Zds1/Zds2–PP2A^{Cdc55} complex specifies signaling output from Rho1 GTPase

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Budding yeast Rho1 guanosine triphosphatase (GTPase) plays an essential role in polarized cell growth by regulating cell wall glucan synthesis and actin organization. Upon cell wall damage, Rho1 blocks polarized cell growth and repairs the wounds by activating the cell wall integrity (CWI) Pkc1–mitogen-activated protein kinase (MAPK) pathway. A fundamental question is how active Rho1 promotes distinct signaling outputs under different conditions. Here we identified the Zds1/Zds2–protein phosphatase 2A^{Cdc55} (PP2A^{Cdc55}) complex as a novel Rho1 effector that regulates Rho1 signaling specificity. Zds1/Zds2–PP2A^{Cdc55} promotes polarized growth and cell wall synthesis by inhibiting Rho1 GTPase-activating protein (GAP) Lrg1 but inhibits CWI pathway by stabilizing another Rho1 GAP, Sac7, suggesting that active Rho1 is biased toward cell growth over stress response. Conversely, upon cell wall damage, Pkc1–Mpk1 activity inhibits cortical PP2A^{Cdc55}, ensuring that Rho1 preferentially activates the CWI pathway for cell wall repair. We propose that PP2A^{Cdc55} specifies Rho1 signaling output and that reciprocal antagonism between Rho1–PP2A^{Cdc55} and Rho1–Pkc1 explains how only one signaling pathway is robustly activated at a time.

Introduction

Budding yeast Rho1 GTPase is an essential regulator of both cell growth and stress response. Active Rho1 serves as an essential regulatory subunit of the β-1,3-glucan synthase complex that has two redundant catalytic subunits, Fks1 and Fks2 (Fig. 1 A; Drgonová et al., 1996; Mazur and Baginsky, 1996; Qadota et al., 1996). In addition, Rho1 regulates the formin Bni1 (Kohno et al., 1996; Tolliday et al., 2002; Dong et al., 2003) and the exocyst subunit Sec3 (Guo et al., 2001), both of which are essential for polarized cell growth and morphogenesis. Rho1 is also essential for the cell wall integrity (CWI) stress response pathway, where it binds and activates Pkc1 (Nonaka et al., 1995; Kamada et al., 1996; Schmitz et al., 2002). Pkc1 terminates polarized cell growth in part by inducing proteasomal degradation of both Bni1 and Sec3 (Kono et al., 2012). Pkc1 also activates a MAPK cascade that leads to the activation of Mpk1/Slt2 to control the transcription of genes important for stress resistance (Fig. 1 A; Lee and Levin, 1992; Irie et al., 1993; Lee et al., 1993; Martín et al., 1993; Kamada et al., 1995; Nonaka et al., 1995; Schmitz et al., 2002).

The spatiotemporal regulation of Rho GTPase signaling is governed by the tight control of key regulators that directly affect the GTPase activation cycle, such as guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs). Compared with GEFs, regulation of Rho1 GAP activity is not well understood. There are two major Rho1 GAPs: Lrg1 and Sac7 (Lorberg et al., 2001; Watanabe et al., 2001). Rho1 GAPs have selectivity in downregulating specific Rho1 targets. Specifically, *lrg1*Δ cells have increased glucan synthesis without affecting Pkc1–Mpk1 activity (Lorberg et al., 2001; Watanabe et al., 2001), and conversely, *sac7*Δ cells have hyperactivation of Pkc1–Mpk1 without affecting glucan synthesis (Martín et al., 2000; Watanabe et al., 2001; Schmidt et al., 2002). Thus, Lrg1 selectively antagonizes the Rho1–Fks1/Fks2 glucan synthase complex, whereas Sac7 preferentially antagonizes the Rho1–Pkc1 stress response pathway.

In a genetic screen, we identified the regulatory B-subunit of the protein phosphatase 2A (PP2A), Cdc55, and its cortical anchoring proteins Zds1/Zds2 as novel regulators of Rho1 signaling. We show that PP2A^{Cdc55} controls the outcome of Rho1 activation without affecting global Rho1 activity. Cortical Zds1/Zds2–PP2A^{Cdc55} promotes cell wall glucan synthesis through inhibition of the Rho1 GAP, Lrg1. The cortical localization of Zds1/Zds2 and Cdc55 depend on Rho1 activity, which suggests a positive feedback loop that maintains cell wall synthesis throughout bud growth. We also show that Rho1 activation of the Pkc1-Mpk1 cascade results in the loss of cortical Cdc55 and Zds1. Collectively, these data suggest that Rho1 activation of polarized cell growth or stress response signaling is maintained by a mutual antagonism between the Rho1–PP2A^{Cdc55} and Rho1–Pkc1 pathways.

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Abbreviations used in this paper: CWI, cell wall integrity; GAP, GTPase-activating protein; GEF, guanine nucleotide-exchange factor; PP2A, protein phosphatase 2A; YPD, yeast extract peptone dextrose.

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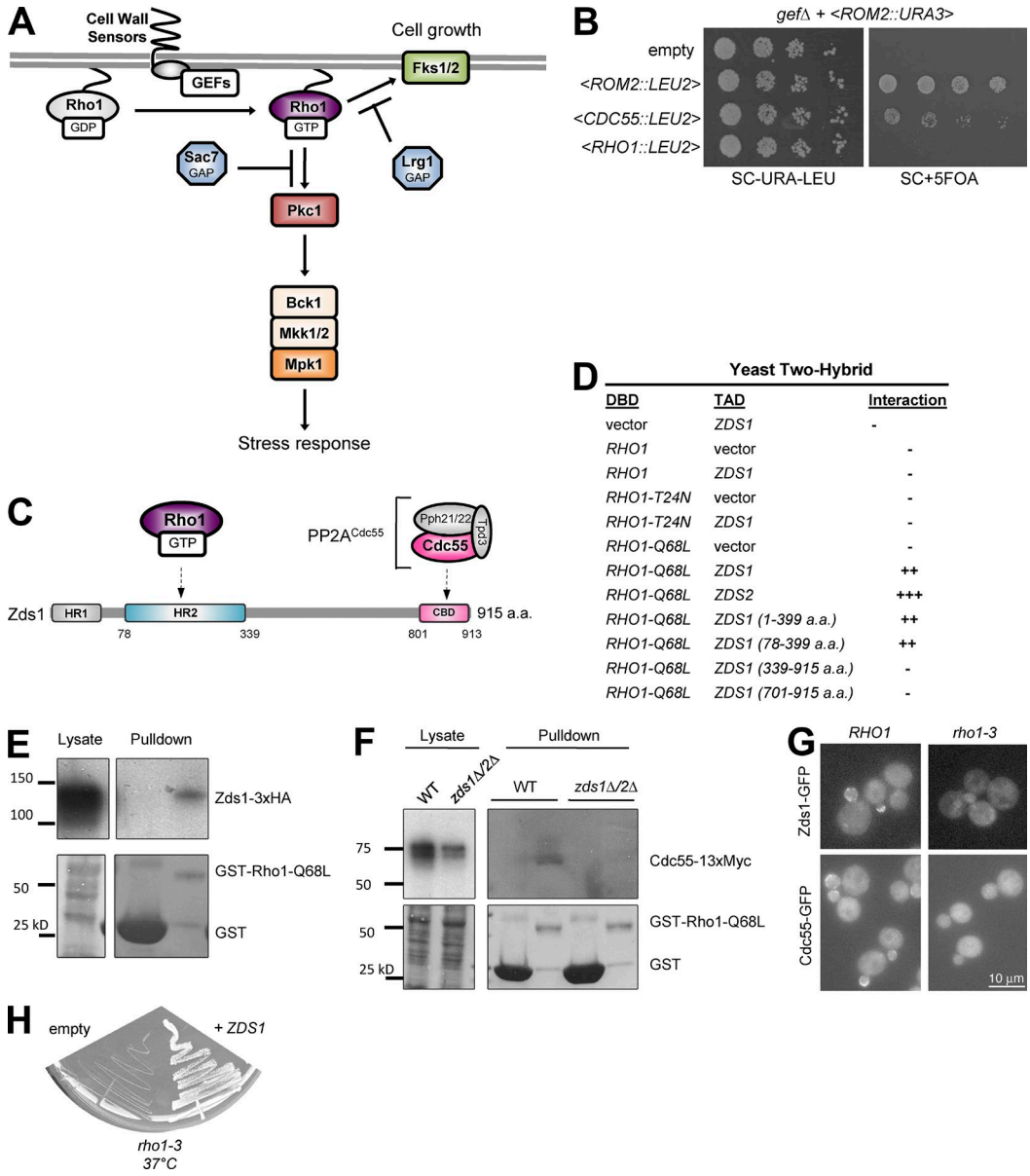

