Nuclear PP2A-Cdc55 prevents APC-Cdc20 activation during the spindle assembly checkpoint

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Summary

Cdc55, a regulatory B-subunit of protein phosphatase 2A (PP2A) complex, is essential for the spindle assembly checkpoint (SAC) in budding yeast, but the regulation and molecular targets of PP2A-Cdc55 have not been clearly defined or are controversial. Here, we show that an important target of Cdc55 in the SAC is the anaphase-promoting complex (APC) coupled with Cdc20 and that APC-Cdc20 is kept inactive by dephosphorylation by nuclear PP2A-Cdc55 when spindle is damaged. By isolating a new class of Cdc55 mutants specifically defective in the SAC and by artificially manipulating nucleocytoplasmic distribution of Cdc55, we further show that nuclear Cdc55 is essential for the SAC. Because the Cdc55-binding proteins Zds1 and Zds2 inhibit both nuclear accumulation of Cdc55 and SAC activity, we propose that spatial control of PP2A by Zds1 family proteins is important for tight control of SAC and mitotic progression.

Key words: Protein phosphatase 2A, PP2A, Spindle assembly checkpoint, SAC, Anaphase-promoting complex, APC, Cdc55, Zds1

Introduction

The PP2A (protein phosphatase 2A) proteins are a conserved family of serine/threonine phosphatases that have many important roles in mitotic progression in eukaryotes (Shi, 2009). The heterotrimeric PP2A consists of a structural A subunit, a regulatory B subunit and a catalytic C subunit. B subunits bind to the AC heterodimer and regulate both the substrate specificity and the localization of the PP2A complexes. In budding yeast, Cdc55 (B) and Rts1 (B′) have been identified as B-regulatory subunits and bind to the core PP2A in a mutually exclusive manner (Jiang, 2006; Shu et al., 1997; Zhao et al., 1997). Recently, Zds1 (zillion different screens) and its paralog Zds2 have been found to specifically bind to PP2A-Cdc55, but not to PP2A-Rts1 (Queralt and Uhlmann, 2008; Wicky et al., 2010; Yasutis et al., 2010), and these two proteins regulate the nucleocytoplasmic distribution of PP2A-Cdc55 (Rossio and Yoshida, 2011), adding a new level of complexity.

PP2A-Cdc55 is involved in the stress response, polarized growth, meiosis and mitotic progression (Jiang, 2006). In the cytoplasm, PP2A-Cdc55, in complex with Zds1 or Zds2, promotes mitotic entry (Rossio and Yoshida, 2011). *cdc55Δ* and *zds1Δ zds2Δ* cells exhibit abnormally elongated cell morphology as a consequence of prolonged G2-phase delay (Bi and Pringle, 1996; Healy et al., 1991). The mitotic entry defect of *cdc55Δ* cells is due to inhibitory phosphorylation of Cdc28 (budding yeast Cdk1) on Tyr19 by the kinase Swe1 – the elongated morphology of *cdc55Δ* is rescued either by deletion of *SWE1* (McMillan et al., 1999a; McMillan et al., 1999b; Rossio and Yoshida, 2011; Wang and Burke, 1997; Wicky et al., 2010; Yang et al., 2000) or by introduction of the *cdc28-Y19F* mutation.

In the absence of Zds1 and Zds2, PP2A-Cdc55 accumulates in the nucleus and prevents mitotic exit (Rossio and Yoshida, 2011). It is known that PP2A-Cdc55 inhibits mitotic exit because overexpression of Cdc55 is toxic to the mutants defective in mitotic exit (Wang and Ng, 2006) and because deletion of *CDC55* rescues variety of mutants defective in mitotic exit (Clift et al., 2009; Queralt et al., 2006; Wang and Ng, 2006; Yellman and Burke, 2006). The target of PP2A-Cdc55 in mitotic exit is the phosphatase Cdc14, which is essential for mitotic exit in budding yeast (Visintin et al., 1998). Cdc14 is kept inactive by its inhibitor Net1 (also known as Cfi1) in the nucleolus (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Cdc14 release from the nucleolus requires Cdk-dependent phosphorylation of Net1 (Azzam et al., 2004). PP2A-Cdc55 counteracts Net1 phosphorylation and prevents mitotic exit (Queralt et al., 2006; Queralt and Uhlmann, 2008). Thus, it has been proposed that PP2A-Cdc55 is inactivated at anaphase onset either by nuclear exclusion (Rossio and Yoshida, 2011) or by inhibition of PP2A phosphatase activity (Calabria et al., 2012; Queralt and Uhlmann, 2008).

PP2A-Cdc55 is also an essential regulator of the spindle assembly checkpoint (SAC) (Clift et al., 2009; Evans and Hemmings, 2000; Koren et al., 2004; Minshull et al., 1996; Wang and Burke, 1997; Wang and Ng, 2006; Yellman and Burke, 2006) although the target of PP2A that is relevant for the SAC is not known. *cdc55Δ* cells, when challenged with microtubule-depolymerizing drugs, fail to arrest the cell cycle in mitosis and re-enter next round of the cell cycle and die. In *cdc55Δ* cells, the securin Pds1 is degraded in an anaphase-promoting complex

(APC)-dependent manner and sister chromatids separate as in other well-characterized SAC mutants (Minshull et al., 1996; Wang and Ng, 2006; Yellman and Burke, 2006), such as *mad1Δ* or *mad2Δ* (Alexandru et al., 1999). Interestingly, in *cdc55Δ* cells, the mitotic cyclins Clb2 and Clb3 remain stable, but Cdk1 activity declines and cells exit from mitosis (Chiroli et al., 2007; Minshull et al., 1996; Yellman and Burke, 2006). Recent papers (Wang and Ng, 2006; Yellman and Burke, 2006) proposed that the target of Cdc55 in the SAC is Cdc14, because Cdc14 is precociously released in *cdc55Δ,* and the Cdc14 activator Tem1 is dephosphorylated in a manner dependent on Cdc55. However, activation of Cdc14 does not explain why *cdc55Δ* cells undertake Pds1 degradation and show precautious sister chromatid separation upon spindle damages.

Here, we propose that the major target of PP2A-Cdc55 in the SAC is the APC bound to its regulatory subunit Cdc20 (APC-Cdc20). It is known that Cdk1-dependent phosphorylation of at least three APC subunits (Cdc16, Cdc23 and Cdc27) is required for optimal activity of APC-Cdc20 (Rudner and Murray, 2000) but the counteracting phosphatase has not been identified. The early finding by Wang and Burke (Wang and Burke, 1997) that *cdc55* deletion rescues the temperature-sensitive growth defect of *cdc20-1* cells, and the finding that Tpd3, an A subunit of PP2A, can be immunoprecipitated with APC (Boronat and Campbell, 2007; Kornitzer et al., 2001) suggest that PP2A-Cdc55 has a direct role in the regulation of APC-Cdc20 activity.

By isolating novel *cdc55* mutants specifically defective in the SAC, but not the other cell cycle steps, we found that mutation of *cdc55* can rescue growth defects in a large variety of APC mutants. We also demonstrate that the APC subunits Cdc16 and Cdc27 are hyperphosphorylated in the absence of Cdc55, and that unphosphorylatable APC mutants rescued the SAC defects of *cdc55Δ*, supporting the hypothesis that APC-Cdc20 is a direct target of Cdc55 in the SAC. In addition, we show evidence that dephosphorylation of APC-Cdc20 is taking place within the nucleus. When treated with nocodazole, both Cdc55 and APC are in the nucleus and APC is inhibited. When nuclear accumulation of Cdc55 is perturbed, as in the *cdc55-101* mutation or upon Zds1 overexpression, APC-Cdc20 is activated and cells become SAC defective. Our results are the first demonstration that APC is a functional target of PP2A-Cdc55 and confirm that regulation of nucleocytoplasmic distribution of PP2A-Cdc55 is important for proper cell cycle control.

