versions of truncated Zds1p under the control of the *ZDS1* promoter and then introduced them into a *zds1Δ* strain. We

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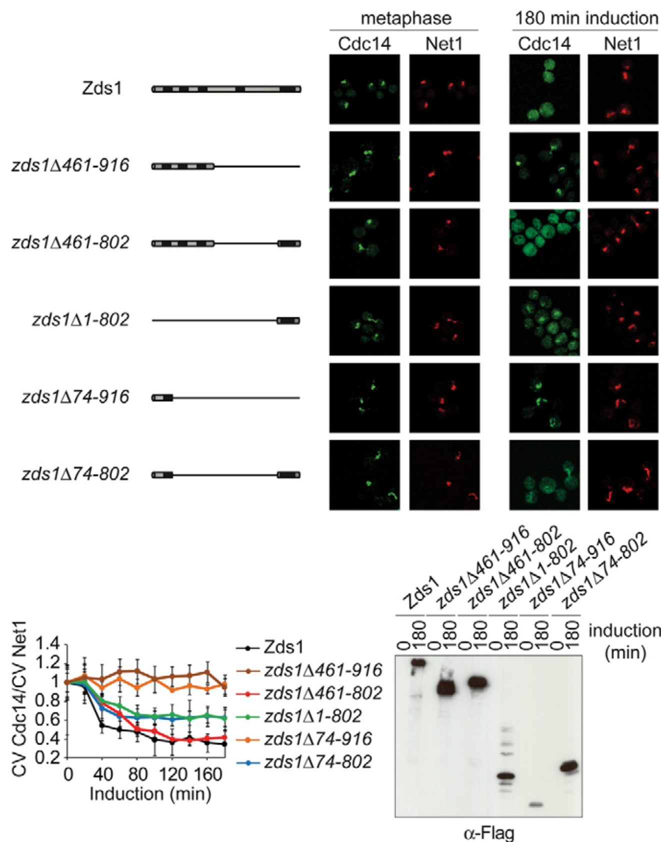

Fig. 4. Quantification of Cdc14p release. Strains Y715 (*MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-eGFP NET1-mCherry*), Y712 (as Y715, but *GAL1-Flag$_3$-zds1Δ461–916*), Y713 (as Y715, but *GAL1-Flag$_3$-zds1Δ461–802*), Y709 (as Y715, but *GAL1-Flag$_3$-zds1Δ1–802*), Y711 (as Y715, but *GAL1-Flag$_3$-zds1Δ74–916*) and Y716 (as Y715, but *GAL1-Flag$_3$-zds1Δ74–802*) were arrested in metaphase by Cdc20p depletion, and then treated with galactose to induce Zds1p expression. Cdc14p was quantified at each time point as described in Materials and Methods. At least 30 cells were measured for the quantification at each time point. Representative images of cells in metaphase and at 180 minutes after Zds1p induction are shown. Zds1p expression levels were checked by western blotting.

quantified release of Cdc14p from the nucleolus relative to an internal marker for mitotic progression: anaphase spindle length. Cdc14p was released in 60% of the cells expressing the full-length Zds1p protein at a spindle length of 3–4 μm (Fig. 5A), which is similar to wild-type cells, as we previously reported (Queralt and Uhlmann, 2008b). By contrast, Cdc14p was only released in 26% of control *zds1Δ* cells bearing an empty plasmid (pYCplac22) – again, consistent with what we previously reported (Queralt and Uhlmann, 2008b). Cdc14p was released in 22% and 28% of *zds1Δ* cells containing the N-terminal truncated Zds1p proteins *zds1Δ74–916* and *zds1Δ461–916*, respectively; these values are similar to the ones that we obtained with the *zds1Δ* mutant cells. Cdc14p was released in 36% of the cells expressing the Zds_C motif, either alone (*zds1Δ1–802*) or fused to the Zds1p N-terminal region between residues 1 and 73 (*zds1Δ74–802*). Interestingly, Cdc14p was released in 52% of the cells containing the Zds1p N-terminal region from residues 1 to 460 fused to the Zds_C motif (*zds1Δ461–802*), similar to what we observed with the wild-type protein at endogenous levels. Thus, both the N-terminal and the C-terminal region of Zds1p participate in timely Cdc14 activation in early anaphase. However, the Zds_C motif appears to contribute more strongly than the N-terminal does: *zds1Δ* cells expressing the Zds_C motif alone or fused to the Zds1p N-terminal region from residues 1 to 73 presented modest but reproducible release of Cdc14p from the nucleolus in early anaphase cells (spindle length of 3 to 4 μm).