Figure 1. **PP2A^{Cdc55} is a novel Rho1 effector.** (A) Model of yeast Rho1 signaling pathways. Activated Rho1 activates either Fks1 for cell growth or Pkc1 for stress response. (B) Overexpression of *ROM2* and *CDC55* but not wild-type *RHO1* from *LEU2* harboring plasmids rescued the lethality of *gefΔ* strain after counterselection of *ROM2-URA3* plasmids by 5-FOA. (C) Domain structure of Zds1 and Zds2. Highly conserved region among fungal species (HR1 and HR2) and Cdc55-binding domain (CBD) are boxed. (D) Yeast two-hybrid assay mapped the Rho1 binding site of Zds1 to amino acids 78–339. Strength of the interaction was judged by LacZ assay. DBD, DNA-binding domain; TAD, transcriptional activation domain. (E) In a pull-down assay, Zds1-3xHA can bind to purified GST-Rho1-Q68L. (F) Cdc55-myc physically associates with Rho1-Q68L, and this interaction requires Zds1/Zds2. WT, wild type. (G) The cortical localization of Zds1-GFP and Cdc55-GFP depends on Rho1. Wild-type (*RHO1*) and *rho1-3* mutant cells expressing Zds1-GFP or Cdc55-GFP were incubated at 37°C for 2 h, and GFP signal was imaged. (H) The growth defect of *rho1-3* was suppressed by overexpression of *ZDS1*. Photo was taken after incubation for 2 d at 37°C.

Results and discussion

Cdc55 and Zds1/Zds2 are novel Rho1 effectors

To identify novel regulators of Rho1 signaling, we performed a multicopy suppressor screen using a yeast genomic DNA library for suppressors of the lethality of a yeast strain lacking all three Rho1 GEFs, *ROM1*, *ROM2*, and *TUS1* (*gefΔ*; Yoshida et al., 2009). In this screen, we identified *CDC55* (Fig. 1 B). *CDC55* encodes the regulatory B-subunit of PP2A, which has multiple roles in cell cycle progression and polarized growth (Jiang, 2006). Here, we focused our analysis on Cdc55, because we had previously identified the Cdc55 binding proteins, Zds1 and Zds2, as multicopy suppressors of the temperature-sensitive growth and glucan synthesis defects of the *rho1-2* mutant (Sekiya-Kawasaki et al., 2002).

Because PP2A^{Cdc55} and the Cdc55 binding domain of Zds1/Zds2 form a substoichiometric complex in vivo (Wicky et al., 2011), and because Zds1/Zds2 are reported to show two-hybrid interaction with Rho1-GTP (Fig. 1 C; Drees et al., 2001), we first examined if active Rho1, Cdc55, and Zds1/Zds2 could form a complex. We confirmed by two-hybrid assay that both Zds1 and Zds2 specifically interacted with the GTP-locked *RHO1-Q68L* mutant, but not with type *RHO1* or with

the nucleotide-free *RHO1-T24N* mutant (Fig. 1 D). We also found that the highly conserved homology region (HR2) domain (78–339 aa) of Zds1 was sufficient for two-hybrid interaction with *RHO1-Q68L* (Fig. 1 D). In a GST–pull-down assay, we confirmed the interaction of *GAL1*-expressed HA-tagged Zds1 in yeast extract with purified GST-Rho1-Q68L (Fig. 1 E). Furthermore, we were able to pull down Cdc55-myc from the yeast cell extract with GST-Rho1-Q68L (Fig. 1 F). The interaction between Rho1 and Cdc55 depended on the presence of Zds1/Zds2, because Cdc55-myc was not associated with GST-Rho1-Q68L in the yeast cell lysates from a *zds1Δ zds2Δ* double-mutant strain (Fig. 1 F). Thus, Zds1/Zds2 serves as a link between Rho1-GTP and Cdc55.

Because active Rho1, Zds1/Zds2, and Cdc55 all localize to the bud cortex during polarized cell growth (Yamochi et al., 1994; Bi and Pringle, 1996; Rossio and Yoshida, 2011), we also examined their localization dependence. In a temperature-sensitive *rho1-3* strain, the cortical localization of Zds1-GFP and Cdc55-GFP was lost at the restrictive temperature, 37°C (Fig. 1 G). Thus, complex formation between active Rho1 and Zds1 may be required for stable localization of $PP2A^{Cdc55}$ at the bud cortex. We found that overexpression of *ZDS1* efficiently suppressed the growth defect of the *rho1-3* strain at 37°C (Fig. 1 H), suggesting that defects associated with *rho1-3* are caused by mislocalization of Zds1. *rho1-3* is known to be severely impaired in glucan synthesis, but not in the Pkc1–Mpk1 pathway (Saka et al., 2001), which suggests that Zds1 and Cdc55 function together with Rho1-GTP at the cell cortex to promote cell wall synthesis. Collectively, the GTP-dependent complex formation, localization dependence, and genetic suppression of a *rho1* mutant strongly suggest that Cdc55–Zds1/Zds2 is a novel effector of Rho1.

Our attempts to demonstrate direct interaction between bacterially purified Rho1-Q68L and the HR2 domain of Zds1 failed (unpublished data), suggesting that stable interaction between Rho1 and Cdc55-Zds1 may require posttranslational modifications, a hypothesis that is consistent with previous studies that Zds1 is a phosphoprotein (Wicky et al., 2011). Alternatively, Rho1-GTP and Zds1/Zds2 may interact indirectly as part of a larger macromolecular complex, an idea that is suggested by the observation that several other effectors and regulators of Rho GTPases such as Pkc1, Bni1, Gic1/Gic2, Boi1/Boi2, Cla4, and Bem3 were identified as Zds1- or Zds2-binding proteins in the same screen that identified Rho1-GTP (Drees et al., 2001).