Results

Isolation of novel *cdc55* alleles specifically defective in the SAC

tmr4 mutants have been identified as recessive-revertant suppressor mutants of the temperature-sensitive mitotic growth arrest seen in the *tom1* mutant (Sasaki et al., 2000; Utsugi et al., 1999). On the basis of the benomyl sensitivity of *tmr4* mutants, we screened plasmids from a genomic library for the ability to complement the benomyl sensitivity of *tmr4* mutants. Next, we verified whether the plasmids inhibited the growth of each *tmr4 tom1-2* double mutant at 35℃. By sub-cloning and sequence analysis, we identified that *tmr4* is allelic to *CDC55* and confirmed the mutant alleles by backcrossing. We then mapped the mutation sites of *tmr4* mutants (hereafter we designate them *cdc55-101, cdc55-102* and *cdc55-103*) and found that *cdc55-101* has the single mutation Gly43 to Asp, *cdc55-102* has the mutation Asp319 to Asn and *cdc55-103* has the mutation Gly47 to Arg. These mutated residues are highly conserved in the PP2A-B55 family from yeast to human (Fig. 1A). On the basis of a recent structural study of the PP2A complex, Gly43 and Gly47 are located in the β1B sheet and Asp319 is in the β5C sheet (Li and Virshup, 2002; Xu et al., 2008) (Fig. 1B). Because these mutation sites are far away from binding sites between the B-subunit and the AC complex and from putative substrate-binding regions, they are unlikely to be affecting the structural integrity of PP2A complex or enzymatic activity.

It is known that *cdc55Δ* cells are defective in mitotic entry and have abnormally elongated morphology. In a clear contrast to the *cdc55Δ* cells, the newly isolated *cdc55* mutants did not show elongated morphology or cold sensitivity (Fig. 1C), suggesting that these mutants are not defective in mitotic entry. However, all three *cdc55* mutants showed clear sensitivity to the microtubule-depolymerizing drug benomyl that was almost comparable to *cdc55Δ* (Fig. 1C). The benomyl sensitivity of *cdc55Δ* was associated with misregulation of catalytic activity of PP2A because overexpression of any of the catalytic subunits, *PPH21*, *PPH22* and *PPH3*, partially rescued the benomyl sensitivity (Fig. 1D). We also found that overexpression of *SIT4*, a homolog of the phosphatase PP6, can rescue the benomyl sensitivity of *cdc55Δ* although the functional relationship between Cdc55 and Sit4 is not clear.

The benomyl sensitivity of *cdc55-101*, *cdc55-102* and *cdc55-103* mutants prompted us to examine the functionality of SAC in these mutants. Indeed, they failed to maintain mitotic arrest in the presence of nocodazole, and rebudded and lost viability with similar kinetics to *mad1Δ* (a known SAC mutant) (Hoyt et al., 1991; Li and Murray, 1991) and *cdc55Δ* (Fig. 2A,B). In addition, we observed that the *cdc55-101* mutant failed to maintain sister chromatid cohesion after nocodazole-induced arrest, as with *mad1Δ* (Fig. 2C) (Hoyt et al., 1991; Stearns et al., 1990; Straight et al., 1996). Pleiotropic cell cycle defects associated with *cdc55Δ* were one reason that analysis of Cdc55 function in the SAC has been difficult. Thus, we took advantage of our newly isolated *cdc55* mutants that were only defective in the SAC but not mitotic entry. Because all three *cdc55* mutants showed similar SAC defects, we focused our analysis on *cdc55-101*.

To further characterize the SAC defects of *cdc55-101*, we synchronized yeast cells in G1 phase by use of mating pheromone and released them into the medium containing nocodazole. In wild-type cells, the securin Pds1, S-phase cyclin Clb5 and mitotic cyclin Clb2 were all stabilized (Fig. 2D; supplementary material Fig. S1) owing to SAC-dependent inhibition of APC-Cdc20 (Visintin et al., 1997). In the SAC-defective *mad2Δ* strain, Pds1 is precociously degraded (Fig. 2D). In addition, Clb2 is gradually degraded as a consequence of mitotic exit and activation of APC-Cdh1 (Zachariae et al., 1998). In *cdc55-101*, as in *mad2Δ*, Pds1 and Clb5 were precociously degraded. Consistent with previous reports (Chiroli et al., 2007; Minshull et al., 1996; Yellman and Burke, 2006), we did not see clear effects on Clb2 degradation (Fig. 2D) suggesting that Cdc55-101 has a specific effect on APC-Cdc20 but not APC-Cdh1. We also confirmed that the timing of Pds1 and Clb2 degradation in *cdc55-101* is very similar to that of *cdc55Δ* but distinct from that of *mad2Δ* (Fig. 2D). Thus, the *cdc55-101* mutant is completely defective in Cdc55 function in the SAC.

Cdc55 regulates APC-Cdc20

It is known that the *cdc20-1* temperature sensitivity is suppressed by *cdc55Δ* (Wang and Burke, 1997). We found that *cdc55-101*,

