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Comparative genetic analysis of PP2A-Cdc55 regulators in budding yeast

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Abbreviations: SAC, spindle assembly checkpoint

Cdc55, a regulatory B subunit of the protein phosphatase 2A (PP2A) complex, plays various functions during mitosis. Sequestration of Cdc55 from the nucleus by Zds1 and Zds2 is important for robust activation of mitotic Cdk1 and mitotic progression in budding yeast. However, Zds1-family proteins are found only in fungi but not in higher eukaryotes. In animal cells, highly conserved ENSA/ARPP-19 family proteins bind and inhibit PP2A–B55 activity for mitotic entry.

In this study, we compared the relative contribution of Zds1/Zds2 and ENSA-family proteins Igo1/Igo2 on Cdc55 functions in budding yeast mitosis. We confirmed that Igo1/Igo2 can inhibit Cdc55 in early mitosis, but their contribution to Cdc55 regulation is relatively minor compared with the role of Zds1/Zds2. In contrast to Zds1, which primarily localized to the sites of cell polarity and in the cytoplasm, Igo1 is localized in the nucleus, suggesting that Igo1/Igo2 inhibit Cdc55 in a manner distinct from Zds1/Zds2.

Our analysis confirmed an evolutionarily conserved function of ENSA-family proteins in inhibiting PP2A-Cdc55, and we propose that Zds1-dependent sequestration of PP2A-Cdc55 from the nucleus is uniquely evolved to facilitate closed mitosis in fungal species.

Introduction

In eukaryotic cells, activation of cyclin-dependent kinase 1 (Cdk1) complexed with mitotic cyclins promotes mitosis.[1] Regulatory mechanisms of Cdk1 activation have been studied extensively;[2] however, far less is understood about the control of the protein phosphatases that counteracts the mitotic Cdk1 activity. A key phosphatase that antagonizes Cdk1 activity and dephosphorylates Cdk1 substrates is the protein phosphatase 2A (PP2A) in complex with its regulatory subunit B55 (Cdc55 in budding yeast).[3]

The PP2A is a trimeric complex and consists of a scaffolding A subunit, a regulatory B subunit, and a catalytic C subunit.[4] Among them, the regulatory B subunit is largely responsible for the subcellular localization and substrate specificity of PP2A.[4]

In budding yeast, cells lacking Cdc55 (a regulatory B subunit) exhibit multiple cell cycle defects, including mitotic entry,[5-10] spindle assembly checkpoint (SAC),[5,11-13] and precocious mitotic exit.[12,14-18] We have proposed previously that PP2A-Cdc55 activity is largely controlled by its subcellular distribution.[13,16] Cdc55 binding protein Zds1 (zillion different screens) and its homolog Zds2 are cytoplasmic proteins and sequester Cdc55 from the nucleus during mitosis.[13,16] Sequestration of PP2A-Cdc55 in the cytoplasm by Zds1/Zds2 promotes mitotic entry and allows full activation of mitotic Cdk1 in the nucleus. Removal or inhibition of nuclear Cdc55 is also necessary for robust activation of APC-Cdc20 during metaphase–anaphase transition, as well as for the robust activation of Cdc14 phosphatase essential for mitotic exit.[13,16]

Because Zds1/Zds2 are important regulators of PP2A-Cdc55, *zds1*Δ *zds2*Δ cells exhibit pleiotropic cell cycle defects. First, *zds1*Δ *zds2*Δ cells are severely impaired in mitotic entry, and, as a consequence, they exhibit hyper-elongated cell morphology and cold temperature sensitivity.[19-21] Second, *zds1*Δ *zds2*Δ cells are also impaired in mitotic exit, because nuclear PP2A-Cdc55 activity prevents activation of a Cdc14 phosphatase.[14,15] These phenotypes of *zds1*Δ *zds2*Δ cells are due to nuclear accumulation of Cdc55, because either deletion or nuclear exclusion of Cdc55 rescues both phenotypes.[8,14,15]

On the other hand, overexpression of *ZDS1* (or *ZDS2*) can inhibit nuclear functions of PP2A-Cdc55 by sequestering Cdc55 in the cytoplasm. For example, overexpression of *ZDS2* promotes precocious mitotic entry.[8] Overexpression of *ZDS1* activates APC-Cdc20 and, as a consequence, causes SAC defects similar to *cdc55*Δ cells.[13] Furthermore, overexpression of *ZDS1* rescues the growth defect of late mitotic mutants such as *cdc15–2*.[15] Although Zds1/Zds2 inhibit nuclear functions of PP2A-Cdc55,

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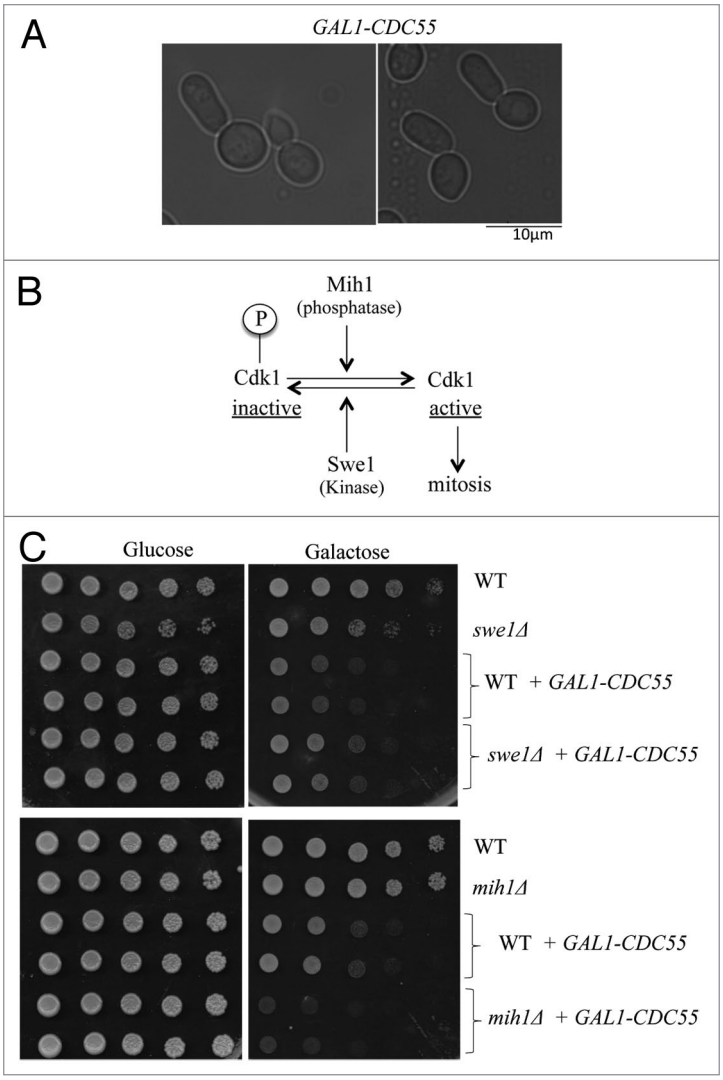

Figure 1. Overexpression of *CDC55* delays mitotic entry (**A**) Representative images of cells overexpressing *CDC55* under the strong *GAL1* promoter. Cells were grown in YEP medium containing galactose (2%) for 3 h at 24 °C. (**B**) Schematic model of mitotic entry in budding yeast. (**C**) Wild-type (WT), *swe1*Δ or *mih1*Δ cells were transformed with either empty vector or *GAL1-CDC55*-containing plasmid. Saturated cultures were serially (10 x) diluted and spotted on SC (synthetic complete)-URA media containing either glucose (*CDC55* off) or galactose (*CDC55* on). Images were taken after 3 d at 24 °C.