To determine whether there was any delay in Cdc14p activation with the truncated Zds1p proteins relative to the wild-type protein, we studied single cells by time-lapse microscopy. We determined Cdc14p–eGFP localization and compared it with the nucleolar Net1p–mCherry signal in synchronized cells at the metaphase-to-anaphase transition, by depletion and subsequent re-induction of Cdc20p. The *zds1Δ* cells containing a plasmid expressing the full-length Zds1p protein released Cdc14p on average 22±2.6 minutes after entering synchronous anaphase. Approximately 17 minutes later, Cdc14p–eGFP was re-sequestered into the nucleolus (Fig. 5B,D). By contrast, in the *zds1Δ* control cells (bearing the empty vector pYCplac22), mean Cdc14p release occurred 29±2.0 minutes after entering synchronous anaphase. Cells expressing the Zds_C motif alone (*zds1Δ1–802*) released Cdc14p approximately 27±1.8 minutes after entering synchronous anaphase. Remarkably, the Zds1p fusion construct *zds1Δ461–802*, which encodes the Zds1p N-terminal region from residues 1 to 460, bound to the Zds_C motif, promoted release of Cdc14p from the nucleolus at the same time (mean: 22.5±1.8 minutes) as the full-length protein did. These results confirmed that *zds1Δ* cells experience delayed Cdc14p activation (of approximately 5–9 minutes) compared with wild-type cells. All these findings further corroborated that both the N-terminal and the C-terminal region of Zds1p are necessary for timely release of Cdc14p from the nucleolus in early anaphase.

We previously demonstrated that control of mitotic exit by PP2A^{Cdc55} is independent of Cdc28p inhibitory phosphorylation. In *S. cerevisiae* and in higher eukaryotes mitotic entry is driven by Cdk1 activation. Full Cdk1 activation is prevented by phosphorylation of Cdc28 Y19 by the kinase Wee1 (Swe1p in *S. cerevisiae*). Cdc25 phosphatase (Mih1p in *S. cerevisiae*) eliminates the inhibitory signal by dephosphorylating Cdc28 Y19, thereby enabling mitotic entry. Introduction of the *CDC28^{Y19F}* allele is a good experimental technique for distinguishing between PP2A^{Cdc55} functions in mitotic entry (by Swe1p regulation) and in mitotic exit (by controlling Cdc14p activation) (Queralt et al., 2006). *zds1Δ zds2Δ* double-mutant cells are highly elongated because of prolonged G2 delay (Pal et al., 2008; Rossio and Yoshida, 2011). *zds1Δ zds2Δ* cells are difficult to synchronize, and grow very slowly, and consequently, lose synchrony quickly. Therefore, we introduced the *CDC28^{Y19F}* allele, which is refractory to inhibition by phosphorylation at Cdc28-Y19 and rescues the elongated morphology phenotype of the *zds1Δ zds2Δ* double mutant. We previously reported that *zds1Δ zds2Δ* double-mutant cells experience an even longer delay in Cdc14p activation than the *zds1Δ* single-mutant cells (Queralt and Uhlmann, 2008b). We synchronized cells at the metaphase-to-anaphase transition by Cdc20p depletion. Following entry into anaphase (triggered by Cdc20p re-addition), the kinetics of release of Cdc14p from the nucleolus in *zds1Δ zds2Δ* cells were indistinguishable from those in *zds1Δ zds2Δ CDC28^{Y19F}* cells (supplementary material Fig. S3). Next,