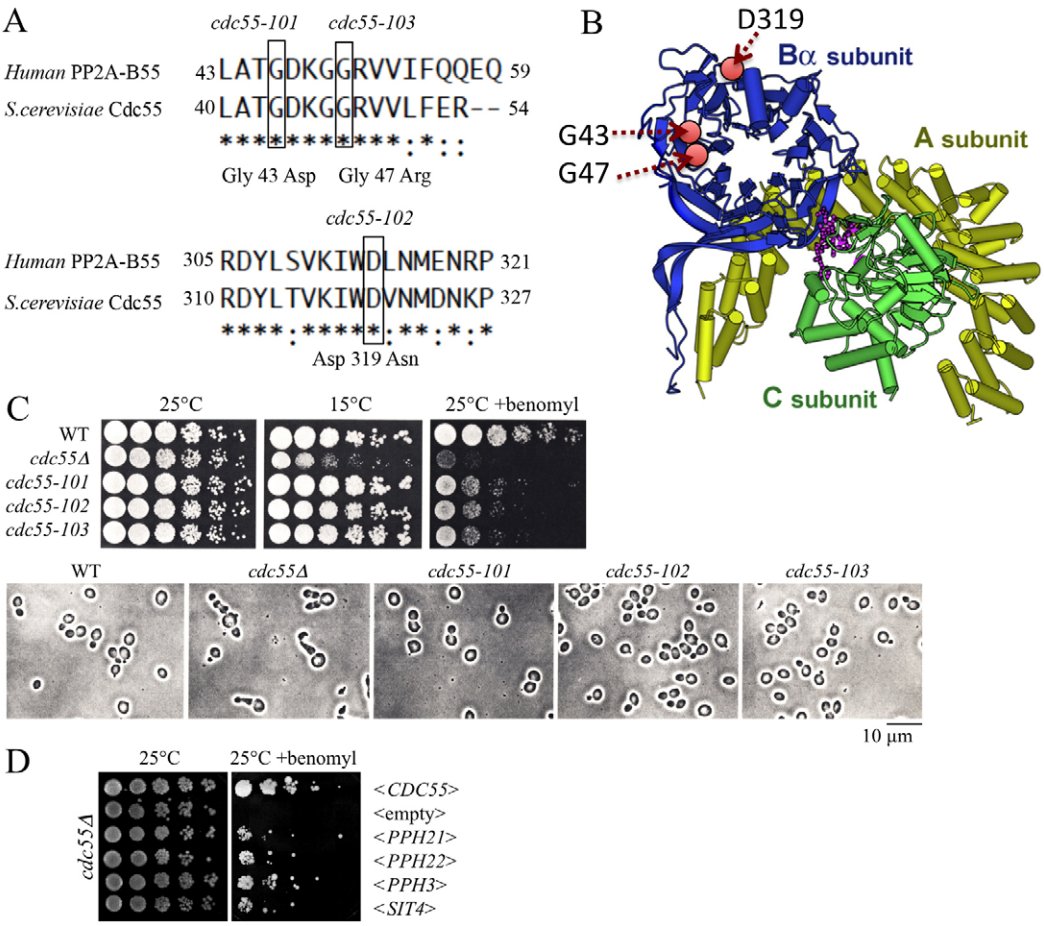

Fig. 1. Isolation of novel *cdc55* alleles. (A) Sequence alignment of yeast Cdc55 and human PP2A-B55. Mutations resulting in amino acid substitutions in *cdc55-101, cdc55-102* and *cdc55-103* (G43D, G47R, D319N) are boxed. The T-Coffee (Notredame et al., 2000) alignment program was used. Asterisks indicate conserved residues, colons indicate semi-conserved residues. (B) Structure of the human PP2A holoenzyme modified from Xu et al. (Xu et al., 2008). The scaffold (A subunit), catalytic (C subunit) and regulatory B (Bα subunit) subunits are shown in yellow, green and blue, respectively. The positions of the novel *cdc55* mutations are indicated with dotted arrows. (C) Growth phenotype of *cdc55* mutants. Top: *cdc55-101, cdc55-102* and *cdc55-103* are sensitive to anti-microtubule drug benomyl. WT, wild-type. In contrast to *cdc55Δ*, these mutants did not exhibit cold sensitivity at 16℃ and did not show abnormal morphogenesis. Serial dilutions are shown from left to right. Bottom: representative images of the *cdc55* mutants cells grown at 16℃. (D) Benomyl sensitivity of *cdc55Δ* is rescued by overexpression of each catalytic subunit of PP2A-related phosphatases from 2-micron plasmids. Serial dilutions are shown from left to right.

cdc55-102 and *cdc55-103* also rescued the temperature sensitivity of *cdc20-3* (Fig. 3A). The suppression of *cdc20-3* is not simply due to a SAC defect because neither *mad1Δ* nor *bub2Δ* rescued *cdc20-3* (Fig. 3A). Furthermore, not only *cdc20-3*, but also several other APC mutants, including *cdc16-1*, *cdc26Δ* and *apc1-1* were rescued by *cdc55-101* mutation (Fig. 3B). These genetic data suggest that Cdc55 has a specific function in controlling APC-Cdc20 activation or localization.

It is known that activation of APC-Cdc20 requires Cdk-dependent phosphorylation (Rudner et al., 2000; Rudner and Murray, 2000). At least three subunits of APC (Cdc16, Cdc23 and Cdc27) are phosphorylated both *in vitro* and *in vivo* by Cdk, and mutating these phosphorylation sites (Cdc16-6A, Cdc23-A and Cdc27-5A) impairs APC functions *in vivo* (Rudner and Murray, 2000). Because Cdk1 and PP2A counteract each other, we hypothesized that PP2A-Cdc55 inhibits APC-Cdc20 through dephosphorylation. We found that Cdk-dependent phosphorylation of Cdc16 and Cdc27 is controlled by Cdc55. Cdc16, Cdc23 and Cdc27 are known to be phosphorylated by Cdk and migrate slowly in the SDS-PAGE after Cdk-dependent phosphorylation (Rudner and Murray, 2000). The amount of the slow-migrating species of Cdc16–HA and Cdc27–HA seen in western blots is more abundant in *cdc55Δ* when cells were arrested by nocodazole, resulting in SAC activation (Fig. 4A), indicating that PP2A-Cdc55 is required for dephosphorylation of Cdc16 and Cdc27 in this condition. We also found that the slow-migrating form of Cdc16–HA accumulated in *cdc55-101*, similar to in *cdc55Δ* after nocodazole treatment (Fig. 4C). We also tested Cdc23 in the same conditions, but were not able to see an obvious mobility shift in the *cdc55Δ* cells (data not shown), probably because there are fewer phosphorylation sites on Cdc23 than on Cdc16 and Cdc27 (Rudner and Murray, 2000). To confirm that the SAC defect of *cdc55Δ* is due to hyperphosphorylation of APC, we combined *cdc55* deletion with the unphosphorylatable APC mutants (Cdc16-6A, Cdc23-A and Cdc27-5A) and found that the SAC defects of *cdc55* were almost completely rescued by these mutations (Fig. 4B). This genetic data suggests that the major target of PP2A-Cdc55 in the SAC is the APC.