$PP2A^{Cdc55}$ is important for glucan synthesis

Because Rho1 has essential roles in cell wall biogenesis and integrity, we next examined the effects of various stresses on cells lacking each subunit of PP2A. Cdc55 is known to function in the cytoplasm and at the bud cortex in a complex with Zds1/Zds2 (Gentry and Hallberg, 2002; Yasutis et al., 2010; Rossio and Yoshida, 2011; Wicky et al., 2011; Rossio et al., 2014). Budding yeast contain two redundant catalytic subunits for PP2A: Pph21 and Pph22. Sit4, a PP6 phosphatase, has been implicated in the regulation of Pkc1-MAPK signaling and is reported to function with Cdc55 (Angeles de la Torre-Ruiz et al., 2002; Jiang, 2006). We found that deletions of *CDC55*, *ZDS1/ZDS2*, *PPH21/PPH22*, and *SIT4* were all hypersensitive to the cell wall–damaging reagent SDS as well as low temperature (Fig. 2 A). Although the mechanism of the cold sensitivity phenotype is not well understood, abnormal activation of Pkc1–Mpk1 signaling has been implicated (Schmidt et al., 2002; Córcoles-Sáez et al., 2012; Lockshon et al., 2012).

It is known that *cdc55Δ* and *zds1Δ zds2Δ* mutants exhibit a hyperelongated bud morphology caused by a cell cycle defect in progression through the G_2/M phase (Yang et al., 2000). To exclude possible side effects caused by the G_2 delay, we deleted the mitotic inhibitor *SWE1* in all cytological analyses that followed. As has been reported, deletion of *SWE1* rescued the morphological defects of *cdc55Δ* and *zds1Δ zds2Δ* (Yang et al., 2000; Rossio and Yoshida, 2011), but not the SDS sensitivity of these mutants (Fig. 2 B). We also found that the mutants were sensitive to low glucose (0.1%; Fig. 2 B), which suggests a possible problem in cell wall glucan production (Inoue et al., 1999; Douglas, 2001; Roh et al., 2002; Sekiya-Kawasaki et al., 2002).

To closely examine the cell wall defects of *cdc55Δ* and *zds1Δ zds2Δ* mutants, we first stained the cell wall glucan and chitin with aniline blue and calcofluor white, respectively. Aniline blue staining of glucan was not severely affected in *cdc55Δ* and *zds1Δ zds2Δ* mutants grown in the presence of high glucose (2% in the standard rich media), but we found significantly reduced glucan staining in the bud when the mutants were grown in the medium containing low glucose (0.1%; Fig. 2, C and D). Consistent with these results, the total amount of alkaline-extractable glucan was also significantly reduced in *cdc55Δ* and *zds1Δ zds2Δ* mutants (Fig. 2 E). We also noticed that there was a significant accumulation of chitin in the cell wall, possibly through a compensation mechanism for maintaining CWI (Fig. 2 C; Valdivia and Schekman, 2003; Lesage et al., 2004). To directly assess glucan synthesis, we measured the incorporation of ^{14}C-labeled glucose into β-1,3-glucan and found that *zds1Δ zds2Δ* double-mutant cells exhibited a severe reduction in glucan synthesis (Fig. 2 F). These data suggest that Zds1/Zds2 and Cdc55 are positive regulators for Rho1 in glucan synthesis.

Mpk1 is hyperactivated in the absence of *CDC55*

We examined Pkc1-MAPK activity, another major downstream target of Rho1, in *cdc55Δ* cells using a phospho-specific antibody that specifically recognizes the dual phosphorylation of Mpk1 (pT190 and pY192), a hallmark of active Mpk1 (Cobb and Goldsmith, 1995; Kamada et al., 1995; Martín et al., 2000). Under normal growth conditions, Mpk1 was hyperphosphorylated in *cdc55Δ* cells compared with wild-type cells (Fig. 2 G), which suggests that Cdc55 has a negative role in the Rho1–Pkc1–Mpk1 stress response pathway. Alternatively, it is possible that the hyperactivation of the Rho1–Pkc1–Mpk1 pathway is a consequence of the cell wall defects in *cdc55Δ* cells. The addition of osmotic stabilizer, 1 M sorbitol, partially suppressed the hyperactivation of Mpk1 in *cdc55Δ* cells (Fig. 2 J), which suggests that hyperactivation of Mpk1 in these cells was, at least partially, caused by cell wall problems. Indeed, Mpk1 was important for *cdc55Δ* cell viability at high and low temperatures (Fig. 2 H). We further found that deletion of *MPK1* from *cdc55Δ* and *zds1Δ zds2Δ* cells caused significant cell wall defects, as these mutants were hypersensitive to the cell wall–digesting enzyme Zymolyase (Fig. 2 I). These results revealed that $PP2A^{Cdc55}$ mutants require the Pkc1–Mpk1 pathway for maintaining cellular integrity but did not necessarily rule out a possible effect of Cdc55 in inhibition of the Pkc1–Mpk1 pathway.