Zds1/Zds2 are not necessarily inhibitors of PP2A-Cdc55 catalytic activity; rather, it is formally possible that Zds1/Zds2 promote cytoplasmic functions of PP2A-Cdc55.

In animal cells, inhibition of PP2A–B55 activity is a prerequisite for mitotic entry.[22] However, animal cells lack an apparent homolog of Zds1. Recent studies have identified α-endosulfine (ENSA) and Arpp19 as potent inhibitors of PP2A-B55.[23,24] ENSA-family proteins bind directly to PP2A-B55 and inhibit its activity after phosphorylation by the Greatwall kinase.[23,24]

ENSA family proteins are widely conserved among eukaryotes. In budding yeast, Rim15 (a homolog of Greatwall kinase) phosphorylates ENSA family proteins Igo1 and Igo2 (initiation of G_0) and promotes their binding to PP2A–Cdc55 complex. Rim15 and Igo1/Igo2 were originally identified as key regulators of entry into quiescence (G_0) state.[25] A recent study has shown that Rim15-Igo1/Igo2 can directly inhibit PP2A-Cdc55 activity in vitro,[26,27] and that *igo1*Δ *igo2*Δ cells are defective in mitotic entry.[27]

However, relative contribution of ENSA-family proteins and Zds1-family proteins in regulation of PP2A-Cdc55-associated processes during mitosis and why Zds1-family proteins are uniquely evolved in fungal species remain interesting questions.

In this study, we performed a comparative genetic analysis of Zds1/Zds2 and Igo1/Igo2 to elucidate their relative contribution to mitotic progression via Cdc55 and to evaluate their differences. Our genetic analysis suggests that Igo1 and Igo2 serve as inhibitors of PP2A-Cdc55 in vivo, but their contribution is relatively minor compared with Zds1 and Zds2. We also found that Igo1 is enriched in the nucleus, in contrast to Zds1/Zds2, which are exclusively cytoplasmic. This suggests that, in agreement with the genetic data provided, Igo1/Igo2 are controlling PP2A-Cdc55 in a distinct mechanism from Zds1/Zds2.

Results

Zds1/Zds2 and Igo1/Igo2 prevent the toxic effects caused by *CDC55* overexpression

It has been proposed that both Zds1/Zds2 and Igo1/Igo2 are inhibitors of PP2A–Cdc55 activity.[13,16,27] To test this hypothesis in vivo, we examined the effects of *CDC55* overexpression in the presence or absence of these proteins. We expected that overexpression of *CDC55* is more toxic in the absence of its inhibitors.

Overexpression of *CDC55* under strong *GAL1* promoter was highly toxic to wild-type cells (**Fig. 1C**). This toxicity was most likely due to defects in mitotic entry, since cells overexpressing *CDC55* exhibited elongated bud morphology (**Fig. 1A**). In budding yeast, Cdk1 activation upon mitotic entry is negatively regulated by Swe1 (wee1 homolog) and positively regulated by Mih1 (cdc25 homolog) (**Fig. 1B**). We found that deletion of *SWE1* partially rescued *GAL1–CDC55* toxicity, while deletion of *MIH1* rendered the cells more sensitive to *GAL1-CDC55* (**Fig. 1C**). These results suggest that *GAL1-CDC55* delays mitotic entry in a manner dependent both on Swe1 and Mih1.

We found that the toxicity of *GAL1-CDC55* is much stronger in *zds1*Δ *zds2*Δ cells compared with wild-type cells. This is consistent with the idea that *Zds1/Zds2* can act as in vivo inhibitors of Cdc55 activity or function (**Fig. 2A**). We also found that overexpression of *ZDS1* suppressed the toxicity of *GAL1-CDC55* (**Fig. 2B**). Importantly, overexpression of *ZDS1* lacking the Cdc55-binding domain (*ZDS1ΔC800*)[16] failed to suppress the toxic effects of *GAL1-CDC55* (**Fig. 2B**), confirming that Zds1 inhibits abnormal Cdc55 activity by direct interaction. These results suggest that Zds1/Zds2-dependent retention of Cdc55 in the cytoplasm is critical when Cdc55 is overexpressed.