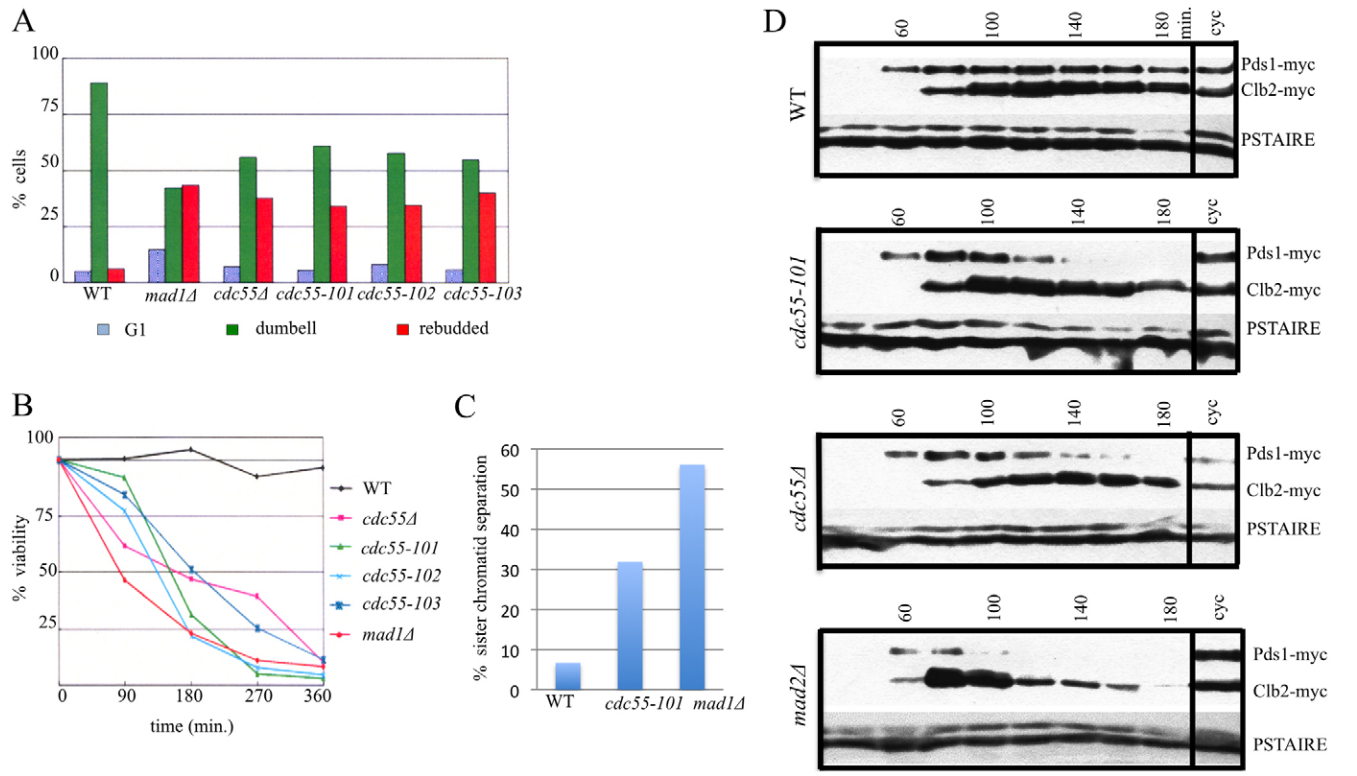

Fig. 2. *cdc55* mutants are defective in the SAC. (A) Log-phase cells of the indicated strains were diluted in YPD containing 15 μg/ml nocodazole. After 4 hours the cells were briefly sonicated and the percentage of G1, large budded (dumbell) and rebudded cells (rebudding assay) was determined by microscopy. (B) Log phase cells of the indicated strains were treated with 15 μg/ml nocodazole in YPD at 25℃ and the viability of the cells was measured at different time point as percentage of cells able to form colonies on YPD medium (viability assay). (C) After 3 hours of nocodazole treatment, loss of sister chromosome cohesion of cen V was visualized and quantified in the indicated strains (Straight et al., 1996). (D) Cultures of cells with the indicated genotypes were arrested in G1 by 0.5 μg/ml α-factor for 180 minutes and then released in the YPD medium containing 15 μg/ml nocodazole (t=0) and α-factor was re-added after 90 minutes to prevent cells from entering in the next cell cycle. Samples were collected at the indicated times for western blot analysis of cell extracts with anti-Myc antibody. Anti-PSTAIRE was used as a loading control. WT, wild-type.

We also examined localization of the APC subunit Cdc23. As it has been reported previously, Cdc23–GFP is constitutively in the nucleus and sometimes localized to the spindles (Jaquenoud et al., 2002; Melloy and Holloway, 2004) in both wild-type and in *cdc55Δ* cells (supplementary material Fig. S4). Activation of the SAC, by addition of nocodazole, had no clear effect on general nuclear Cdc23–GFP localization (supplementary material Fig. S2). Thus, phosphorylation of APC subunits does not affect APC localization. This is consistent with previous data that indicated that localization of Cdc23 is not altered in a strain with all Cdk consensus sites mutated in the APC/C subunits Cdc23, Cdc27 and Cdc16 (Melloy and Holloway, 2004).

It is formally possible that the APC is not the only target of Cdc55 in the SAC. To examine the contribution of Cdc14 release

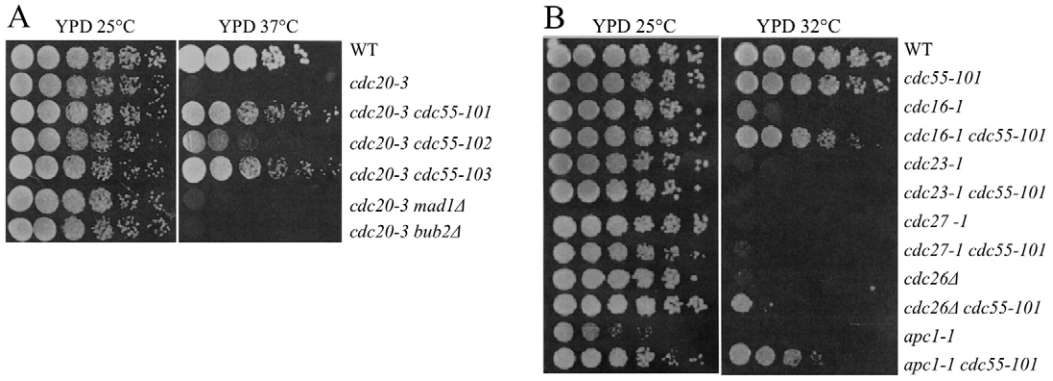

Fig. 3. *cdc55-101* mutants suppress the temperature-sensitivity of *cdc20-3* cells and of different APC mutants. (A,B) Serial dilutions of the strains with the indicated genotypes were spotted on YPD at the indicated temperatures. WT, wild-type.

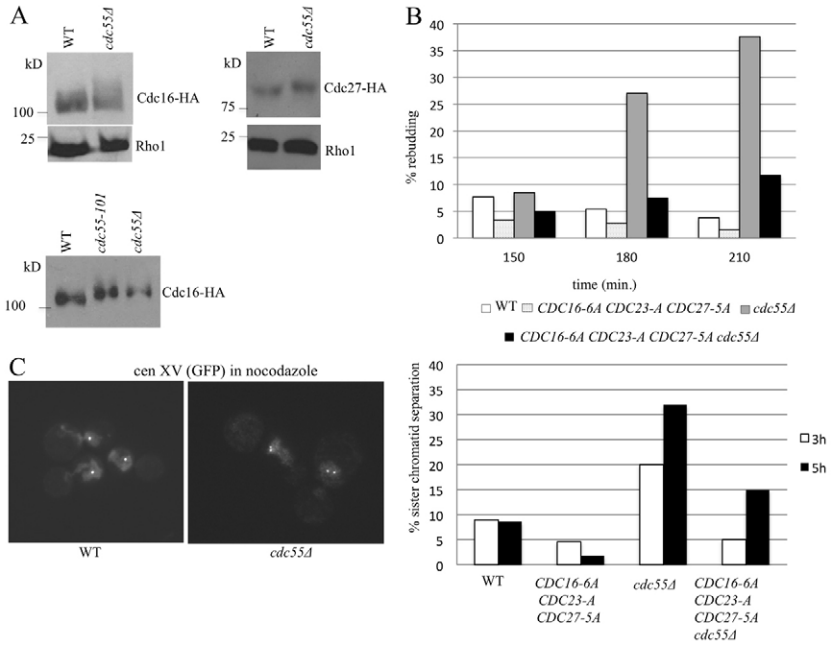

Fig. 4. APC-Cdc20 is a target of PP2A-Cdc55 in the SAC. (A) Cdc55 affects the mobility of Cdc16 and Cdc27 in SDS-PAGE gels. Wild-type (WT) and *cdc55Δ* cells expressing either Cdc16–3HA or Cdc27–3HA were treated with 15 μg/ml nocodazole for 2 hours. Total cellular proteins were separated by SDS-PAGE followed by western blotting using anti-HA antibody. In the same assay, slower migration of Cdc16–HA was also observed in *cdc55-101*, as in *cdc55Δ*. Rho1 was used as a loading control. (B) Unphosphorylatable APC mutations prevent rebudding of *cdc55Δ*. The indicated strains were released in YPD medium containing 15 μg/ml nocodazole (t=0). At the indicated time point, at least 100 cells for each strain were scored to determine the percentage of G1, dumbell and rebudded cells. The experiment has been repeated two times and one representative data set is shown. (C) Unphosphorylatable APC mutations prevent sister chromatid separation of *cdc55Δ* in nocodazole. Cells of the indicated genotypes were released in YPD medium containing 15 μg/ml nocodazole. After 3 and 5 hours, at least 150 cells of each strain were examined for sister chromatid separation. The experiment was been repeated two times and one representative data set is shown. Left: representative images of sister chromatid separation after nocodazole treatment in WT and in *cdc55Δ* cells. Right: quantitative summary of the sister separation defect.

by phosphorylation of Net1 in the SAC defect of *cdc55Δ*, we tested whether unphosphorylatable Net1 (*NET1-6cdk-9myc*) (Azzam et al., 2004) could rescue the SAC defects of *cdc55Δ*. Indeed, *NET1-6cdk-9myc* partially rescued the SAC defects of *cdc55Δ*, however, *NET1-9myc*, which was used as a negative control, also rescued the SAC defects of *cdc55Δ* (supplementary material Fig. S3). This result suggests that 9xmyc tag of Net1 has an unexpected dominant role in delaying mitotic exit and the contribution of Cdk-dependent phosphorylation of Net1 in the SAC remains unclear.