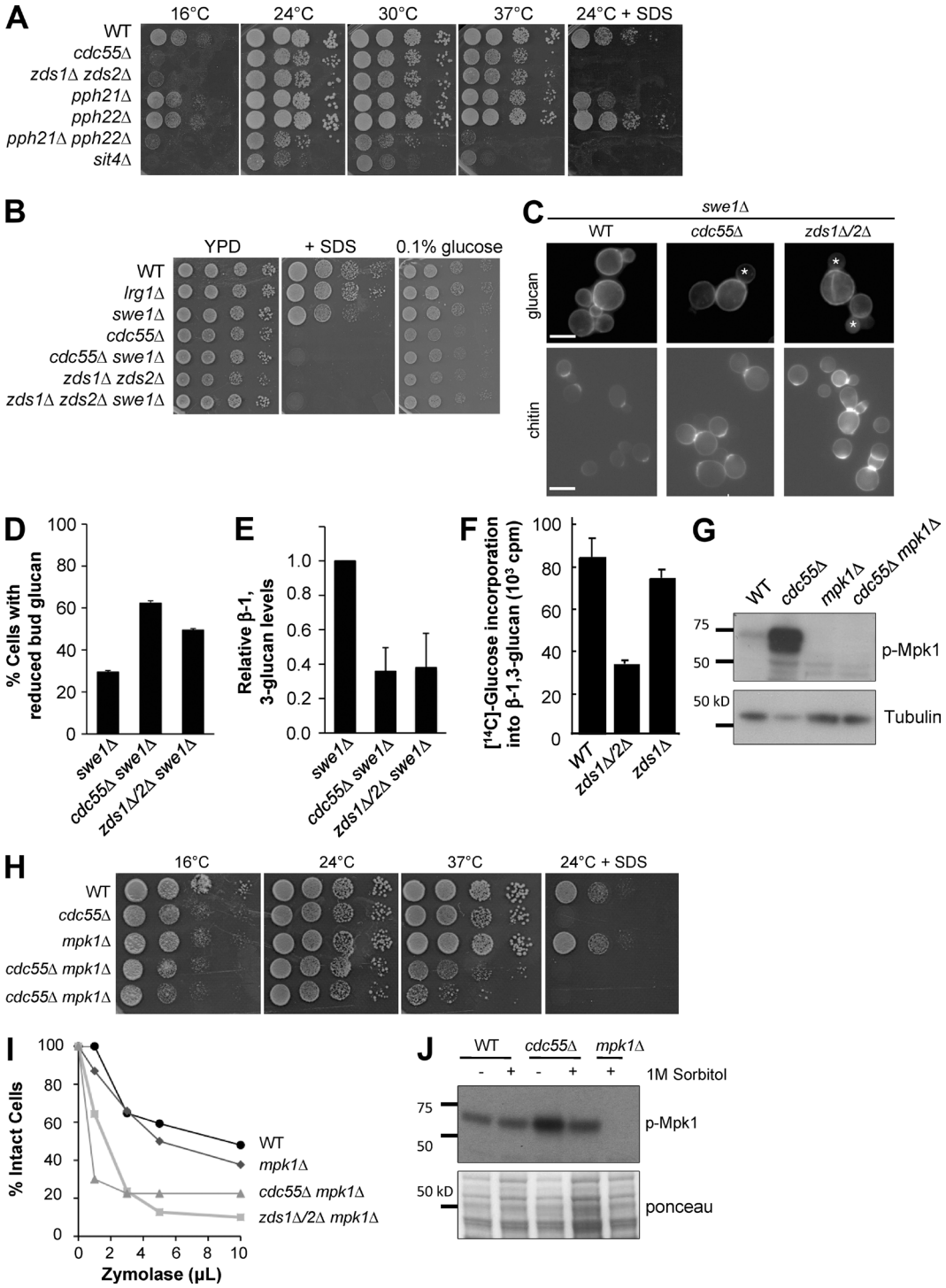

Figure 2. ***cdc55Δ* cells have reduced glucan synthesis and require Mpk1 activity for cell integrity.** (A) Serial dilutions of yeast strains deleted for PP2A subunits are spotted on YPD with or without 0.03% SDS plates and grown at different temperatures. (B) Deletion of *SWE1* did not rescue the stress sensitivity of PP2A mutants. (C) Abnormal cell wall in *cdc55Δ* and *zds1Δ zds2Δ* cells grown at 25°C. β-1,3-Glucan and chitin were stained with aniline blue and calcofluor white, respectively. Note that this and the following experiments were performed in *swe1Δ* strains. (D) Quantification of small budded cells with reduced anilin blue staining in the bud. (E) Quantification of the total amount of alkaline-soluble glucan synthesized in low-glucose media. (F) *zds1Δ zds2Δ* cells are defective for incorporation of glucose into β-1,3-glucan. Cells were incubated at 25°C, labeled with [^{14}C]glucose for 2 h, and measured for incorporation of glucose into β-1,3-glucan. (G) Mpk1 is hyperphosphorylated in *cdc55Δ* cells grown at 25°C in YPD. (H) *cdc55Δ* cells require Mpk1 for growth under cell wall–stressing conditions (37°C and SDS). (I) In the absence of Mpk1, *cdc55Δ* and *zds1Δ zds2Δ* cells exhibit a severe cell lysis defect when challenged with Zymolyase. Loss of OD600 in the presence of Zymolyase for 60 min at 37°C was monitored as an indicator of cell lysis. (J) Osmotic stabilization by addition of 1 M sorbitol partially reduced the high phospho-Mpk1 levels of *cdc55Δ* cells. Ponceau staining is shown as a loading control. WT, wild type.

Rho1 activity and localization is not affected in PP2A^{Cdc55} mutants

To understand the mechanism by which PP2A^{Cdc55} regulates Rho1 signaling, we hypothesized that PP2A^{Cdc55} directly regulates either Rho1 or the Rho1 GAPs, because overexpression of *CDC55* rescued the *gef1Δ* strain, but not a *rho1Δ* strain (unpublished data), which suggests that PP2A^{Cdc55} is neither functioning through the known Rho1 GEFs nor bypassing the essential requirement of Rho1. It is unlikely that PP2A^{Cdc55} regulates the only Rho guanosine nucleotide dissociation inhibitor, Rdi1, because deletion of *RDI1* failed to rescue the *gef1Δ* strain (Yoshida et al., 2009). We first tested if PP2A^{Cdc55} affected Rho1 activity or localization. To quantify total cellular Rho1 activity, we used the Rho-binding domain of rhotekin in a pull-down assay to measure Rho1-GTP levels (Kono et al., 2008; Yoshida et al., 2009). In this assay, loss of the Rho1 GAP *LRG1* resulted in a significant increase in the total amount of Rho1-GTP (Fig. S1 A); however, we were not able to detect a significant effect on Rho1-GTP levels in the absence of *CDC55* (Fig. S1 A). The cortical localization of Rho1 is dependent on membrane trafficking and Rdi1 (Abe et al., 2003; Tiedje et al., 2008; Boulter et al., 2010). Because Cdc55 and Rho1 display polarized localization to the bud tip and bud neck, we also examined the localization of GFP-Rho1 and GTP-locked GFP-Rho1-Q68L in *cdc55Δ* and *zds1Δ zds2Δ* and did not find a significant change (Fig. S1, B and C). Together, these results suggest that neither activity nor localization of Rho1 is largely affected by PP2A^{Cdc55}.

PP2A^{Cdc55} downregulates Lrg1 GAP activity

Previously it was shown that deletion of the Rho1 GAPs, either *LRG1* or *SAC7*, can rescue the *gef1Δ* strain (Yoshida et al., 2009). We hypothesized that Cdc55 may regulate Rho1 GAPs, because overexpression of *CDC55* also rescued the *gef1Δ* strain lethality (Fig. 1 B). To monitor the total cellular amount of activated GAP (competent for Rho1-GTP binding), we developed an affinity-based assay by taking advantage of the fact that both Lrg1 and Sac7 bind to the active Rho1-Q68L, but not with wild-type or GDP-locked Rho1, in a two-hybrid assay (Lorberg et al., 2001; Watanabe et al., 2001; Schmidt et al., 2002). Using a constitutively active Rho1 mutant, Rho1-Q68L, we were able to detect robust interaction of Lrg1 and Sac7 GAPs with GST-Rho1-Q68L beads, but not with GST control beads (Fig. 3 A).

Having established a method to quantify the amount of activated GAPs from yeast cell lysates, we first tested if loss of *CDC55* affected Lrg1 GAP activity, because reduction in glucan synthesis in *cdc55Δ* implicates hyperactivation of Lrg1. In cells lacking *CDC55*, Lrg1 was equally expressed in cell lysates (Fig. 3 B) and localized normally to the bud cortex and neck (Fig. S1 D); however, more HA-tagged Lrg1 was bound to GST-Rho1-Q68L beads in *cdc55Δ* cell lysates compared with wild type (Fig. 3 B). Thus, the total cellular Lrg1 GAP activity was significantly increased in the absence of Cdc55. The glucan synthesis defect of *cdc55Δ* was indeed caused by hyperactivity of Lrg1. Deletion of *LRG1* almost completely rescued the glucan synthesis defect of *cdc55Δ* (Fig. 3 C). Furthermore, expression of GTP-locked *RHO1-Q68H* restored glucan synthesis defects of *cdc55Δ* and *zds1Δ zds2Δ* (Fig. 3, D–F). These results, together with the physical interaction with Rho1-GTP and localization dependence, strongly suggest that the Zds1/Zds2–PP2A^{Cdc55} complex is a new effector of Rho1 that promotes cell wall biogenesis via inhibition of Lrg1 (Fig. 3 H).