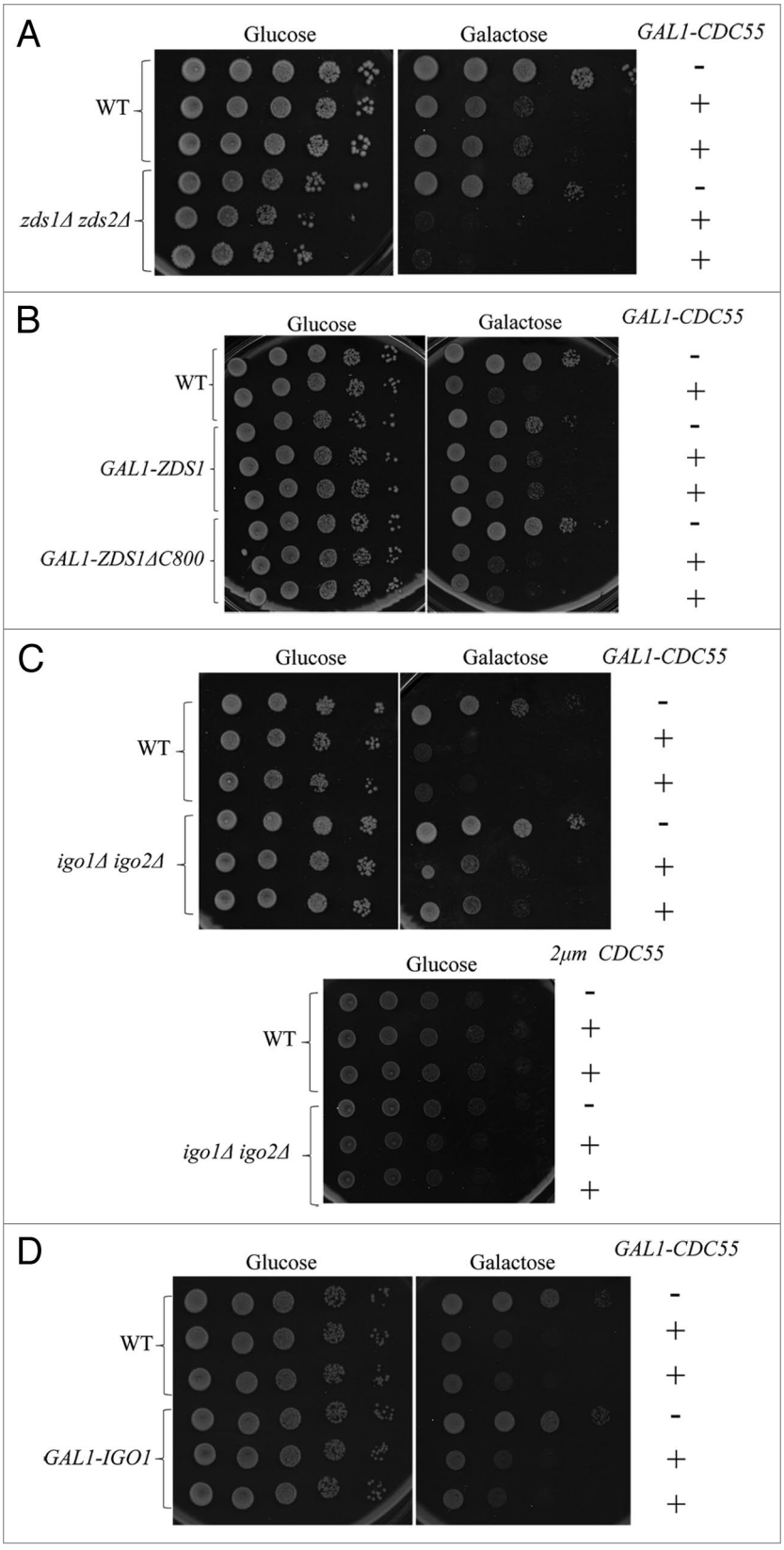

Figure 2. *ZDS1/2* and *IGO1/2* counteract with *CDC55* overexpression Each saturated culture was serially (10x) diluted and spotted on glucose (*CDC55* off) or galactose (*CDC55* on). Images were taken after 3 d at room temperature. (**A**) *GAL1-CDC55* is more toxic in *zds1*Δ *zds2*Δ cells. (**B**) Overexpression of *ZDS1* rescued the toxicity caused by *GAL1-CDC55*. Note that overexpression of *ZDS1ΔC800* lacking Cdc55-binding domain failed to rescue the toxicity of *GAL1-CDC55*. (**C**) Top: *igo1*Δ *igo2*Δ cells grew better than wild-type cells in galactose media. *igo1*Δ *igo2*Δ cells expressing *GAL1-CDC55* grew better than wild-type cells with *GAL1-CDC55*. However, the better growth in the galactose media was associated with *igo1*Δ *igo2*Δ cells, not with *GAL1-CDC55*. Bottom: Overexpression of Cdc55 under its endogenous promoter with a multicopy plasmid is slightly more toxic to *igo1*Δ *igo2*Δ cells compared with the wild-type cells. (**D**) Overexpression of *IGO1* did not rescue the toxicity caused by *GAL1-CDC55*.

In contrast to our prediction, *GAL1-CDC55* was less toxic to *igo1*Δ *igo2*Δ cells compared with the wild-type cells in BY4741 strain background (**Fig. 2C**). Reduced toxicity of *GAL1-CDC55* in *igo1*Δ, *igo2*Δ, and *rim15*Δ cells has also been previously reported in w303 background.[27] In addition, overexpression of *IGO1* failed to rescue the toxicity of *GAL1-CDC55* (**Fig. 2D**). These results are inconsistent with our hypothesis that Igo1/Igo2 are inhibitors of PP2A-Cdc55.

Because we noticed that *igo1*Δ *igo2*Δ cells grow much better than wild-type cells in galactose media, we re-evaluated the effect of *CDC55* overexpression in glucose media using a multicopy vector containing *CDC55* under the control of its endogenous promoter. *CDC55* expressed from a multicopy plasmid was slightly more toxic to *igo1*Δ *igo2*Δ cells compared with wild-type cells (**Fig. 2C**, bottom). We do not know why *igo1*Δ *igo2*Δ cells can grow better than wild-type cells in galactose, but it is interesting to note that *cdc55*Δ grows poorly in the galactose media (data not shown). The opposite phenotype of *igo1*Δ *igo2*Δ cells and *cdc55*Δ suggests that regulation of Cdc55 by Igo1/Igo2 is important for cell growth in different carbon sources. The reduced toxicity of *GAL-CDC55* in *igo1*Δ *igo2*Δ cells was due to different carbon source in the media, thus we concluded that Igo1/Igo2 are serving as inhibitors of Cdc55 function in vivo.

IGO1/IGO2 have minor roles in mitotic entry and function in parallel with *ZDS1/ZDS2*

To gain insights into the role and relative contributions of Igo1/Igo2 in PP2A–Cdc55 regulation during the cell cycle, we re-evaluated the phenotype of *igo1*Δ *igo2*Δ cells in comparison to *zds1*Δ *zds2*Δ cells in our strain background (BY4741). *zds1*Δ *zds2*Δ cells are known to exhibit abnormally elongated cell morphology and show cold-sensitive growth defects.[5,8,16,20] These phenotypes are associated with mitotic entry defects, because both abnormal morphology and cold sensitivity of *zds1*Δ *zds2*Δ cells are rescued either by deletion of *SWE1*[16] or

by introduction of *CDC28Y19F* mutation.[5] Moreover, we proposed that these phenotypes are due to an abnormal presence of Cdc55 in the nucleus. In our strain background (BY4741), *igo1*Δ *igo2*Δ cells did not exhibit any obvious morphological defects (**Fig. 3B**) or cold sensitivity (**Fig. 3A**) in contrast to *zds1*Δ *zds2*Δ cells.[16] However, in agreement with the study using w303 background,[27] *igo1*Δ *igo2*Δ cells caused synthetic morphological and growth defects when combined with the *zds1*Δ *zds2*Δ in BY4741 background (**Fig. 3A and B**).