Cdc55-101 is cytoplasmic

Cdc55 localizes to the polarized growth sites, as well as the cytoplasm and nucleus (Gentry and Hallberg, 2002). We have recently shown that Cdc55 has compartment-specific functions (Rossio and Yoshida, 2011); for example, cytoplasmic Cdc55 promotes mitotic entry, whereas nuclear Cdc55 delays mitotic exit. It is known that APC-Cdc20 and its substrates are in the nucleus (Jaquenoud et al., 2002; Melloy and Holloway, 2004); hence, we anticipated that nuclear Cdc55 is responsible for APC dephosphorylation. Because Cdc55-101 is competent for mitotic entry (Fig. 1C) but defective in the SAC (Fig. 2) we hypothesized that Cdc55-101 was excluded from the nucleus.

Cdc55–GFP was localized to both in the nucleus and the cytoplasm, as judged by Nup159–mCherry staining of nuclear envelope (Fig. 5A), in contrast Cdc55-101–GFP was excluded from the nucleus throughout the cell cycle (Fig. 5A). Loss of nuclear signal was not due to reduced expression or instability of Cdc55-101–GFP protein because Cdc55-101–GFP was expressed to similar levels as wild-type Cdc55–GFP as judged by western blotting (Fig. 5B). Similar nuclear exclusion was observed with Cdc55-103–GFP (supplementary material Fig. S4). Cdc55-102–GFP was also

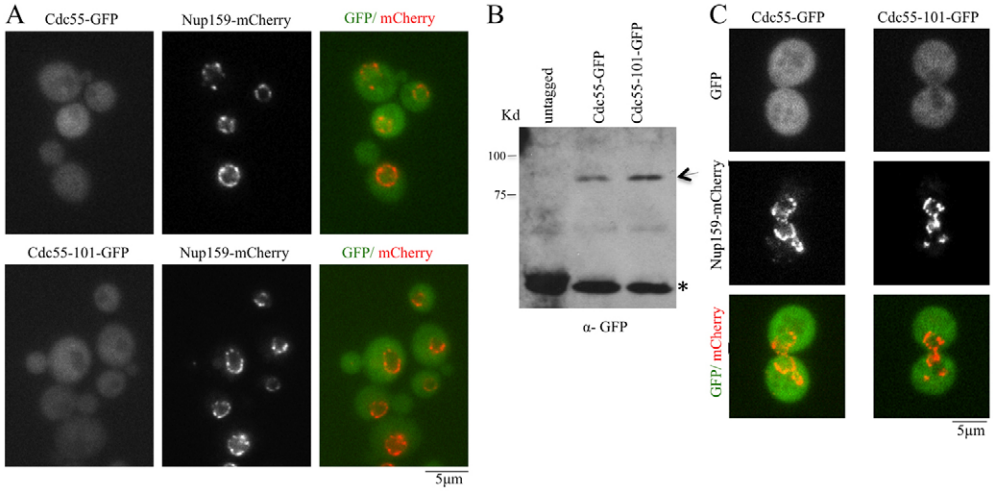

Fig. 5. Localization of Cdc55–GFP and Cdc55-101–GFP. (A) Localization of Cdc55–GFP and Cdc55-101–GFP in rapidly growing culture. Nup159–mCherry was used as a marker of the nuclear envelope. 84.3% cells showed nuclear Cdc55–GFP signal, whereas 3.9% cells showed Cdc55-101–GFP signal in the nucleus (n>100). (B) Western blotting confirmed that Cdc55–GFP and Cdc55-101–GFP (arrow) were expressed to a similar level. The asterisk corresponds to a non-specific band used as a loading control. (C) Cdc55–GFP and Cdc55-101–GFP localization in nocodazole-treated cells. 89% cells showed nuclear Cdc55–GFP signal, whereas 3.5% cells showed Cdc55-101-GFP signal in the nucleus (n>100).

partially excluded from the nucleus, but the effect was not as strong as Cdc55-101 and Cdc55-103, suggesting that this mutant might only show a minor localization defect. Importantly, when the SAC is activated by nocodazole, Cdc55 was found both in the nucleus and in the cytoplasm (Fig. 5C). By contrast, Cdc55-101 barely accumulated in the nucleus (3.5% of the cells have Cdc55-101 in the nucleus compared with 89% of the control). These results are consistent with our hypothesis that nuclear Cdc55 is responsible for its role in the SAC.

Overexpression of Zds1 impairs the SAC

To test whether nuclear exclusion of Cdc55 results in the activation of APC-Cdc20 and the SAC defect, we examined the effect of Zds1 overexpression. Zds1 forms a stoichiometric complex with PP2A-Cdc55 (Queralt and Uhlmann, 2008; Wicky et al., 2010; Yasutis et al., 2010) and enhances the cytoplasmic functions of Cdc55 but inhibits the nuclear functions of Cdc55 (Rossio and Yoshida, 2011).

We first confirmed that Zds1 is a cytoplasmic protein (Bi and Pringle, 1996; Rossio and Yoshida, 2011) and that it is always excluded from the nucleus by using the nuclear envelope marker Nup159–mCherry (supplementary material Fig. S5A). We also confirmed that Cdc55 was excluded from the nucleus after *ZDS1* overexpression (Rossio and Yoshida, 2011) by using Nup159–mCherry (supplementary material Fig. S5B).

We found that the temperature-sensitive growth defect of *cdc20-3* was effectively suppressed by overexpression of *ZDS1* (Fig. 6A). Thus, Zds1 overexpression caused a similar effect to the *cdc55-101* mutation or to *cdc55Δ*. To assess the effect of *ZDS1* overexpression on Cdc55 in the SAC, we used *zds1Δc800*, which lacks Cdc55-binding domain (CBD) as a negative control. Note, we found highly conserved residues within the CBD of fungal Zds1 family proteins and the vertebrate cortactin-binding proteins CTTNBP2 and CTTNBP2NL (Fig. 6B), which have been recently identified as PP2A-B55 (PP2A-Striatin)-binding proteins (Goudreault et al., 2009) and a crucial regulator of PP2A-B55 localization to the dendritic spines (Chen et al., 2012).

Overexpression of *ZDS1* but not *zds1Δc800* caused sensitivity to benomyl (Fig. 6C). Cells overexpressing *ZDS1* were not able to arrest in nocodazole and rebudded, which is characteristic of SAC mutants, and this effect was dependent on the ability of Zds1 to bind to Cdc55 because overexpression of *zds1Δc800* did not cause these defects (Fig. 6C,E), although Zds1Δc800 was expressed to a similar level to that of the full-length Zds1 (Fig. 6D). Thus, overexpression of Zds1 has a similar effect to loss of Cdc55 and causes SAC defects that are probably due to depletion of nuclear Cdc55.