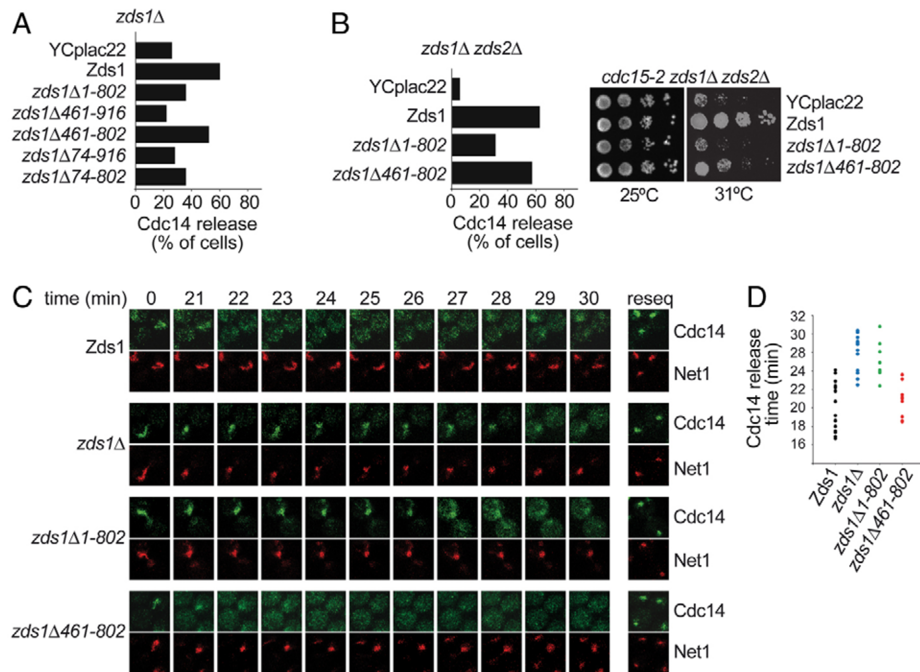

Fig. 5. Delayed release of Cdc14p from the nucleolus in the absence of the N-terminal and the C-terminal domains of Zds1p. (**A**) Analysis of Cdc14p release in the different truncated Zds1p proteins at endogenous levels. Strains Y668 (*MATa MET-CDC20 CDC14-Pk$_9$ NET1-myc$_{18}$ zds1Δ* pYCplac22), Y702 (as Y668, but pYCplac22-*Flag$_3$-ZDS1*), Y674 (as Y668, but pYCplac22-*Flag$_3$-zds1Δ1–802*), Y703 (as Y668, but pYCplac22-*Flag$_3$-zds1Δ461–916*), Y706 (as Y668, but pYCplac22-*Flag$_3$-zds1Δ461–802*), Y708 (as Y668, but pYCplac22-*Flag$_3$-zds1Δ74–916*) and Y701 (as Y668, but pYCplac22-*Flag$_3$-zds1Δ74–802*) were arrested in metaphase by Cdc20p depletion and then brought into synchronous anaphase. Release of Cdc14p from the nucleolus was quantified in at least 50 cells with a spindle length of 3–4 µm. As control, strain Y746 (*MATα CDC14-HA$_6$ ZDS1-Flag$_3$*), epitope-tagged in the C-terminus at the endogenous locus, was used. (**B**) The truncated protein *zds1Δ461–802* functions similarly to full-length Zds1p in terms of mitotic exit. Left panel: the *zds1Δ461–802*-truncated Zds1p timely release of Cdc14p from the nucleolus. Strains Y765 (*MATα MET-CDC20 CDC14-HA$_6$ CDC28^{Y19F} zds1Δ zds2Δ* pYCplac22), Y766 (as Y765, but pYCplac22-*Flag$_3$-ZDS1*), Y767 (as Y765, but pYCplac22-*Flag$_3$-zds1Δ1–802*) and Y768 (as Y765, but pYCplac22-*Flag$_3$-zds1Δ461–802*) were arrested in metaphase by Cdc20p depletion, and release of Cdc14p from the nucleolus was quantified as in A. Right panel: suppression of the synthetic growth defect of the *cdc15-2* and *zds1Δ zds2Δ* mutations by the *zds1Δ461–802* truncated Zds1p protein. Viability of the strains Y769 (*MATa CDC14-Pk$_9$ NET1-myc$_9$ cdc15-2 zds1Δ zds2Δ* pYCplac22), Y770 (as Y769, but pYCplac22-*Flag$_3$-ZDS1*), Y771 (as Y769, but pYCplac22-*Flag$_3$-zds1Δ1–802*) and Y772 (as Y769, but pYCplac22-*Flag$_3$-zds1Δ461–802*) was determined by spotting tenfold serial dilutions onto YPD plates and incubating at 31℃ for 2 days. (**C**) Time-lapse microscopy of Cdc14p release in cells with the truncated Zds1p proteins. Strains Y761 (*MATa MET-CDC20 CDC14-eGFP NET1-mCherry zds1Δ* pYCplac22-*Flag$_3$-ZDS1*), Y764 (as Y761, but pYCplac22), Y762 (as Y761, but pYCplac22-*Flag$_3$-zds1Δ1–802*) and Y763 (as Y761, but pYCplac22-*Flag$_3$-zds1Δ461–802*) were brought into synchronous anaphase by Cdc20p re-addition. Representative time-lapse images of the CDC14p–GFP and Net1p–mCherry experiments are shown for each strain. (**D**) Release of Cdc14p from the nucleolus was timed in at least ten cells.

we introduced the aforementioned centromeric plasmids (encoding the different truncated Zds1p proteins), and again studied Cdc14p activation relative to anaphase spindle length (as an internal marker for mitotic progression). Most *zds1Δ zds2Δ CDC28^{Y19F}* control cells (with an empty vector) released Cdc14p only at spindle lengths of 6–7 µm and longer, as we previously described (Queralt and Uhlmann, 2008b): only 13% of the cells released Cdc14p at spindle lengths of 3–4 µm (Fig. 5B, left panel). Cdc14p was released in 64% of the cells expressing the full-length Zds1p protein, which is similar to what we had previously observed in wild-type and *zds2Δ* cells (Queralt and Uhlmann, 2008b). By contrast, Cdc14p was only released in 26% of the cells expressing the Zds_C motif alone (*zds1Δ1–802*). Interestingly, Cdc14p was released in 58% of the cells with the fusion construct *zds1Δ461–802* (containing the Zds1p N-terminal region from residues 1–460 fused to the Zds_C motif), which is similar to what we observed for the cells with the wild-type protein. All these results further support the premise that both the N-terminal and C-terminal regions of Zds1p participate in timely Cdc14p activation in early anaphase.