Because Rim15 is known to phosphorylate Igo1/Igo2 to promote their binding to PP2A-Cdc55,[26,27] we also tested the genetic interaction between *RIM15* and *ZDS1*. Deletion of *RIM15* did not give any obvious morphological defects in w303 background; however, *rim15*Δ *zds1*Δ cells exhibited a greater number of abnormally elongated buds (27%) compared with *zds1*Δ cells (10%) (**Fig. 3C**). These genetic interactions suggest that Rim15-Igo1/Igo2 function in a parallel pathway to Zds1/Zds2 for mitotic entry. To understand the relationship between Igo1/Igo2 and Zds1/Zds2, we further examined if Zds1/Zds2 can substitute for Igo1/Igo2 or Rim15. We used w303 background for this analysis, because both *igo1*Δ *igo2*Δ and *rim15*Δ cells exhibit stronger cold-sensitive growth phenotype than in our strain background (BY4741).[27] We found that the cold-sensitive growth defects of *igo1*Δ, *igo2*Δ, and *rim15*Δ cells were efficiently suppressed by overexpression of *ZDS1* (**Fig. 4A**). In contrast, overexpression of *IGO2* failed to rescue the cold-sensitive growth defects of *zds1*Δ *zds2*Δ cells (**Fig. 4B**). These results suggest that although Igo1/Igo2 are potent inhibitors of PP2A-Cdc55 in vitro, *IGO2* has limited or minor effect in vivo compared with *ZDS1/ZDS2*. We also found that cold-sensitive growth defects of *rim15*Δ cells were rescued by overexpression of *IGO2*, consistent with *IGO2* being an important downstream target of the Rim15 kinase.[26-28] The partial rescue of the cold sensitivity of *rim15*Δ cells by overexpression of *IGO2* suggests that phosphorylation of Igo2 by Rim15 is essential for its full activation, or, alternatively, Rim15 may have other downstream targets than Igo1/Igo2.

Igo1/Igo2 can inhibit PP2A–Cdc55 activity in the SAC arrest

Because Cdc55 is essential for mitotic arrest by SAC activation through inhibition of APC-Cdc20,[5,11,13] we tested whether Igo1/Igo2 are involved in the SAC and show genetic interaction with *CDC20*.

To test if Igo1/Igo2 have roles in the SAC, we first examined the effects of spindle damaging drugs. Unlike *cdc55*Δ, *igo1*Δ

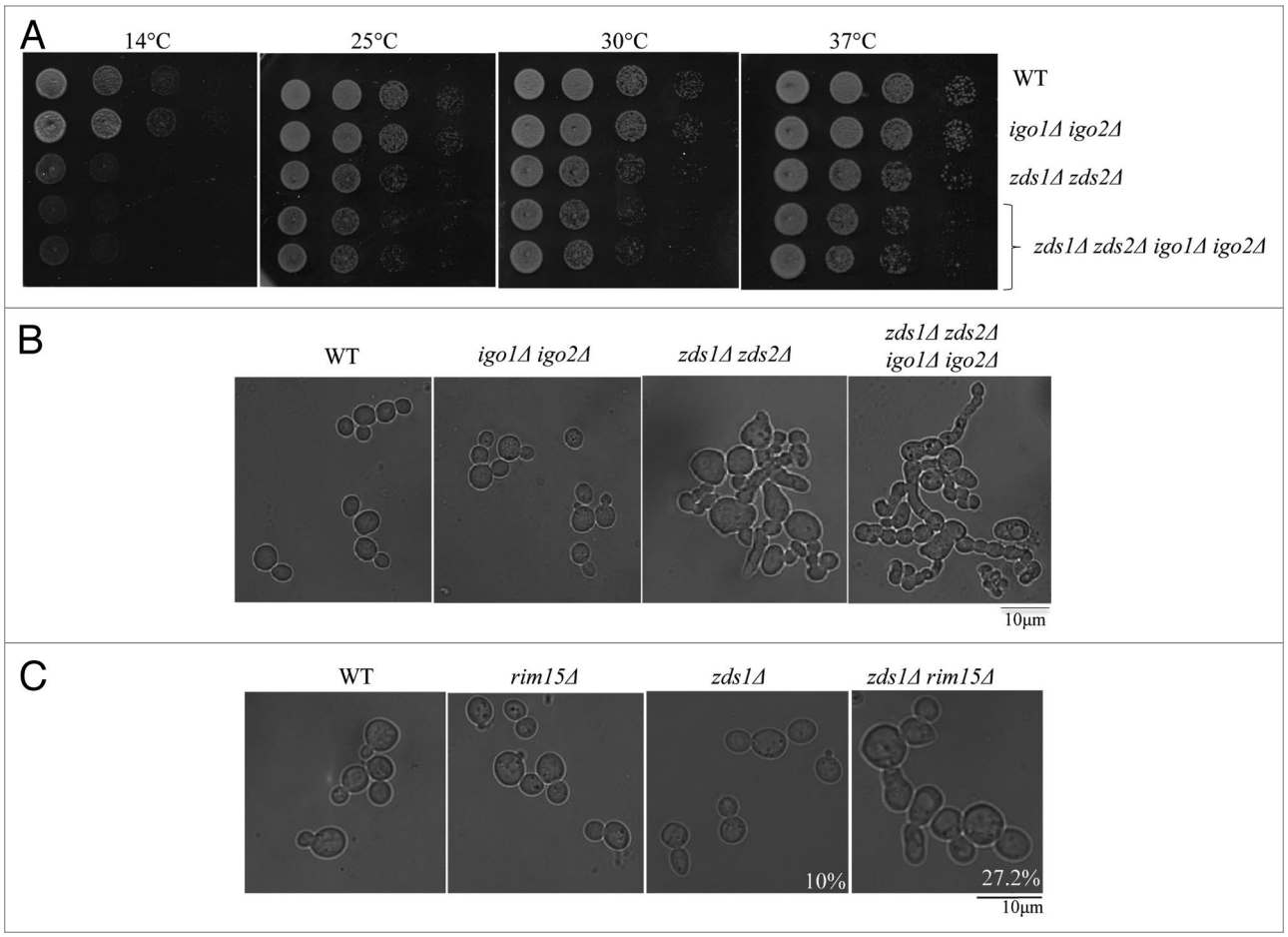

Figure 3. Deletion of *IGO1* and *IGO2* has synthetic growth defects with deletion of *ZDS1* and *ZDS2*. (**A**) Serial dilutions of the indicated strains were spotted on YPD at different temperatures (**B**) and (**C**) Morphology of the cells grown in YPD at 24 °C. The strains used are in BY4741 background (**B**) or in w303 (**C**). The percentage of elongated buds is 10% in *zds1*Δ cells (n = 100) and 27.2% in *zds1*Δ *rim15*Δ (n = 110).