Active Rho1 is harmful to *cdc55Δ* and *zds1Δ zds2Δ*

We also examined whether deletion of *LRG1* rescues any growth defect associated with *cdc55Δ*. Contrary to our expectation, SDS sensitivity of *cdc55Δ* was not rescued by deletion of *LRG1*; rather, *cdc55Δ lrg1Δ* exhibited a synthetic growth defect at high temperature (Fig. 3 G). The observed synthetic sickness is likely caused by misregulation of Rho1 activity, because introduction of GTP-locked *RHO1-Q68H* caused severe growth defects in *cdc55Δ* (Fig. 4 B) and *zds1Δ zds2Δ* (Fig. S2). Because the Pkc1–Mpk1 pathway was hyperactivated in *cdc55Δ* (Fig. 2 G), we suspected that the toxic effect of *RHO1-Q68H* is caused by abnormal activation of the Rho1–Pkc1 pathway. Indeed, expression of an activated mutant form of Pkc1, *PKC1-R398P*, caused severe growth defects in *cdc55Δ* (Fig. 4 B).

Sac7 is unstable in the absence of PP2A^{Cdc55}

To understand why Pkc1–Mpk1 is hyperactivated in *cdc55Δ*, we examined total cellular Sac7 GAP activity in both wild-type and *cdc55Δ* lysates and found a significant reduction in the amount of active Sac7 GAP (Fig. 4 C). The reduction of active Sac7 in *cdc55Δ* was largely caused by the reduced amount of total Sac7 protein compared with a loading control, α-tubulin (Fig. 4 B). We confirmed that Sac7 protein level is dependent on *CDC55,* as expression of *CDC55* from a plasmid fully restored Sac7 protein abundance in *cdc55Δ* (Fig. 4 C). Regulation of Sac7 protein level by Cdc55 was not caused by transcriptional control but rather degradation control, because induction of *SAC7* expression from an inducible *GAL1* promoter failed to overexpress 6xHis-Sac7 in *cdc55Δ* (Fig. 4D). In contrast, Sac7 was stabilized in a proteasome mutant *cim3-1* even in the absence of *CDC55* (Fig. 4 E). These results suggest that Cdc55 prevents Pkc1 activation by stabilizing Sac7 (Fig. 4 F). The molecular mechanisms by which PP2A inactivates Lrg1 and protects Sac7 from proteasomal degradation require further investigation but may involve dephosphorylation by PP2A, because both Lrg1 and Sac7 are phosphoproteins in vivo (Swaney et al., 2013).

Antagonism between PP2A and Pkc1

In a complementary approach, we examined the effect of upregulating PP2A^{Cdc55} activity by overexpressing *ZDS1*. Consistent with our previous result (Sekiya-Kawasaki et al., 2002), overexpression of *ZDS1* efficiently rescued the temperature-sensitive growth defect of the glucan synthase mutant *fks1-1154 fks2Δ* (Fig. 5 A, top). In contrast, overexpression of *ZDS1* was toxic to the temperature-sensitive *pkc1-2* mutant at the semipermissive temperatures of 30°C and 34°C (Fig. 5 A, bottom). Interestingly, we also found that overexpression of *PKC1* was toxic to *fks1-ts fks2Δ*, suggesting a possible antagonism between the PP2A^{Cdc55}–Zds1/Zds2 and Pkc1–Mpk1 pathways.

The inhibitory effects of Zds1 on the Pkc1 pathway predict that PP2A^{Cdc55} needs to be prevented from interacting with Rho1 to allow optimal Pkc1 activation upon cell wall damage (Fig. 5 B). Indeed, both Zds1-GFP and Cdc55-GFP were rapidly delocalized from the bud cortex after cell wall damage by a heat shock (Fig. 5 C). In contrast, Pkc1-GFP was robustly recruited to the cell cortex (Fig. 5 C), as previously described (Andrews and Stark, 2000; Kono et al., 2012). Collectively, these data suggest that Rho1 robustly switches from activation of polarized cell growth effectors to activation of stress response effectors through the removal of cortical polarity factors.