To further ascertain the functions of Zds1p during mitotic exit, we explored the genetic relationship between Zds1p and the MEN. The MEN is essential to maintain Cdc14p activity during mitotic exit and Cdc15p is an essential MEN kinase. The mutants *cdc15-2* and *zds1Δ zds2Δ* exhibit a synthetic growth defect at 31℃ (Queralt and Uhlmann, 2008b). We prepared *cdc15-2 zds1Δ zds2Δ* strains containing different truncated Zds1p proteins at endogenous levels, and then analyzed their growth at 31℃ (Fig. 5B, right panel). Cells expressing full-length Zds1p protein rescued the synthetic growth defect of the *cdc15-2* and *zds1Δ zds2Δ* mutations. Remarkably, the fusion construct *zds1Δ461–802* also rescued the synthetic growth defect of the triple mutant, although to a lesser extent than the full-length protein. These results indicated that during mitotic exit *zds1Δ461–802* (containing the Zds1 N-terminal region, residues 1–460 fused to the Zds_C motif) functions similarly to full-length Zds1p.

The Zds1p Zds_C motif recruits PP2A^{Cdc55} to the nucleolus

Zds1p interacts with PP2A^{Cdc55} throughout mitosis (Queralt and Uhlmann, 2008b), through the Cdc55p subunit (Wicky et al.,

Journal of Cell Science

2011) (our unpublished results). Moreover, the Zds2p C-terminal domain is necessary and sufficient for interaction with Cdc55p (Yasutis et al., 2010). This prompted us to study a possible interaction between Cdc55p and the truncated versions of Zds1p. Thus, we performed co-immunoprecipitation analyses with Cdc55p and each of the truncated Zds1p proteins. We prepared protein extracts from *zds1Δ* cells bearing centromeric plasmids expressing (under control of the *ZDS1* promoter) full-length Zds1p; the Zds_C motif (*zds1Δ1–802*); the N-terminal truncated versions of Zds1p (*zds1Δ74–916* and *zds1Δ461–916*); the Zds_C motif fused to the Zds1p N-terminal region between residues 1 and 73 (*zds1Δ74–802*); and the Zds_C motif fused to the N-terminal region between residues 1 and 460 (*zds1Δ461–802*). None of the truncations that contained the Zds_C motif had any effect on the interaction between Cdc55p and Zds1p (Fig. 6A). However, in immunoprecipitates of the Zds1p N-terminal truncated versions (*zds1Δ74–916* and *zds1Δ461–916*), we were not able to detect co-purification of the Cdc55p subunit. Therefore, we concluded that Zds1p interacts predominantly with Cdc55p through its Zds_C motif.

Cdc55p is located in the nucleolus to keep Net1p under-phosphorylated in metaphase, and it remains in the nucleolus throughout mitosis (Queralt et al., 2006). In order to further corroborate our previous results, we visualized Cdc55p localization from chromosome spreads at the metaphase to anaphase transition. To detect nucleolar localization, the spread chromosomes were washed to remove nucleoplasmic protein, and then immunostained (Fig. 6B). The nucleolar Cdc55p signal was relatively weak, which was probably because of the lower amount of Cdc55p protein remaining in the nucleolus after washing away the nucleoplasmic protein. Thus, we used the in situ proximity ligation assay (PLA) method to stain for Cdc55p protein. Colocalization with Net1p revealed similar nucleolar enrichment of Cdc55p in metaphase and in cells released into synchronous anaphase. In the chromosome spreads, the Cdc55p signal was predominantly nucleolar; this was probably because the nuclear Cdc55p detected in intact cells is a soluble nuclear protein, and therefore, was washed away during spreading of the samples. These results further confirm that Cdc55p is at the right place to keep Net1p under-phosphorylated in metaphase, and