*igo2*Δ cells were not highly sensitive to microtubule-depolymerizing drug benomyl (**Fig. 5A**). SAC-defective mutants fail to maintain mitotic arrest in the presence of microtubule-depolymerizing drug nocodazole and proceed through the next round of the cell cycle, resulting in multi-budded phenotype.[29,30] We confirmed that *igo1*Δ *igo2*Δ cells were tightly arrested in G_2/M phase in the presence of nocodazole (**Fig. 5B**), using *cdc55*Δ *swe1*Δ as a SAC-defective positive control. Thus, *igo1*Δ *igo2*Δ cells are not defective in the SAC similar to *zds1*Δ *zds2*Δ cells.[13] Because PP2A-Cdc55 is essential for the SAC, and because *GAL1-ZDS1* causes SAC defects in a manner dependent on its Cdc55-binding domain (801–915 a.a.) (ref. 13; **Fig. 5C**), we tested whether overexpression of *IGO1* or *IGO2* can impair the SAC, and found that both *GAL1-IGO1* and *GAL1-IGO2* caused benomyl sensitivity (**Fig. 5C**). The rebudding assay confirmed that both *GAL1-IGO1* and *GAL1-IGO2* cells showed SAC defects (**Fig. 5D**), although the effect was milder compared with *GAL1-ZDS1* cells.

The target of PP2A-Cdc55 in the SAC is APC-Cdc20, whose activity is essential for anaphase onset. We have previously demonstrated that deletion of *cdc55* or overexpression of *ZDS1* can rescue the temperature-sensitive growth defects of *cdc20–3*.[13] Although it is less efficient compared with that of *ZDS1*, overexpression of *IGO2* partially rescued the growth defects of *cdc20-3* cells (**Fig. 5E**), suggesting that Igo2 can inhibit PP2A–Cdc55 activity toward APC-Cdc20.

We also found that overexpression of *IGO2* and *ZDS1* partially rescues the temperature-sensitive growth defects of *tom1*Δ cells (**Fig. 5F**). *TOM1* encodes an HECT type E3 ubiquitin ligase, and *tom1*Δ cells arrest in early mitosis at high temperature.[13] Because removal of nuclear Cdc55 rescues the temperature-sensitive growth defect of *tom1*Δ,[13] it is highly likely that overexpression of *IGO2* and *ZDS1* rescues *tom1*Δ by inhibition of nuclear PP2A-Cdc55.

These data suggest that, when overexpressed, Igo2 also can inhibit PP2A–Cdc55 function in early mitosis, and as a consequence, APC-Cdc20 is activated, leading to impairment in the SAC.

Igo1/Igo2 play no detectable role in mitotic exit

It is known that nuclear PP2A-Cdc55 prevents mitotic exit by inhibition of Cdc14.[12,14,16,18] Thus, Cdc55 needs to be inactivated or removed from the nucleus for timely mitotic exit.[14] Because Cdc55 accumulates in the nucleus throughout mitosis, *zds1*Δ *zds2*Δ cells are defective in Cdc14 activation and exhibit synthetic lethality with *lte1*Δ, which encodes an activator of mitotic exit.[16] In contrast to *zds1*Δ *zds2*Δ, *igo1*Δ *igo2*Δ showed no synthetic growth defects with *lte1*Δ even at semi-permissive temperatures (**Fig. 6A**). This result suggests that Igo1/Igo2 are not required for timely mitotic exit and are not involved in inhibition of nuclear Cdc55 in anaphase.

Although *lte1*Δ is non-essential at the room temperature, it is partially defective in mitotic exit and shows synthetic lethality with *spo12*Δ, another mutant partially defective in mitotic exit.[32] The synthetic lethality of *spo12*Δ *lte1*Δ is rescued by additional deletion of *CDC55*.[12] We found that overexpression of *ZDS1* efficiently rescued the lethality of *spo12*Δ*::GAL1-SPO12 lte1*Δ in glucose medium (**Fig. 6B**); however, overexpression of *IGO2* failed to suppress the *spo12*Δ *lte1*Δ lethality. Thus, we conclude that *IGO1/IGO2* do not play any detectable roles in mitotic exit.

Igo1-GFP is enriched in the nucleus

To better understand the function of Igo1, we examined the subcellular localization. In a clear contrast to Zds1-GFP, which localizes to the bud cortex, to the bud neck, and in the cytoplasm, Igo1 was enriched in the nucleus. (**Fig. 7**). This is consistent with the previous work[28] that reported that Igo1/Igo2 proteins localize to both to the nucleus and cytoplasm. Nuclear accumulation of Igo1 is independent of Cdc55 binding, as nuclear localization of Igo1-GFP was unaffected in *cdc55*Δ cells (data not shown). This suggests that Igo1/Igo2 nuclear localization is independent of Cdc55.

Nuclear enrichment of Igo1 is consistent with the idea that Igo1 inhibits PP2A–Cdc55 activity in the nucleus. Little overlap of subcellular localization may explain the difference between Igo1/Igo2 and Zds1/Zds2.

Both Zds1/Zds2 and Igo1/Igo2 control nuclear accumulation of Cdc55, but only Zds1/Zds2 are essential for cortical localization of Cdc55

Lastly we examined if Igo1/Igo2 regulates subcellular localization of Cdc55. We used w303 background for this analysis, because *igo1*Δ *igo2*Δ cells exhibit a more severe phenotype than BY4741, as previously mentioned. In the wild-type cells, Cdc55-GFP is localized both in the nucleus and cytoplasm. In addition, Cdc55-GFP localized to the bud cortex in small to medium budded cells and at the bud neck in late mitotic cells[16,33] as in

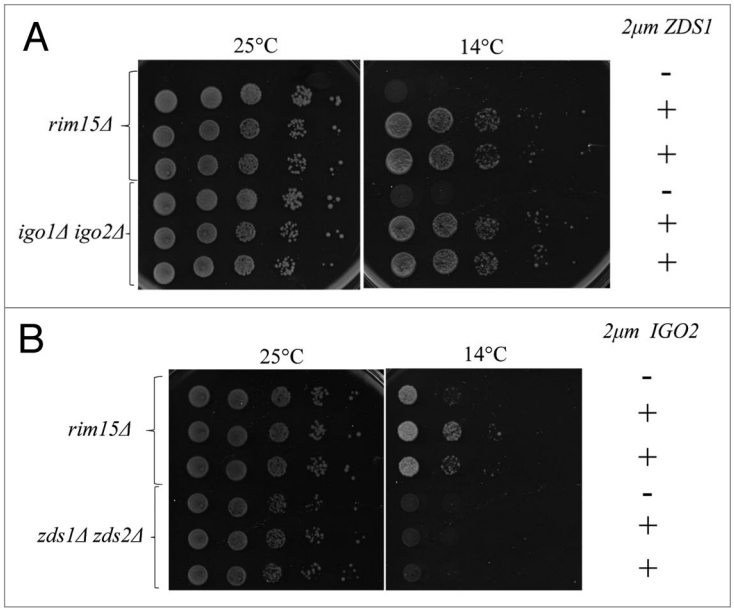

Figure 4. Genetic interactions between *ZDS1/ZDS2* and *RIM15-IGO1/IGO2* (**A**) Overexpression of *ZDS1* rescued the cold-sensitive growth defects of *rim15*Δ and *igo1*Δ *igo2*Δ cells. Serial dilutions of the indicated strains were spotted on SC-URA medium at 14 °C and 24 °C. 2μ*m ZDS1* is a high copy number plasmid expressing *ZDS1*. (**B**) Overexpression of *IGO2* rescued the cold-sensitive growth defect of *rim15*Δ but not *zds1*Δ *zds2*Δ cells. Serial dilutions of the indicated strains were spotted on SC-URA medium at 14 °C and 24 °C. 2μ*m IGO2* is a high copy number plasmid expressing *IGO2*.