Nuclear Cdc55 is essential for the SAC

To further test whether nuclear Cdc55 is responsible for the SAC arrest, we took advantage of engineered *cdc55* mutants, where Cdc55 is either predominantly cytoplasmic (*cdc55-NES*) or predominantly nuclear (*cdc55-NLS*) (NES, nuclear export signal; NLS, nuclear localization signal) (Rossio and Yoshida,

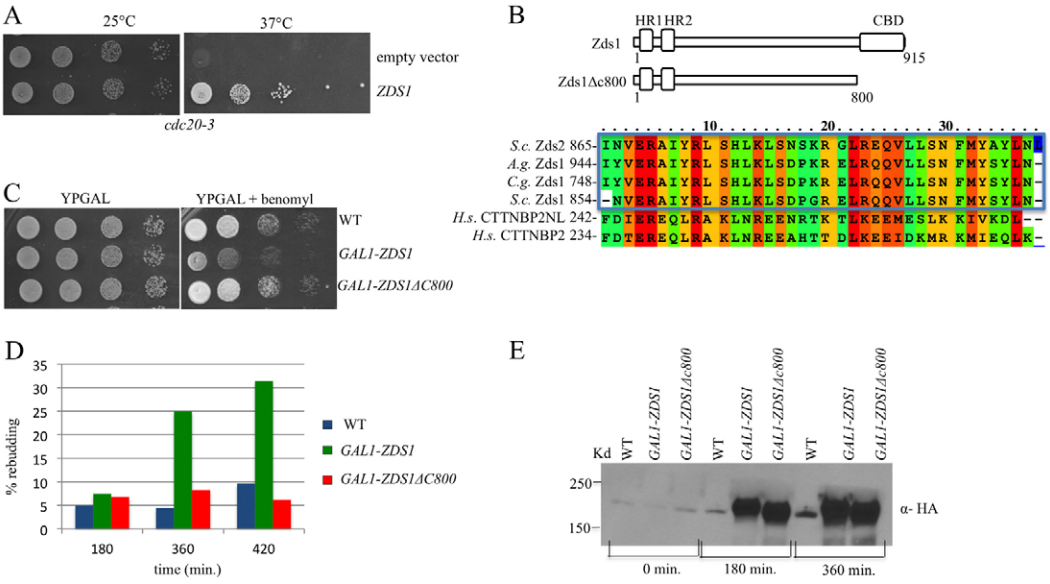

Fig. 6. Overexpression of *ZDS1* rescues the temperature sensitivity of *cdc20-3* cells and causes SAC defects. (A) Serial dilutions of the strains with the indicated genotypes were spotted on SC-URA at the indicated temperatures. (B) Top: schematic representation of the Zds1 constructs: Zds1 and Zds1Δc800. The first and the second homology region (HR1 and HR2, respectively), which are conserved in Zds1 homologues in fungal species, and C-terminal Cdc55-binding domain (CBD; 801–913 a.a.) are depicted. Bottom: alignment of the highly conserved CBD region of fungal Zds1 family proteins and human PP2A B-subunit-binding proteins. *S. cerevisiae* (*S.c.*) Zds1 and Zds2, *C. glabrata* (*C.g.*) Zds1 (EMBL accession number CAG60947), *A. gossypii* (*A.g.*) Zds1 (EMBL accession number AEY94466), *H. sapiens* (*H.s.*) CTCTTNBP2NL (EMBL accession number EAW56517) and CTTNBP2 (EMBL accession number ABC87066). (C) Serial dilutions of the strains with the indicated genotypes were spotted on galactose medium (YPG) and YPG containing 12.5 μg/ml benomyl at 24℃. WT, wild-type. (D) Effect of overexpression of *ZDS1* (*GAL1-ZDS1*) or *ZDS1Δc800* (*GAL-ZDS1Δc800*) on the SAC. Cells were first arrested by with 15 μg/ml (final concentration) of nocodazole in the raffinose medium (YPR). After 180 minutes in the nocodazole, either *ZDS1* or *ZDS1Δc800* was induced by galactose addition. Samples were collected at the indicated time points and at least 150 cells of each strain were examined for the presence of rebudded cells indicative of spindle checkpoint defects. The experiment has been repeated two times and one representative data set is shown. (E) Western blotting confirmed the induction of *GAL1-ZDS1* and *GAL1-ZDS1Δc800* after galactose addition in the experiment shown in D.

2011). We also tested the *zds1Δzds2Δ* mutant, where Cdc55 is predominantly nuclear (Rossio and Yoshida, 2011). First, we found that the *cdc55-NES* strain is sensitive to benomyl, like *cdc55Δ* (Fig. 7A). Note that *cdc55-NES* is not simply a loss of function because *cdc55-NES* has a dominant function in the cytoplasm and can induce mitotic entry in *zds1Δzds2Δ* (Rossio and Yoshida, 2011).

cdc55-NES was indeed defective in the SAC. The *cdc55-NES* strain rebudded in the presence of nocodazole, like *cdc55Δ* (Fig. 7C). In contrast, *zds1Δzds2Δ* was competent in the SAC (Fig. 7C). We also confirmed the SAC defects of *cdc55-NES* by FACS analysis of the DNA contents and found that *cdc55-NES* reduplicated its DNA in the presence of nocodazole, as does *cdc55Δ* (Fig. 7B). In these assays we deleted the *SWE1* gene to exclude the potential G2 phase cell cycle delay in *cdc55* or *zds1Δzds2Δ* strains.

Thus, loss of Cdc55 from the nucleus by *cdc55-NES* causes a similar effect to the *cdc55-101* mutation and to *cdc55Δ* or to *ZDS1* overexpression.

Discussion

Cdc55 inhibits APC-Cdc20

In this study, we have shown that the crucial target of Cdc55 in the SAC is APC-Cdc20 because the phosphorylation status of APC subunits are controlled by Cdc55 *in vivo* and the SAC defects of *cdc55* are largely rescued by preventing APC phosphorylation.

The targets of Cdc55 in the SAC have long been debated. Given that the SAC defect is accompanied by mitotic exit, recent studies suggested that Cdc14 activation is a key step that is prevented by Cdc55 during SAC arrest (Wang and Ng, 2006; Yellman and Burke, 2006). However, we favor APC-Cdc20 activity rather than Cdc14 activity as a key target of Cdc55 phosphatase in the SAC because Cdc14 activates APC-Cdh1, not APC-Cdc20 (Jaspersen et al., 1999; Visintin et al., 1998; Zachariae et al., 1998). The fact that APC-Cdc20 activity but not APC-Cdh1 activity is promoted by Cdk1-dependent phosphorylation (Rudner and Murray, 2000), and our data showing that there is preferential degradation of the APC-Cdc20 substrates Pds1 and Clb5, but not Clb2, a major target of APC-Cdh1, in *cdc55* is consistent with APC-Cdc20 being under the control of Cdc55.

It is important to mention that Cdc14 activation and subsequent APC-Cdh1 activation is under the control of APC-Cdc20. Thus Cdc14 and APC-Cdh1 should be eventually activated in the *cdc55* mutants arrested in spindle damages. Unfortunately, we were not able to address the direct contribution of Cdc14 inhibition by Cdc55 in the SAC because the effects of the 9×Myc tag on *NET1*, not the mutation in the Cdk1-dependent phosphorylation sites of Net1, caused suppression of the SAC defects of *cdc55Δ*.