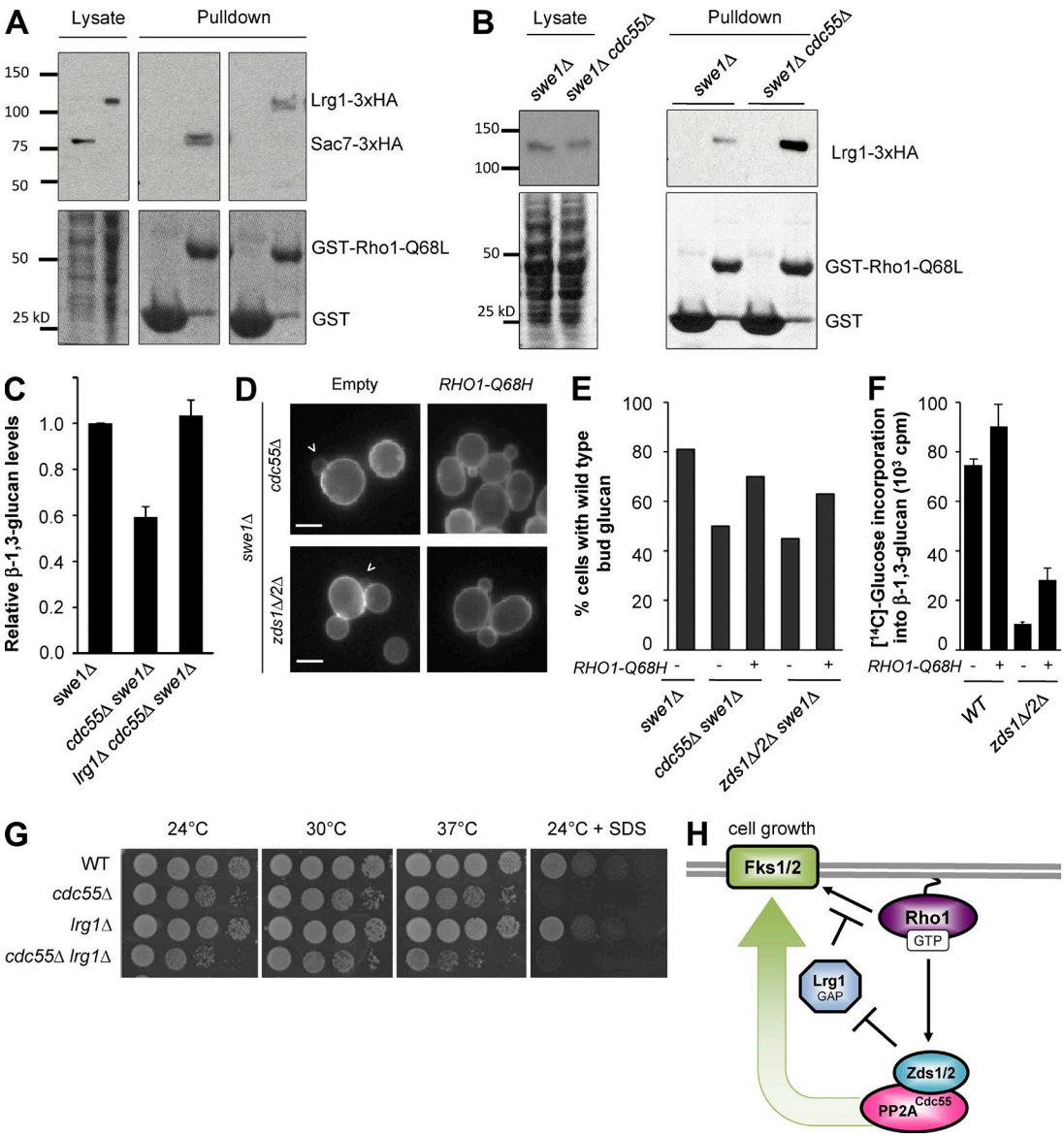

Figure 3. **A Rho1–Zds1/Zds2–PP2A^{Cdc55} positive feedback loop promotes glucan synthesis by inhibition of Lrg1.** (A) Pull-down assay for monitoring active Rho1 GAP. GST-Rho1Q68L was incubated with yeast cell lysates expressing 3XHA-tagged Sac7 or Lrg1. Lysates and bead-bound fractions were subjected to SDS-PAGE and Western blotting with anti-HA antibody. (B) In *cdc55Δ*, more Lrg1-3XHA bound to GST-Rho1Q68L beads compared with wild-type (WT) lysates. (C) Deletion of *LRG1* rescued the glucan synthesis defect of *cdc55Δ* cells. Normalized total glucan synthesis as assayed in Fig. 2 E for indicated strains. (D) Activation of Rho1 can bypass Zds1/Zds2–PP2A^{Cdc55} regulation of glucan synthesis. Arrowhead denotes cells with reduced glucan staining. (E) Quantification of small budded cells with wild-type glucan staining in the bud from D. (F) β-1,3-glucan synthesis defect of *zds1Δ zds2Δ* is partially rescued by *RHO1-Q68H*. (G) Deletion of *LRG1* did not rescue the SDS sensitivity of *cdc55Δ* cells. Serial dilutions of yeast strains spotted on YPD with or without 0.03% SDS plates at the indicated temperature. (H) Model for how Zds1/Zds2–PP2A^{Cdc55} promotes glucan synthesis through the inhibition of Rho1 GAP Lrg1.

We further found that activation of Pkc1 was sufficient to remove Cdc55 and Zds1 from the bud cortex, even in the absence of cell wall stress. Expression of active Pkc1 (Pkc1-R389P) delocalized Zds1-GFP and Cdc55-GFP from the bud cortex (Fig. 5, D and E). Pkc1-induced delocalization of Zds1 depends on having a functional CWI MAPK cascade, because expression of activated MAPK kinase *MKK1-S386P* also caused delocalization of Zds1-GFP (Fig. 5 D). Furthermore, the effect of *PKC1-R398P* and *MKK1-S386P* on Zds1-GFP delocalization was blocked by deletion of the MAPK *MPK1* (Fig. 5 D). Thus, activation of the Pkc1–Mpk1 pathway leads to removal of the PP2A^{Cdc55} complex away from active Rho1 at the cell cortex.

Mutual antagonism between PP2A^{Cdc55} phosphatase and Pkc1 kinase explains how only one signaling pathway is robustly activated at a time

Our genetic and biochemical analysis revealed an interesting role for PP2A^{Cdc55} in Rho1 signaling pathways. Cdc55 promotes cell wall synthesis while antagonizing the activation of Pkc1, without significantly affecting total cellular Rho1 activity. The balance between glucan synthesis and Pkc1 activity is important for cell viability, because simultaneous activation of both pathways by deletion of *LRG1* and *SAC7* results in lethality (Lorberg et al., 2001). Furthermore, reduced glucan synthesis and impaired Pkc1–Mpk1 signaling also results

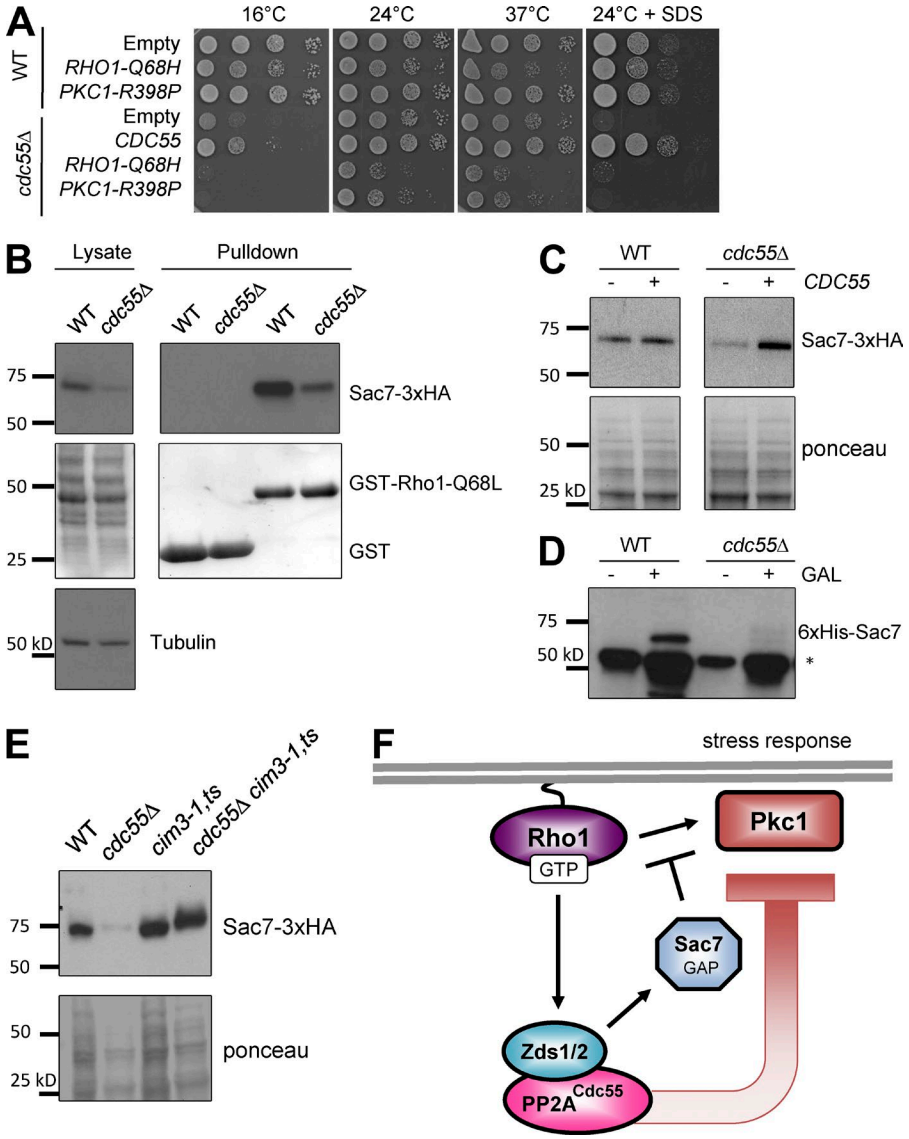

Figure 4. **PP2A^{Cdc55} inhibits Pkc1 activation potentially through stabilization of the Pkc1-specific Rho1 GAP, Sac7.** (A) Expression of *RHO1-Q68H* or *PKC1-R398P* was toxic to *cdc55Δ* cells. (B) Sac7-3XHA is less abundant in the *cdc55Δ* lysates. Lysates and bead-bound fractions were subjected to SDS-PAGE and Western blotting with anti-HA antibody and anti–α-tubulin as a loading control. (C) Expression of *CDC55* restored Sac7-3xHA protein levels in *cdc55Δ* cells to wild-type (WT) levels. (D) The reduction of Sac7 in the *cdc55Δ* lysates is a posttranscriptional mechanism. Expression of 6xHis-Sac7 from an inducible *GAL1* promoter failed to accumulate Sac7 in *cdc55Δ* cells. *, nonspecific band detected by anti-His antibody (Millipore). (E) Sac7 is degraded by the proteasome. Inactivation of the proteasome by a *cim3-1* mutation at the semipermissive temperature of 30°C for 4 h resulted in stabilization of Sac7-3xHA even in the *cdc55Δ* mutants. (F) Model for how Zds1/Zds2–PP2A^{Cdc55} antagonizes Rho1 activation of Pck1 through the stabilization of the Rho1 GAP Sac7.