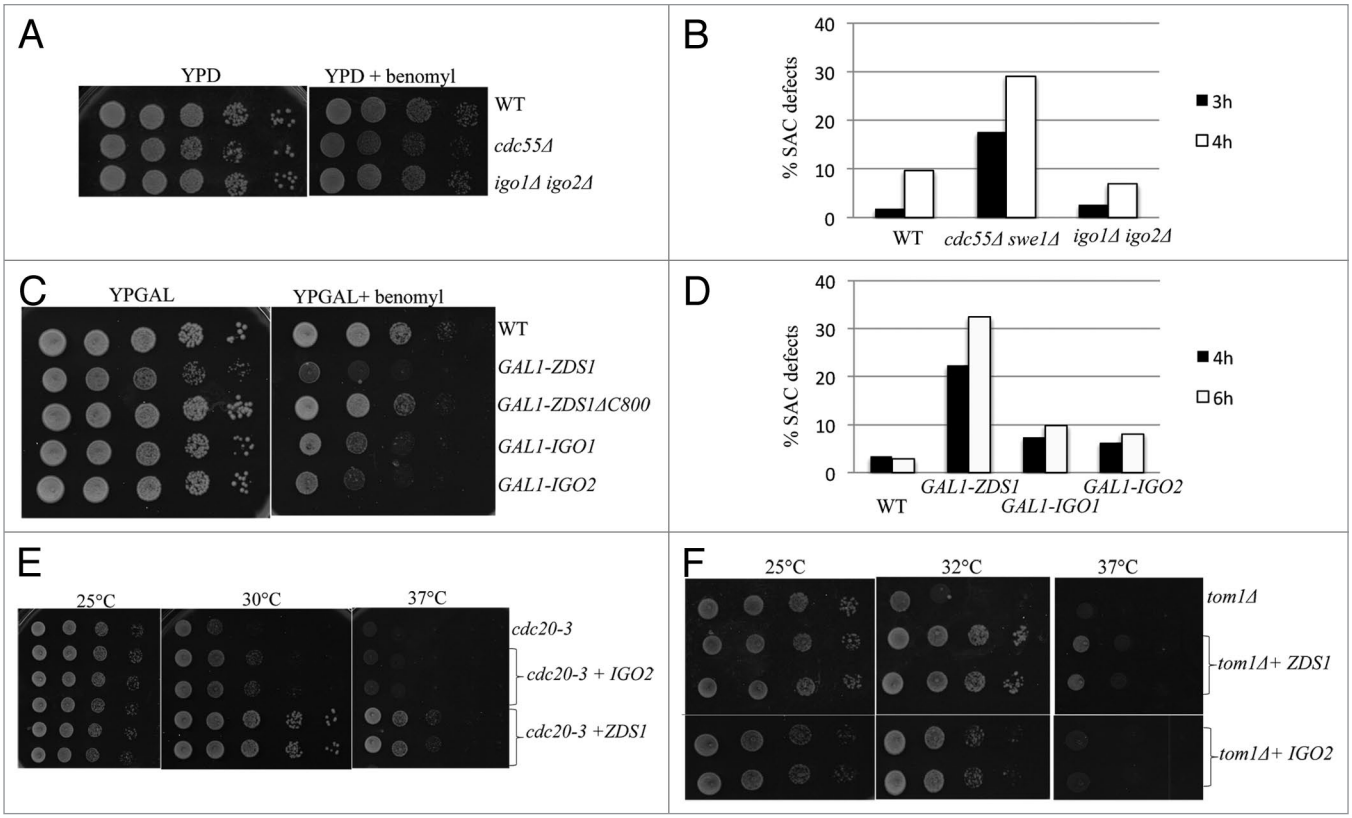

Figure 5. Overexpression of *IGO2* rescues the growth defects of *cdc20–3, and tom1* cells and causes a mild SAC defects. (**A**) *igo1*Δ *igo2*Δ cells were not sensitive to benomyl (20 μg/ml). *cdc55*Δ cells were used as a control. (**B**) *igo1*Δ *igo2*Δ cells were not defective in the SAC. Each strain was grown in YPD medium containing 15 μg/ml of nocodazole. After 3 and 4 h, rebudded cells (indicator of the SAC defects) were counted. At least 150 cells for each strain were scored. (**C**) Overexpression of *IGO1* or *IGO2* caused mild benomyl sensitivity. Serial dilutions of the strains with the indicated genotypes were spotted on YP Galactose with or without benomyl (12.5 μg/ml) at 24 °C. (**D**) Overexpression of *IGO1* or *IGO2* caused mild SAC defects. Each strain was grown in YP Galactose medium containing 15 μg/ml nocodazole. After 4 and 6 h, rebudded cells were counted. At least 100 cells for each strain were scored. (**E**) Overexpression of *IGO2* can weakly rescue the temperature-sensitive growth defects of *cdc20–3* cells. Serial dilutions of the strains with the indicated genotypes were spotted on SC-URA at the indicated temperatures. (**F**) Overexpression of *IGO2* can weakly rescue the temperature-sensitive growth defects of *tom1*Δ cells. Serial dilutions of the strains with the indicated genotypes were spotted on SC-URA at the indicated temperatures.

BY4741. In *zds1*Δ *zds2*Δ cells, consistent with our previous report,[16] both the cortical and bud neck localization of Cdc55-GFP were completely lost, and Cdc55-GFP accumulated in the nucleus (**Fig. 8A**). In *igo1*Δ *igo2*Δ cells, Cdc55 localization to the bud cortex and bud neck was unaffected; however, there was an increase in nuclear Cdc55–GFP signal (**Fig. 8A**). This observation is consistent with the previous report, which used the immunostaining of Cdc55 tagged with HA epitope.[27] Thus, Igo1/Igo2 can partially affect nucleocytoplasmic distribution of Cdc55.