Although deletion of *NET1* rescues various mitotic exit mutants, similar to deletion of *CDC55* (Straight et al., 1999; Visintin et al., 1999; Yellman and Burke, 2006), deletion of *NET1* does not suppress *cdc20-1* (Visintin et al., 1999). Highly specific suppression of APC-Cdc20 function by *cdc55* mutations is consistent with our idea that Cdc55 not only regulates Net1 for Cdc14 release but also APC-Cdc20 for sister chromatid separation.

The roles of PP2A in the regulation of APC and SAC are currently not clear in animal cells because of redundancy of the PP2A subunits (Shi, 2009; Virshup and Shenolikar, 2009) and the pleiotropic defects associated with PP2A inhibition (Janssens and Goris, 2001). Several studies imply that APC activity is negatively regulated by PP2A in animal cells. In *Drosophila*, the metaphase-like arrest of *mks* (a Cdc27 homolog) mutants can be suppressed by mutations in the *twins* (*CDC55* homolog) gene, but not by mutations in SAC genes (Deak et al., 2003). In human, the

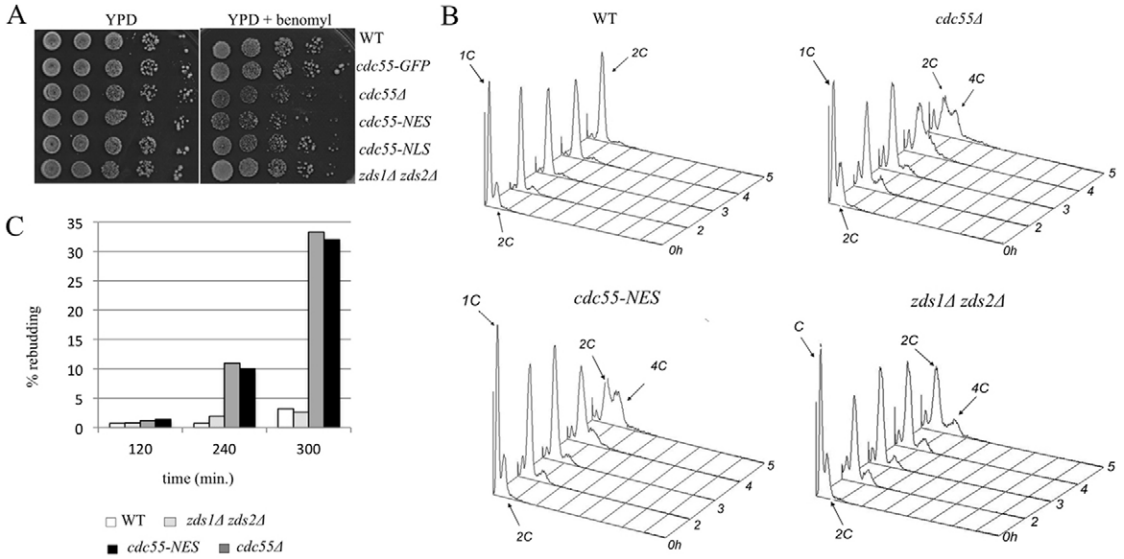

Fig. 7. Nuclear Cdc55 is essential for the SAC. (A) Serial dilutions of the cells with indicated genotypes spotted on YPD and YPD containing 15 μg/ml benomyl at 24℃. (B,C) Cells with the indicated genotypes (all strains are deleted for *SWE1* to avoid mitotic entry delay) were first synchronized in G1 by mating pheromone and then released into medium containing 15 μg/ml nocodazole (t=0). At the indicated time points, samples were withdrawn for FACS analysis of DNA content (B) and at each time point at least 150 cells were scored. The percentage of cells that rebudded is quantified in C. The experiment was repeated three times and one representative data set is shown.

Cdc55 homolog PRB2B regulates the phosphorylation status of Cdc27 and affects the localization of Cdc27 to the mitotic spindle (Torres et al., 2010). Thus it is highly likely that regulation of APC by PP2A is an evolutionarily highly conserved process.

Suppressors of the *tom1* mutant

We have isolated novel *cdc55* mutants as revertant suppressors of the *tom1-2* mutant. *tom1* mutants arrest the cell cycle in G2/M phase, but the substrates and targets of Tom1 important for mitotic progression have not been identified (Sasaki et al., 2000). In contrast to *cdc55Δ*, which shows an abnormally elongated morphology and a cold-sensitive phenotype in addition to the SAC defect, the only phenotype associated with *cdc55-101, cdc-102* and *cdc-103* was the SAC defect. Because Cdc55-101 protein was expressed at the wild-type level and because mutation sites are not located in the binding surface to the PP2A AC subunits, it is unlikely that *cdc55-101* is impaired in PP2A complex assembly or PP2A catalytic activity. The absence of apparent morphological defects in *cdc55-101* also suggests that PP2A activity is not severely impaired. It is formally possible that Cdc55-101 protein has lost the ability to interact with specific substrates such as APC, but we favor the idea that Cdc55-101 is specifically defective in its nuclear localization because the phenotype of *cdc55-101* was very similar to that of *cdc55-NES* strain and that of the cells overexpressing *ZDS1*.

The targets of Tom1 that are crucial for mitotic progression are not understood yet, but it is most likely to be APC-Cdc20 activity. Interestingly, in the same screen as that which identified *tmr4/cdc55*, *tmr1/cyr1* and *tmr2/sch9* were also identified (Sasaki et al., 2000). Cyr1 encodes an adenylate cyclase and Sch9 encodes a kinase antagonizing the protein kinase A (PKA) pathway. In addition, suppression of the PKA pathway, either by overexpression of *BCY1*, a regulatory subunit of PKA or by overexpression of *PDE2*, a phosphodiesterase, can also rescue the growth defect of *tom1* mutants (Sasaki et al., 2000). It is known that PKA activity inhibits APC-Cdc20 activity by phosphorylating Cdc20 (Anghileri et al., 1999; Searle et al., 2004). Because inhibition of PKA activity rescues mitotic defects associated with *tom1* mutation, we suspect that activation of APC-Cdc20 is the key process compromised in the *tom1* mutant.

Compartmentalized function of PP2A-Cdc55 and its regulation by Zds1

We showed that both Cdc55 and APC subunits are in the nucleus in nocodazole-arrested cells. Nuclear localization of APC during mitosis is consistent with the fact that Cdc20, as well as its substrates Pds1 and Clb5, is predominantly nuclear (Jaquenoud et al., 2002).

Three lines of evidence suggest that stable SAC arrest and inhibition of APC-Cdc20 activity requires PP2A-Cdc55 activity in the nucleus: first, a SAC defective Cdc55-101 mutant protein is excluded from the nucleus; second, retention of Cdc55 in the cytoplasm by overexpression of *ZDS1* impaired the SAC; and, third, forced exclusion of Cdc55 from the nucleus by adding a strong nuclear export signal (*cdc55-NES*) results in SAC defects. In these cases, the temperature-sensitive growth defect of *cdc20-3* was also rescued.

In the previous study, we reported that Cdc55 localization changes during the cell cycle and that nuclear Cdc55 signal is high in G1 or small budded cells but is reduced in mitotic cells in a Zds1- or Zds2-dependent manner (Rossio and Yoshida, 2011). Exclusion of Cdc55 from the nucleus during mitosis is consistent with our model that nuclear Cdc55 interferes with APC-Cdc20 activity (this study) and with Cdc14 activation (Rossio and Yoshida, 2011) (Fig. 8).