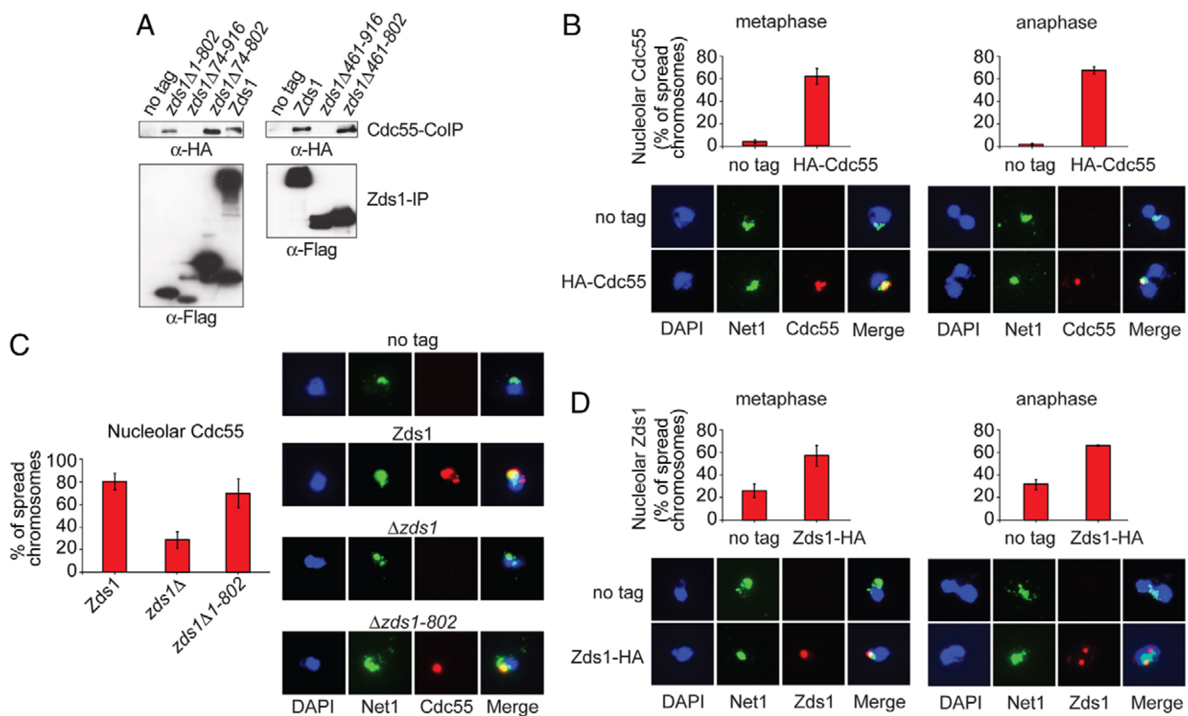

Fig. 6. Zds1p recruits PP2A^{Cdc55} into the nucleolus through its Zds_C motif. (**A**) Zds1p interacts with PP2A^{Cdc55} through its Zds_C motif. Co-immunoprecipitation between Zds1p and Cdc55p was analyzed in protein extracts from strains Y730 (*MATa HA$_3$-CDC55 zds1Δ* pYCplac22-*Flag$_3$-ZDS1*), Y727 (as Y730, but pYCplac22-*Flag$_3$-zds1Δ1–802*), Y728 (as Y730, but pYCplac22-*Flag$_3$-zds1Δ74–916*), Y729 (as Y730, but pYCplac22-*Flag$_3$-zds1Δ74–802*), Y731 (as Y730, but pYCplac22-*Flag$_3$-zds1Δ461–916*) and Y732 (as Y730, but pYCplac22-*Flag$_3$-zds1Δ461–802*). Protein extracts from strain Y756 (*MATa HA$_3$-CDC55 zds1Δ* pYCplac22) lacking a FLAG epitope on Zds1p served as control. IP, immunoprecipitation; CoIP, co-immunoprecipitation. (**B**) Cdc55p localization to the nucleolus. Strain Y692 (*MATa GAL1-CDC20 HA$_3$-CDC55 NET1-GFP*) was arrested in metaphase by the depletion of Cdc20p and released into synchronous anaphase. Chromosome spreading was performed in metaphase, and 20 minutes after release, when 80% of cells were in anaphase (as seen by tubulin staining). Strain Y2959 (*MATa GAL1-CDC20 NET1-GFP*) lacking an HA epitope on Cdc55p served as negative control. Net1p signal was used as a nucleolar marker. (**C**) The Zds_C motif is required for proper localization of PP2A^{Cdc55} in the nucleolus. Strains Y838 (*MATa MET-CDC20 HA$_3$-CDC55 NET1-GFP zds1Δ* pYCplac22-*Flag$_3$-ZDS1*), Y837 (as Y838, but pYCplac22), Y833 (as Y838, but pYCplac22-*Flag$_3$-zds1Δ1–802*) were arrested in metaphase by depletion of Cdc20p. Strain Y818 (*MATa zds1Δ NET1-GFP* pYCplac22-*Flag$_3$-ZDS1*) lacking an HA epitope on Cdc55p served as negative control. (**D**) Zds1p nucleolar localization. Strain Y3227 (*MATa GAL1-CDC20 ZDS1-HA$_6$ NET1-GFP*) was arrested in metaphase and released into synchronous anaphase. Strain Y2959 (*MATa GAL1-CDC20 NET1-GFP*) lacking an HA epitope on Zds1p served as negative control. Net1p signal was used as a nucleolar marker. Staining of the chromosome spreads in B–D was performed using PLA probes, as described in Materials and Methods.

Journal of Cell Science

no obvious localization changes during anaphase have been observed that could explain the downregulation of the PP2A^{Cdc55} specifically at anaphase.

Our results described above suggested that the Zds_C motif is required for the interaction between Cdc55p and Zds1p. Thus, we first wondered whether Zds1p is necessary to maintain the nucleolar localization of PP2A^{Cdc55} (Fig. 6C). *zds1Δ* cells bearing a centromeric plasmid expressing full-length Zds1p at endogenous levels colocalized with Net1p, revealing nucleolar enrichment of Cdc55p, similarly to that previously reported (Queralt et al., 2006) (Fig. 6B). Strikingly, in *zds1Δ* mutant cells (containing an empty vector), Cdc55p nucleolar localization was impaired. This result suggests that in the absence of Zds1p protein, Cdc55p nucleolar localization is dramatically reduced, and therefore, that Zds1p is involved in maintaining Cdc55p in the nucleolus. More interestingly, expression of the Zds_C motif at endogenous levels in the *zds1Δ* mutant cells (*zds1Δ1–802*) rescued the Cdc55p nucleolar localization. Thus, we can conclude that the Zds_C motif is required to interact with Cdc55p and that this interaction is necessary to properly localized Cdc55p to the nucleolus.