in lethality (Levin et al., 1990; Mazur et al., 1995; Saka et al., 2001). We propose that PP2A^{Cdc55} regulates this essential balance of Rho1 signaling output, which is consistent with the fact that *cdc55Δ* cells are hypersensitive to increased dosages of GTP-locked Rho1 (Fig. 4 A).

Based on these data, we propose the following model for PP2A function in Rho1 signaling (Fig. 5 F). During favorable growth conditions, cortical PP2A^{Cdc55} biases Rho1 to activate polarized cell growth effectors (Bni1, Sec3, and Fks1/Fks2) by inactivating the Rho1 GAP Lrg1. Because Cdc55 is delivered to the bud cortex by polarized secretion (Heger et al., 2011), activation of Bni1, Sec3, and Fks1/Fks2 creates a positive feedback loop of cortical PP2A^{Cdc55} activity that sustains polarized cell growth. At the same time, PP2A^{Cdc55} prevents activation of the Pkc1–Mpk1 pathway by stabilizing the Rho1 GAP Sac7 (Fig. 4, C and D). When a cell is exposed to cell wall stresses, Rho1 rapidly activates Pkc1 and blocks polarized secretion by degradation of Bni1 and Sec3 (Kono et al., 2012). Loss of polarized secretion stops the supply of Cdc55 to the bud cortex. Activation of Pkc1 also triggers a removal of PP2A^{Cdc55} from the cell cortex in a Mpk1-dependent manner.

The mutual antagonism between Zds1/Zds2–PP2A^{Cdc55} phosphatase and Pkc1 kinase activities can form a bistable system in which active Rho1 promotes either polarized cell growth or repair of cell wall wounds. Because small GTPases often regulate multiple signal outputs, we speculate that antagonism between effectors is commonly used to allow robust activation of only one signal outcome at a time.

Materials and methods

Plasmids and strains

Standard methods were used for molecular biology procedures and yeast cell growth. All yeast strains were isogenic to BY4741 (Mata *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0* from OpenBiosystems) unless otherwise noted. Yeast strains and plasmids are listed in Tables S1 and S2, respectively. PY strains and PB plasmids were gifts from D. Pellman (Harvard Medical School, Boston, MA). The *SWE1*-disrupting plasmid was a gift from D. Lew (Duke University, Durham, NC); M. Mizunuma (Hiroshima University, Hiroshima, Japan) provided *ZDS1* and *ZDS2* expression plasmids; and K. Irie (Tsukuba University, Tsukuba, Japan) provided pNV7-MKK1P386. Gene deletions