Nuclear accumulation of Cdc55-GFP in *igo1*Δ *igo2*Δ and in *zds1*Δ *zds2*Δ suggests that they may share similar functions in retaining or in exporting Cdc55 to the cytoplasm. We attempted to compare the nuclear localization of Cdc55-GFP in *zds1*Δ *zds2*Δ *igo1*Δ *igo2*Δ and in *zds1*Δ *zds2*Δ, but it was technically challenging because these cells suffered from highly abnormal morphology, and the nuclear signal of Cdc55-GFP is already very strong in *zds1 zds2*Δ cells.

Zds1/Zds2 are possible positive regulators of PP2A-Cdc55 in the cytoplasm

Essential requirement of Zds1/Zds2 in the cortical Cdc55 localization suggests that Zds1/Zds2 are not simply inhibiting nuclear Cdc55, but also do have distinct, positive roles in Cdc55 functions. Consistent with this idea, we found that overexpression of truncated Zds1 lacking the first 400 amino acids (*GAL1-ZDS1*Δ*N400*) caused toxic effects on cell growth (**Fig. 8B**).

The toxic effects of *GAL1-ZDS1*Δ*N400* was due to abnormal PP2A–Cdc55 activation, because the toxicity was cancelled in *cdc55*Δ (**Fig. 8B**; note that *cdc55*Δ grew poorly in galactose media). Furthermore, the toxicity of *GAL1-ZDS1*Δ*N400* was cancelled by the removal of its Cdc55-binding domain (*GAL1-ZDS1*Δ*N400*Δ*C800*; **Fig. 8B**). These results suggest that overexpression of active Zds1 cause hyperactivation of PP2A-Cdc55 that leads to growth defects. (**Fig. 8B**)

Discussion

Physiological function of ENSA-family proteins in budding yeast cell cycle

Our genetic data are in agreement with the idea that Igo1/Igo2 can function as inhibitors of PP2A-Cdc55. Although *igo1*Δ *igo2*Δ cells don't have obvious defects in mitotic progression,

they showed synthetic growth defects and prolonged G_2 phase in combination with deletion of *ZDS1* and *ZDS2*. Thus Igo1/Igo2 is regulating Cdc55-PP2A during mitotic entry in parallel with Zds1 and Zds2. A role of Igo1 and Igo2 in mitotic entry is consistent with the recent finding in a different strain background,[27] although the phenotype of *igo1*Δ *igo2*Δ cells in our background was much weaker. The idea that Igo1/Igo2 and Zds1/Zds2 are sharing overlapping functions is also supported by our finding that the cold-sensitive growth defects of *igo1*Δ *igo2*Δ cells and *rim15*Δ (in w303) were rescued by overexpression of *ZDS1*. Other than in mitotic entry, we were not able to find detectable mitotic defects in *igo1*Δ *igo2*Δ. For example, *igo1*Δ *igo2*Δ cells are competent in the SAC arrest and in mitotic exit. These results suggest that Igo1/Igo2 are active toward Cdc55 only at the time of mitotic entry. Consistent with this idea, it has been reported that in vivo complex formation of Igo1 and Cdc55 is maximum at G_2/M phase.[27] Thus, compared with Zds1/Zds2, physiological contribution of Igo1/Igo2 in Cdc55 regulation during mitosis is either functionally or temporally limited.

Overexpressions of *IGO1* and *IGO2* also suggest that these proteins can inhibit nuclear functions of PP2A-Cdc55. We found that overexpression of *IGO1* or *IGO2* causes weak SAC defects and suppresses the temperature-sensitive growth defects of *cdc20–3* and *tom1*Δ cells. These genetic interactions are similar to *CDC55* deletion or overexpression of *ZDS1*. However, overexpression of *IGO1* or *IGO2* is not as effective as *ZDS1* overexpression. Furthermore, overexpression of *IGO1* or *IGO2* failed to promote mitotic exit in *GAL1-spo12 lte1*Δ cells and failed to rescue the cold-sensitive growth defects of *zds1*Δ *zds2*Δ cells.

One possible explanation why overexpression of *IGO1* and *IGO2* has only a weak effect on Cdc55 function is because Igo1/Igo2 require Rim15-dependent phosphorylation for their full activity. It is of interest to test co-expression of Rim15 and Igo1/Igo2 or to overexpress a phosphomimetic mutant form of Igo1/Igo2.

Above all, our genetic data suggests that Igo1/Igo2 function as inhibitors of Cdc55 in vivo, but their effect is limited compared with that of Zds1 and Zds2.

How do Igo1/Igo2 inhibit Cdc55?

We confirmed that Igo1 is enriched in the nucleus but does not localize to the bud cortex or the bud neck. Given that Igo1/Igo2 can directly inhibit PP2A–Cdc55 phosphatase activity in vitro,[26,27] it is highly likely that Igo1/Igo2 are mainly inhibiting nuclear PP2A-Cdc55 by direct binding. It is also possible that Igo1/Igo2 have additional regulatory function in PP2A-Cdc55. In *igo1*Δ *igo2*Δ cells, we found that cortical localization of Cdc55 was unaffected, but Cdc55 was accumulated in the nucleus. A similar observation was reported in the recent paper,[26] suggesting that Igo1/Igo2 are not only inhibiting Cdc55 activity, but also regulating Cdc55 localization.

It is interesting that the localization pattern of Igo1/Igo2 is largely different from that of Zds1/Zds2, which localize to the bud cortex and the neck and are excluded from the nucleus.[16,34] Distinct localization pattern predicts that the molecular mechanisms by which Igo1/Igo2 and Zds1/Zds2 regulate Cdc55 are different. If these proteins are regulating nucleocytoplasmic distribution of Cdc55, we speculate that the nuclear proteins Igo1/Igo2 can promote the export of Cdc55 from the nucleus,

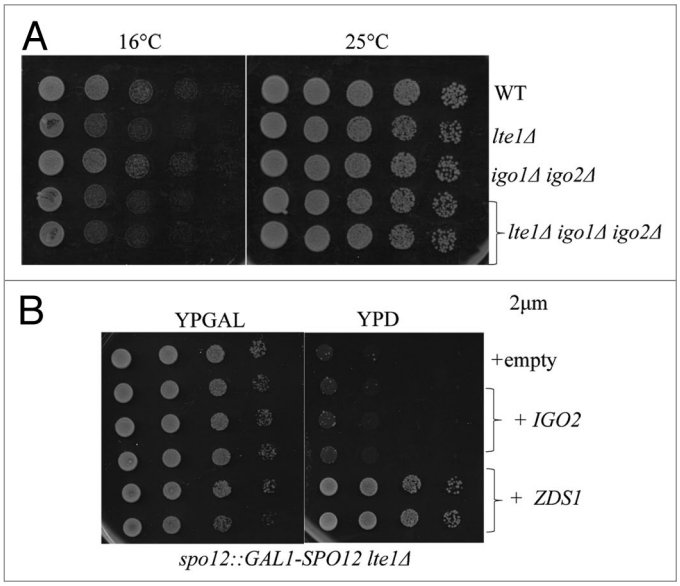

Figure 6. Igo1/Igo2 have no obvious defects in mitotic exit. (**A**) The deletions of *IGO1* and *IGO2* were not synthetic lethal with *lte1*Δ. Serial dilutions of the indicated strains were spotted on YPD medium at 14 °C and 24 °C. (**B**) Overexpression of *ZDS1* but not *IGO2* can rescue synthetic lethality of *spo12*Δ *lte1*Δ. Serial dilutions of the *spo12* Δ::*GAL1-SPO12 lte1* Δ strain whose viability is dependent on galactose. Indicated strains overexpressing either *IGO2* or *ZDS1* were spotted on SC-URA medium containing glucose or galactose medium at 24 °C.