Our above results suggest that the interaction between Zds1p and Cdc55p is necessary to stably maintain Cdc55p in the nucleolus or to recruit Cdc55p into the nucleolus. Alternatively, the Zds1p and Cdc55p interaction could occur in the cytoplasm but it could be required for the posterior transport of Cdc55p into the nucleolus. In order to distinguish between these two possibilities we studied the localization of Zds1. Zds1p has been previously described to be a cytoplasmic protein (Bi and Pringle, 1996; Rossio and Yoshida, 2011). However, nuclear and nucleolar functions have been previously described for Zds1p and Zds2p. For instance, Zds1p and Zds2p physically interact with Sir proteins and with telomeric proteins such as Rap1p (Roy and Runge, 1999), and Zds1p and Zds2p are involved in chromatin silencing at the rDNA (Roy and Runge, 2000). Therefore, a putative Zds1p nuclear localization was expected. We first checked the Zds1p localization in metaphase cells by immunofluorescence (supplementary material Fig. S5). A Zds1p staining was observed throughout the whole cell, suggesting a cytoplasmic and nuclear localization of Zds1. In addition, by *z*-axis projection of confocal images, no nuclear exclusion was observed in mitotic cells. Next, to detect nucleolar localization we immunostained chromosomal spreads and colocalization with nucleolar Net1 was determined (Fig. 6D). Because Zds1p signal was low, we had to concentrate more on the primary antibody (see Materials and Methods). There was some signal from the negative control, but a nucleolar Zds1p signal was detectable and significant. Therefore, we can conclude that Zds1p is present in the nucleolus in mitotic cells, suggesting that Zds1p and Cdc55p interaction is important to recruit or maintain Cdc55p in the nucleolus.

Discussion

The budding yeast phosphatase Cdc14p is a key regulator of mitotic exit. Cdc14p is kept inactive in the nucleolus during most of the cell cycle, but is released throughout the cell to contribute to Cdk downregulation. Cdc14p is activated early in anaphase, as soon as sister chromatids split, and its activation is a prerequisite for successful chromosome segregation. Cdc14p is crucial to successful anaphase progression: it leads to stabilization of microtubules in the elongating anaphase spindle and to assembly of a spindle midzone structure, and is required for condensation and resolution of the late-segregating rDNA locus (D'Amours et al., 2004; Higuchi and Uhlmann, 2005; Khmelinskii et al., 2007; Pereira and Schiebel, 2003; Sullivan et al., 2004; Woodbury and Morgan, 2007). Cdk substrates that are dephosphorylated in these processes include the microtubule regulators Ase1p, Ask1p, Fin1p and Sli15p (D'Amours et al., 2004; Khmelinskii et al., 2007; Pereira and Schiebel, 2003; Woodbury and Morgan, 2007). During anaphase progression in budding yeast, at least two pathways are required to activate Cdc14p during states of high and low Cdk activity: the FEAR and the MEN, respectively.

At the onset of anaphase, release of separase from its inhibitor securin plays a crucial role in Cdc14p activation as part of the FEAR pathway (Queralt et al., 2006; Stegmeier et al., 2002; Sullivan and Uhlmann, 2003). Separase-dependent PP2A^{Cdc55} downregulation at anaphase onset allows Cdk-dependent phosphorylation of the nucleolar Cdc14p inhibitor Net1p, thus promoting Cdc14p release (Queralt et al., 2006). We have previously shown that separase cooperates with two PP2A interactors, Zds1p and Zds2p, to induce Cdc14p activation (Queralt and Uhlmann, 2008b). Zds1p is required for timely Cdc14p activation during anaphase. Ectopic expression of Zds1p or Zds2p is sufficient to trigger PP2A^{Cdc55} downregulation, Cdk-dependent Net1p phosphorylation, and release of Cdc14p from the nucleolus. All the experiments in which we ectopically expressed Zds1p were done in the presence of wild-type Zds2p protein. Therefore, we cannot rule out the possibility that an interaction between Zds1p and Zds2p could be required.