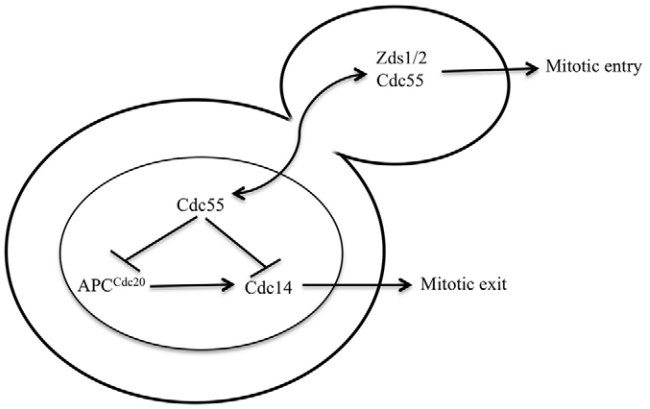

Fig. 8. Regulation of mitosis by PP2A-Cdc55. PP2A-Cdc55 promotes mitotic entry in the cytoplasm. Cytoplasmic localization of PP2A-Cdc55 requires binding to Zds1 or Zds2 proteins. Nuclear PP2A-Cdc55 inhibits APC-Cdc20 when the spindle assembly checkpoint is active. Nuclear PP2A-Cdc55 also interferes with Cdc14 release and prevents mitotic exit. See the Discussion for more details.

The mechanism by which Zds1 and/or Zds2 exports the Cdc55-containing PP2A complex and how interaction between Zds1 and/or Zds2 and Cdc55 is regulated during the cell cycle and upon SAC activation is a very important question. We did not clearly see an obvious change in the nuclear localization of Cdc55 upon SAC activation (data not shown), suggesting that PP2A-Cdc55 is not specifically responding to spindle damage, but rather setting a high threshold for Cdk1-dependent activation of APC to prevent hyperactivation of APC-Cdc20.

Although Zds1 is highly conserved in almost all fungal species, no obvious homolog is found in animals or plants. By carefully examining the CBD sequence of Zds1, we found some conversation between this region and the PP2A-binding region of human CTTNBP2NL and CTTNBP2. Importantly, CTTNBP2NL and CTTNB2 specifically bind to the regulatory B-subunit of PP2A in the region with high similarity to the CBD of Zds1 and this interaction is essential for PP2A complex to localize to the dendritic spines (Chen et al., 2012). Thus Zds1 and CTTNBP2 are functioning in a similar manner to recruit PP2A complex to specific subcellular components. We propose that spatial control of PP2A complex by Zds1-like regulatory proteins is a widely conserved mechanism used to compartmentalize PP2A activity.

Materials and Methods

Yeast genetics

All yeast strains used in this study were isogenic or congenic to BY4741 (*MATa leu2Δ0 his3Δ1 met15Δ0 ura3Δ0*, obtained from Thermo Fisher Scientific) or to W303 (*Mata, ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3, ssd1* obtained from Thermo Fisher Scientific). Standard yeast genetics was used to generate the strains. Yeast strains are listed in supplementary material Table S1. PY3295 and SY strains were gifts from D. Pellman (Dana-Farber Cancer Institute, Boston, MA). D. Lew (Duke University, Durham, NC) provided a *SWE1* gene knockout plasmid. Gene deletions or modifications were performed with PCR-mediated one-step gene replacement using pFA6a vectors provided by J. Pringle (Stanford University, Stanford, CA) (Longtine et al., 1998) and were confirmed by PCR.

The LacO/LacI system for monitoring sister chromatid cohesion was a gift from D. Koshland (UC Berkeley, CA). The *ZDS1* plasmid was purchased from the National Bio-Resource Project (NBRP). α-factor was used at 2 μg/ml. Nocodazole was used at 15 μg/ml. Benomyl was used at 12.5 μg/ml, 7.5 μg/ml or 15 μg/ml as indicated in the figure legends. For galactose induction, 2% galactose was added to the medium.

Biochemistry

Protein extracts were prepared by trichloroacetic acid (TCA) precipitation as previously described (Piatti et al., 1996). Mouse anti-HA (16B12, Roche and Covance), mouse anti-GFP antibody (Millipore), mouse anti-Myc (9E10, Wako) and anti-PSTAIRE (sc-53, Santa Cruz Biotechnology) antibodies were used. Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Millipore, and proteins were detected by an enhanced chemiluminescence system (ECL Prime; GE Healthcare).

FACS analysis

For FACS (fluorescence-activated cell sorting) analysis, cells were collected by centrifugation and then fixed in 70% ethanol. Cells were then washed once with 1 ml of 50 mM Tris-HCl pH 7.5, and the pellet was then resuspended in 0.5 ml of 50 mM Tris-HCl pH 7.5 containing 1 mg/ml RNase. After incubation overnight at 37℃, cells were collected by centrifugation and were then washed once with 1 ml of FACS buffer (200 mM Tris-HCl pH 7.5, 200 mM NaCl, 78 mM $MgCl_2$) and resuspended in the same buffer containing 50 μg/ml propidium iodide.

Viability assay

Logarithmically growing cells were collected and resuspended in YPD containing nocodazole (15 μg/ml). At total of 200 cells were plated on a YPD plate at each time point after nocodazole addition. Once colonies had formed, viability was calculated by dividing the number of colonies formed at the different time points by the number of colonies formed at time 0. The rate of death in the presence of nocodazole is an excellent indicator of checkpoint deficiency (Hoyt et al., 1991).

Rebudding and sister chromatid separation assays

Wild-type cells arrest in response to SAC activation as large budded cells in mitosis. The checkpoint mutants continue through the cell cycle and they eventually pass through the subsequent G1 generating a new bud. Logarithmically growing cells were treated with nocodazole (15 μg/ml). At each indicated time point, cells were fixed in 70% ethanol at room temperature. Cells were then washed two times in PBS, vortexed vigorously, examined by brightfield microscopy and categorized into G1 (unbudded), dumbell (large budded) and rebudded cells (more than one bud). Sister chromatid separation was monitored by visualizing the right arm of chromosome XV using the LacO/green fluorescent protein (GFP)-LacI system (Straight et al., 1996).

Fluorescence microscopy

Fluorescence images were acquired with a fluorescence microscope (Eclipse E600; Nikon) equipped with a charge-coupled device camera (DC350F; Andor) with 100×, 1.45 NA, or 60×, 1.4 NA, oil objectives. The images were captured and analyzed with NIS-Elements software (Nikon), and the figures were processed and assembled in Photoshop (Adobe) or Image J. Fluorescence images of Zds1–GFP in the presence of Nup159–mCherry (supplementary material Fig. S2) were acquired with a spinning disk confocal microscope (Zeiss AxioObserver) with a Plan Apochromat 63×, 1.4 NA oil M27 lens with a DIC prism. The images were captured, analyzed with SlideBOOK™ software and the figures were processed and assembled in ImageJ or Photoshop.

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Author contributions

The study was conceived and designed by V.R., Y.K. and S.Y. Experiments were performed by V.R. (Fig. 2C and Fig. 4, 5, 6), T.M. (Fig. 1, 2, 3), T.S. (Fig. 1), I.W. (Fig. 2), and analyzed by V.R., Y.K. and S.Y. V.R. and S.Y. wrote the paper.

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Note added in proof

While this paper was under revision, a related paper showing dephosphorylation of APC by PP2A-Cdc55 has been published (Lianga et al., 2013).

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.127365/-/DC1

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