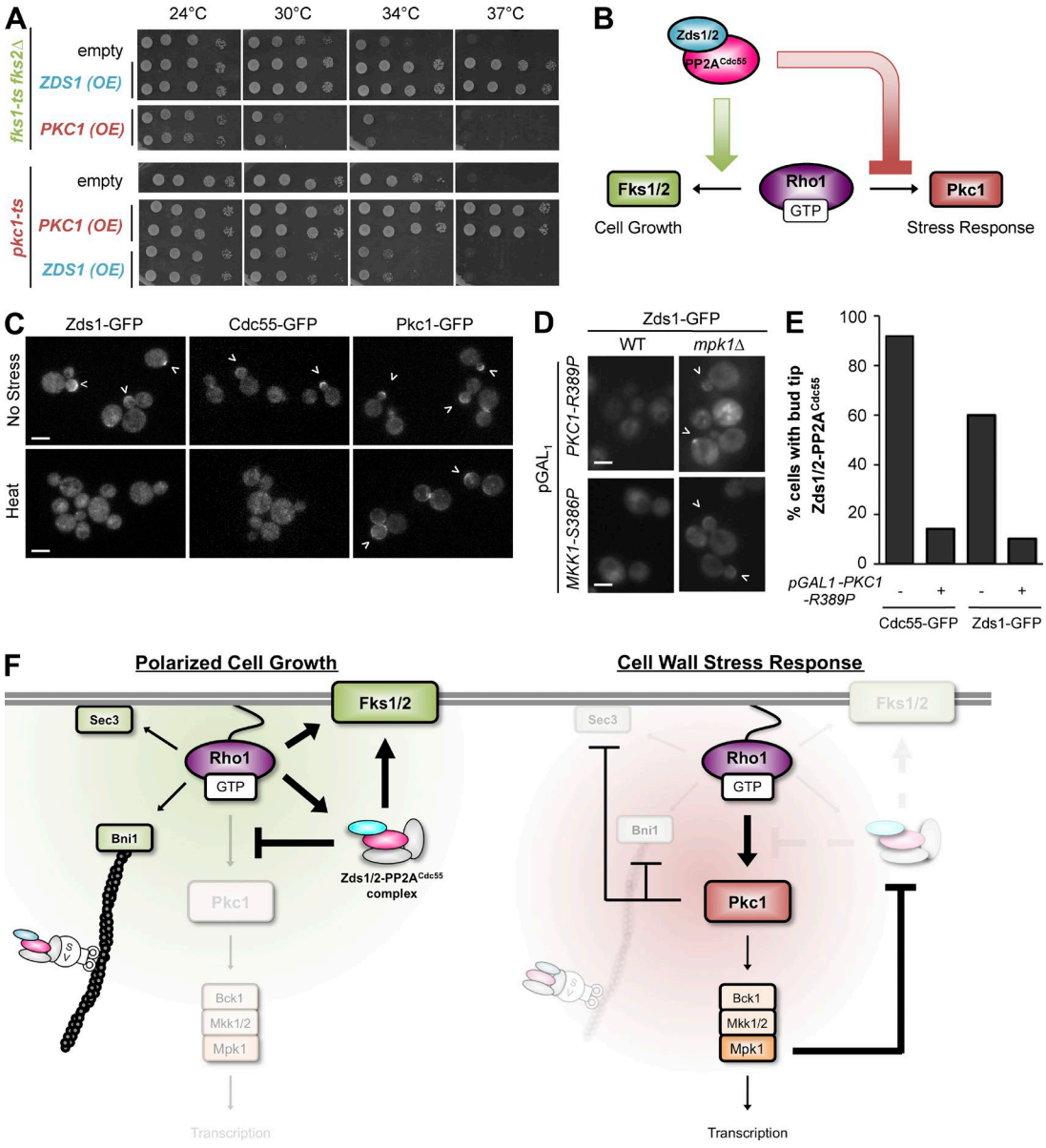

Figure 5. **Mutual antagonism between Zds1/Zds2–PP2A^{Cdc55} and Pkc1 controls Rho1 activation of polarized cell growth or stress response.** (A) Overexpression of *ZDS1* rescued glucan synthase mutant *fks1-ts fks2Δ* but inhibited growth of *pkc1-ts* mutant at higher temperature. (B) Working model for Zds1/Zds2–PP2A^{Cdc55} function. Zds1/Zds2–PP2A^{Cdc55} promotes Rho1–Fks1/Fks2 glucan synthesis while inhibiting the Rho1–Pkc1 stress pathway. (C) Cell wall stress removes Zds1/Zds2–PP2A^{Cdc55} complex from the bud cortex. Zds1-GFP and Cdc55-GFP but not Pkc1 are delocalized from the bud cortex (arrowheads) after a heat shock at 39°C. (D) Artificial activation of Pkc1 or Mkk1 is sufficient to remove Zds1 from the cortex in a *MPK1*-dependent manner. WT, wild type. (E) Quantification of cells with cortically localized Cdc55-GFP or Zds1-GFP before (–) and after (+) *GAL* induction of the activated *PKC1-R389P* mutant. More than 50 cells were counted for each strain and condition. (F) Model for the Zds1/Zds2–PP2A^{Cdc55} complex regulation of Rho1 signaling during polarized cell growth (left) and cell wall stress response (right). Two mutually exclusive Rho1 signaling states are maintained by an antagonism between the Zds1/Zds2–PP2A^{Cdc55} complex and Pkc1 kinase.

and modification were constructed by recombination using *KAN MX6 or HIS3MX6* cassettes provided by J. Pringle (Stanford University, Stanford, CA; Longtine et al., 1998), and accurate integrations were confirmed by PCR. For C-terminal tagging of Lrg1 and Sac7 with 3XHA, a flexible linker (GGSGGS) was introduced between the ORF and tag.

For the yeast two-hybrid β-galactosidase assay, yeast cells were cotransformed with a plasmid containing the LexA DNA binding domain (pBMT116-derived) and a plasmid containing a gene fused to the *GAL4* transcriptional activating domain (pACTII-HK derived; Watanabe et al., 2001). For quantitative analysis for β-galactosidase activity, the transformants were cultured in SC-TRP-LEU, and β-galactosidase activity was measured according to the ortho-nitrophenyl-β-galactoside assay method (Guarente, 1983).

Biochemistry

Whole-cell protein extracts were prepared as described previously (Kushnirov, 2000). In brief, cells were pelleted, treated with 0.1 N NaOH, and incubated for 5 min on ice. Afterward, they were pelleted, resuspended in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, and 0.005% bromophenol blue), boiled for 5 min, and pelleted. The supernatants were loaded in a 4–15% mini-gel (Bio-Rad), and Western blotting was performed with indicated antibodies. Commercially available antibodies used were

rabbit anti–phospho-p42/44 MAPK (T202/Y204) antibody (Cell Signaling Technology), rat anti–tubulin-α antibody (AbD Serotec), mouse anti–6XHis-HRP antibody (Millipore), and mouse anti–HA 12CA5 antibody (Roche). HRP-conjugated secondary antibodies were obtained from Millipore, and proteins were detected with an enhanced chemiluminescence system (ECL plus; Amersham).

The Rho1-Q68L pull-down assays for Zds1 and Cdc55 interaction and Rho1 GAPs Lrg1 and Sac7 were performed as follows. GST-Rho1-Q68L was purified from *Escherichia coli* as previously described (García-Mata et al., 2006). Yeast cell pellets from 50-ml cultures were lysed by a glass bead beater method in 400 µl lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, and standard protease inhibitors [antipain, leupeptin, pepstatin A, aprotinin, and chymostatin]), and lysates were cleared by low-speed (13,000 rpm) centrifugation for 15 min at 4°C. Supernatants were incubated with a bead-bound GST-Rho1-Q68L fusion for 1 h rotating at 4°C and washed three times, and bound fractions were subjected to SDS-PAGE. Bound Zds1-3XHA or Cdc55-13myc was detected by Western blot analysis with mouse anti–HA 12CA5 antibody (Roche) or mouse anti–myc 9E10 antibody (Millipore).

Quantification of Rho1-GTP by rhotekin pull-down assay was performed as previously described (Yoshida et al., 2006; Kono et al., 2008). Yeast cells were lysed by bead-beating method in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 12 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 0.6% CHAPS, and standard protease inhibitors), and lysates were cleared by low-speed centrifugation. Cleared supernatants were incubated with commercially available GST-rhotekin beads (Cytoskeleton) for 1 h rotating at 4°C and washed twice, and bound fractions were subjected to SDS-PAGE and Western blotting with a custom made rabbit anti–Rho1 Y1486 antibody.

Fluorescence microscopy

To examine cell wall phenotype, yeast cells were grown overnight in yeast extract peptone dextrose (YPD; 0.5% glucose), refreshed in YPD (0.1% glucose), and grown to early log-phase. Staining of cell wall glucan and chitin was performed as previously described (Watanabe et al., 2001). For glucan staining, yeast cells grown in low-glucose media (0.1% glucose) were washed twice with PBS and incubated with 0.01% aniline blue for 5 min at RT, washed twice with PBS, and imaged. For chitin staining, yeast cells grown in low-glucose media (0.1%) were fixed in 4% formaldehyde for 10 min at RT, washed twice with PBS, incubated with 10 µg/ml calcofluor white for 5 min at RT, washed twice with PBS, and imaged. Images were acquired using a fluorescence microscope (Eclipse E600; Nikon) equipped with a CCD camera (DC350F; Andor) and a 63× (NA 1.4) oil objective. All images were captured and analyzed with NIS-Elements software (Nikon).

Quantification of β-1,3-glucan

Total soluble β-1,3-glucan was quantified in a microtiter-based assay with aniline blue as described in Watanabe et al. (2001). Cells were grown overnight in YPD containing 0.5% glucose, refreshed in YPD containing 0.1% glucose, and allowed to grow for 4 h. Cells were normalized to a final OD600 of 0.2 in 0.5 ml Tris-EDTA buffer, and NaOH was added to a final concentration of 1 N. β-1,3-Glucan was solubilized by incubation for 30 min at 80°C. 2.1-ml aniline blue buffer (0.03% aniline blue, 0.18 N HCl, and 0.5 M glycine/NaOH, pH 9.5) was added, and cells were incubated for 30 min at 50°C. Reactions were allowed to cool for 30 min at RT before measuring fluorescence using an Infinite M200 plate reader (Tecan) at excitation and emission wavelengths of 400 and 460 nm, respectively.

Incorporation of [^{14}C]glucose into β-1,3-glucan was performed as previously described (Sekiya-Kawasaki et al., 2002). Early log-phase cultures were grown to OD600 0.5 in 1 ml of 0.5% glucose media containing 10 µCi [^{14}C]glucose and incubated for 2 h at RT. After labeling, β-1,3-glucan was extracted as described earlier for the microtiter assay.

Online supplemental material

Tables S1 and S2 list yeast strains and plasmids, respectively. Fig. S1 shows Rho1 activity and localization of Rho1 and Lrg1 In *cdc55Δ*. Fig. S2 shows toxicity of GTP-locked Rho1 in *zds1Δ zds2Δ*. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201508119/DC1.

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