One of our objectives was to study how Zds1p regulates PP2A^{Cdc55} activity. To this end, we performed a functional domain study of Zds1p in order to investigate the mechanism by which it compromises PP2A^{Cdc55} activity. Our results suggest that the functional domain involved in Cdc14p activation is the C-terminal domain (the Zds_C motif, Pfam086032), which comprises residues 803 to 916. The Zds_C motif is required for Zds1p-induced release of Cdc14p from the nucleolus, when *ZDS1* is overexpressed from the *GAL1* promoter. Ectopic expression of the N-terminal region of Zds1p alone does not promote Cdc14p activation. However, a fusion construct containing the Zds1p N-terminal region from residues 1 to 460 fused to the Zds_C motif induces release of Cdc14p from the nucleolus to the same degree as the wild-type protein does. Altogether, we concluded that the Zds_C motif is the most important region of Zds1p for release of Cdc14p from the nucleolus and that the N-terminal region helps regulate Zds1p activity during mitotic exit. More interestingly, when we analyzed the different truncated Zds1p proteins at endogenous levels, using a centromeric plasmid that expresses Zds1p at similar levels as it does the endogenous epitope-tagged protein (supplementary material Fig. S4), the Zds_C motif alone could not induce timely release of Cdc14p from the nucleolus in the *zds1Δ* or *zds1Δ zds2Δ* background strains. Furthermore, the Zds_C motif cannot suppress the synthetic growth defect of the *cdc15-2* and *zds1Δ zds2Δ* mutations. Strikingly, the fusion construct containing the Zds1p N-terminal region from residues 1 to 460 fused to the Zds_C motif (*zds1Δ461–802*) induces timely nucleolar Cdc14 release at endogenous levels and can suppress the synthetic growth defect of the *cdc15-2* and *zds1Δ zds2Δ* mutations. Therefore, both the N-terminal and the C-terminal regions of Zds1p are required for timely Cdc14p activation at anaphase onset. All these results are consistent with the

Journal of Cell Science

C-terminal Zds_C motif being more important for the activity of the Zds1p protein, and with the possibility that the N-terminal region of the protein helps regulate this activity.

Zds1p forms a complex with PP2A^{Cdc55} (Queralt and Uhlmann, 2008b) and interaction of Zds1p with PP2A^{Cdc55} depends on the presence of the Cdc55p regulatory subunit (Wicky et al., 2011). We observed that all the Zds1p constructs containing the Zds_C motif physically interact with Cdc55p in co-immunoprecipitation experiments. Consistent with these results, Yasutis et al. demonstrated that Zds2p directly binds to Cdc55p in vitro through its conserved C-terminal region (Yasutis et al., 2010). Moreover, we have shown that Zds1p regulates the localization of Cdc55p through the Zds_C motif. Thus, in the absence of Zds1p, Cdc55p nucleolar localization is impaired. These results suggest that Zds1p exerts its biological function as PP2A regulator by controlling the PP2A^{Cdc55} localization into the nucleolus, through their physical interaction through the Zds_C motif. Nevertheless, expression of the Zds_C motif at endogenous levels does not induce timely release of Cdc14p from the nucleolus, despite the proper localization of Cdc55p (in the nucleolus). This suggests that the activity of PP2A^{Cdc55} cannot be modulated solely through regulation of its localization, and therefore, that an additional step of regulation might control full PP2A^{Cdc55} activation. Consistently with these observations, we have described that in anaphase cells, when PP2A^{Cdc55} activity is downregulated, Cdc55p remains in the nucleolus (Queralt et al., 2006) (Fig. 6B). This observation argues against the possibility that changes in Cdc55p localization are the primarily regulatory mechanism inactivating PP2A^{Cdc55} during anaphase. Moreover, like separase, Zds1p and Zds2p are relatively low-abundance proteins (Ghaemmaghami et al., 2003). Therefore, it seems unlikely that Zds1p and Zds2p act as direct inhibitory components of the PP2A^{Cdc55} complex. Instead, they might be involved in regulating the status of post-translational modifications or in controlling conformational changes in the PP2A^{Cdc55} holoenzyme that compromises the activity of the complex (Hombauer et al., 2007).

Here, we have shown that Zds1p physically interacts with, and regulates the localization of, Cdc55p through the Zds_C motif. These results show that regulation of PP2A by Zds1p occurs through a physical interaction between these two proteins, which involves the Zds_C motif of the latter. Zds1p and Zds2p have been implicated in various cellular processes that are or might be influenced by PP2A, including cell cycle regulation, cell polarity establishment and chromatin silencing. In fact, Zds1p and Zds2p have been revealed as PP2A regulators, not only during mitotic exit, but also in diverse cellular settings. Fission yeast zds1, similar to its budding yeast ortholog, has been found to contribute to myriad cellular processes, including sexual differentiation, cell wall integrity and cell morphology (Yakura et al., 2006). To date, no homologs or orthologs of the Zds proteins in mammalian cells have been characterized; however, some weak sequence homology between the Zds proteins and the pseudouridine synthase 10 family of proteins has been found (Yasutis et al., 2010). Future work will be required to elucidate the mechanism by which separase and Zds1p regulate PP2A^{Cdc55} activity to promote progression through anaphase, which might serve as a paradigm for regulation of PP2A by Zds1p and Zds2p in other cellular contexts. Recently, a role for PP2A^{Cdc55} during meiosis in budding yeast has been described (Bizzari and Marston, 2011; Kerr et al., 2011). Whether Zds1p also acts as a regulator of PP2A during meiosis would be interesting to determine.

Materials and Methods

Yeast strains, plasmids and cell cycle synchronization procedures

All yeast strains used in this study were derivatives of W303. Epitope tagging of endogenous genes and gene deletions were performed by gene targeting using polymerase chain reaction (PCR) products. For ectopic expression of truncated Zds1p proteins, the different *ZDS1* fragments were generated by PCR, introduced into a YIp204-GAL1 plasmid, and induced by addition of galactose. To obtain the various *ZDS1* fragments, the following amplified products were obtained: *zds1Δ804–916* (comprising nucleotides 1–2409); *zds1Δ461–916* (1–1380); *zds1Δ1–72* (217–2748); *zds1Δ1–802* (2407–2748); *zds1Δ1–840_Δ894–916* (2521–2679); and *zds1Δ74–916* (1–219). Next, the truncated Zds1 proteins containing internal deletions, *zds1Δ74–802* and *zds1Δ461–802,* were created by cloning a PCR fragment from nucleotides 2407 to 2748 into the previously described *zds1Δ74–916* and *zds1Δ461–916*, respectively. Three tandem FLAG epitopes were inserted in front of the open reading frame, and plasmids were integrated into the yeast genome after linearization within the *TRP1* marker gene. For endogenous expression of the truncated Zds1 proteins, a fragment of the *ZDS1* promoter from −314 was generated by PCR and subsequently introduced into the centromeric plasmid YCplac22, and the different *ZDS1* fragments were added by subcloning from the aforementioned integrative plasmids. Three tandem FLAG epitopes were inserted between the promoter and the open reading frame, and yeast strains containing the *ZDS1* or *ZDS1 ZDS2* deletions were transformed and grown in synthetic medium lacking tryptophan. N-terminal tagging of endogenous *CDC55* was performed as previously described (Queralt et al., 2006). *CDC28*^{Y19F} mutant strains were created by integration and loop-out, using 5-fluoroorotic acid selection, of the linearized pJM1054 plasmid (a gift from D. J. Lew) (McMillan et al., 1999). Ectopic expression of Zds1p and each truncated Zds1p protein in cells that had been arrested in metaphase by Cdc20p depletion was performed as previously described (Queralt and Uhlmann, 2008b). Metaphase arrest by Cdc20p depletion and entry into synchronous anaphase by Cdc20p re-induction were also performed as previously described (Uhlmann et al., 1999). Functionality of all the epitope-tagged proteins used in this work were checked by analyzing the kinetics of Cdc14 release from the nucleolus during mitosis.

In vivo imaging and image analysis

Time-lapse microscopy was performed on a Leica TCS SP5 inverted confocal spectral fluorescence microscope equipped with an environmental chamber. Images were acquired every minute with LAS AF software (Leica Microsystems). *Z*-stacks at 0.7 μm intervals were taken for each fluorescence channel and projected onto a single image per channel. For quantitative analysis of fluorescence microscopy, ImageJ software was used. Release of Cdc14p from the nucleolus was quantified as the ratio between the coefficient of variation (CV; standard deviation divided by mean) of the Cdc14p–eGFP signal and the CV of the Net1p–mCherry signal (Lu and Cross, 2009; Lu and Cross, 2010).

Immunoprecipitation assay

The immunoprecipitation assay was performed as previously described (Queralt and Uhlmann, 2008b). Protein extracts were prepared by mechanical lysis using glass beads. The clarified extracts were incubated with antibody, and the immunocomplexes were adsorbed onto magnetic protein-A Dynabeads® (Invitrogen). The beads were washed in extraction buffer and boiled with SDS-PAGE loading buffer. The antibody used for immunoprecipitation was anti-FLAG clone M2 (Sigma).

Other techniques

Protein extracts for western blots were obtained by TCA protein extraction. The antibodies used for western blots were anti-HA clone 12CA5 (Roche), anti-myc clone 9E10 (Babco), anti-FLAG clone M2 (Sigma) and anti-FLAG polyclonal (Sigma). The primary antibodies used for immunofluorescence were anti-HA clone 16B12 (Babco), anti-Pk clone SV5-Pk1 (Serotec) and anti-α-tubulin clone YOL1/34 (Serotec). The secondary antibodies were Cy3-labeled anti-mouse (GE Healthcare) and fluorescein-conjugated anti-rat (Millipore). Cells were imaged at room temperature using a DM6000B Leica microscope (Leica Microsystems) equipped with a 63× objective and a DFC 360FX camera. The spindle lengths at anaphase were measured with the quantitative analysis tools of the LAS AF software (Leica Microsystems). Chromosome spreads were performed as previously described (Michaelis et al., 1997). The primary antibody used for the chromosome spreads was anti-HA clone 16B12 (Babco) at 1:500 for Cdc55p and at 1:100 for Zds1p, and the secondary antibodies were Cy3-labeled anti-mouse-PLA probes (Olink Bioscience). The in situ PLA method can be used to detect and quantify low-abundance proteins. A pair of oligonucleotide-labeled secondary antibodies (PLA probes) generates a signal only when the two PLA probes have bound, in close proximity, the same primary antibody; the signal from each detected pair is visualized as an individual fluorescent spot. Secondary antibody

Journal of Cell Science

staining and detection were performed using Duolink II reagents (Olink Bioscience), following the manufacturer's instructions.

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