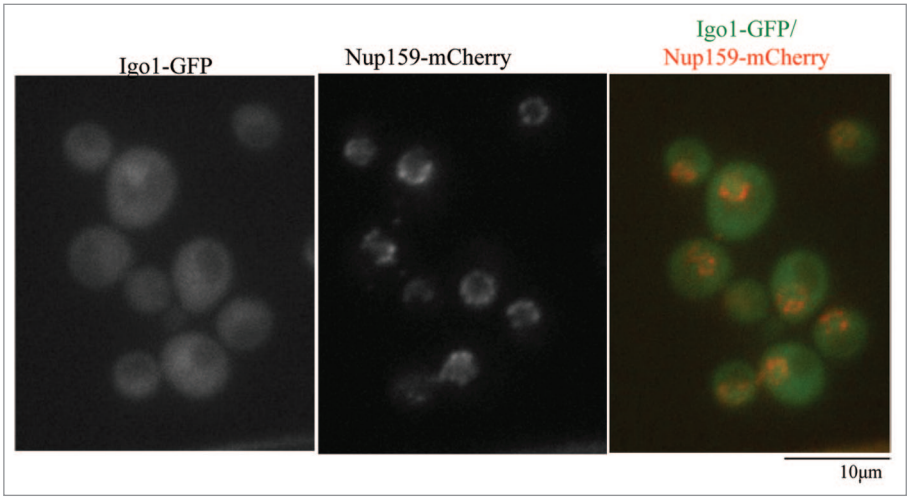

Figure 7. Igo1-GFP is enriched in the nucleus and is not localized at the bud cortex or at the bud neck. (**A**) Igo1-GFP expressed at the endogenous locus was visualized together with a nuclear envelope marker Nup159-mCherry. Merged image is shown in the right panel.

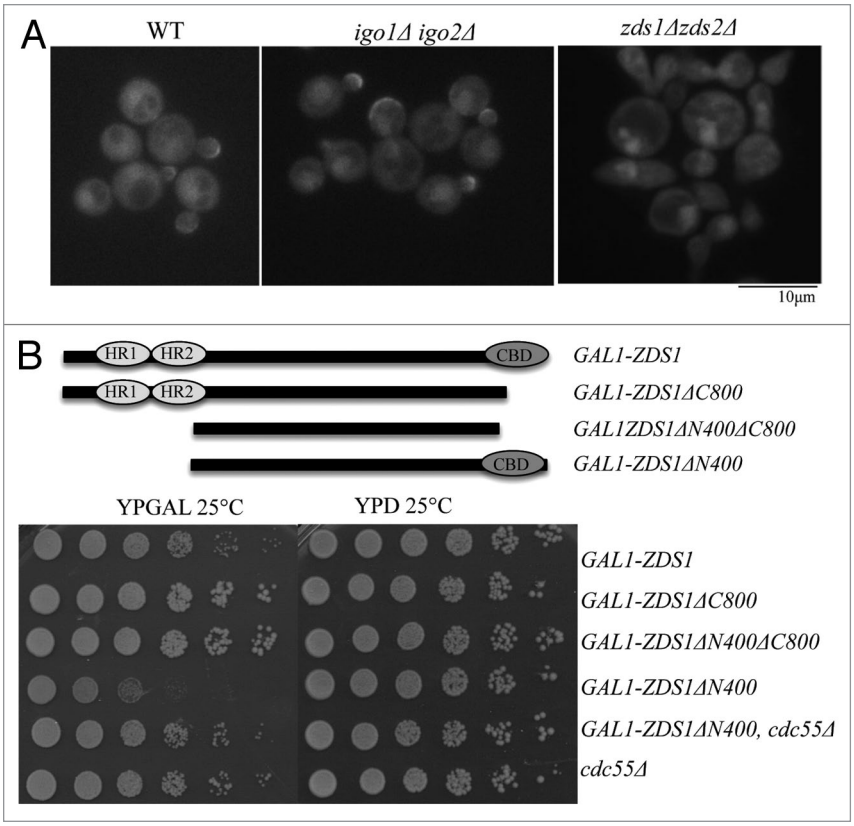

Figure 8. *ZDS1* is essential for Cdc55 localization to the sites of cell polarity and can induce Cdc55-dependent toxicity. (**A**) Localization of Cdc55-GFP in *igo1*Δ *igo2*Δ and in *zds1*Δ *zds2*Δ. Note that cortical localization of Cdc55 was unaffected in *igo1*Δ *igo2*Δ but was completely lost in *zds1*Δ *zds2*Δ. (**B**) Top: Domain structure of the Zds1: HR, homology region; CBD, Cdc55-binding domain. Truncated constructs are as follows: full-length Zds1 (1–915 a.a.), Zds1Δ*C800* (1–800 a.a.), Zds1Δ*N400ΔC800* (401–800 a.a.), Zds1Δ*N400* (401–915 a.a.). All the constructs were expressed under the control of the strong *GAL1* promoter. Bottom: Each saturated culture were serially (5×) diluted and spotted on glucose (*ZDS1* off) or galactose (*ZDS1* on).

while cytoplasmic proteins Zds1/Zds2 retain Cdc55 in the cytoplasm by sequestration. Further analysis is required to fully understand the molecular mechanisms by which these Cdc55 interacting proteins regulate PP2A–Cdc55 functions, but we propose that although they share overlapping functions, Zds1/Zds2 and Igo1/Igo2 are controlling PP2A-Cdc55 in a distinct manner.

Zds1 is not necessarily an inhibitor of PP2A-Cdc55

We have previously proposed that Zds1/Zds2 inhibit nuclear Cdc55 functions by sequestrating Cdc55 in the cytoplasm.[16] This proposal is based on the observation that Cdc55 accumulates in the nucleus in *zds1*Δ *zds2*Δ cells, and that overexpression of *ZDS1* excludes Cdc55 from the nucleus. In our model, Zds1/Zds2 are not necessarily inhibitors of Cdc55; rather, Zds1/Zds2 should behave as activators of cytoplasmic Cdc55 functions.

Consistent with this model, we found that overexpression of *GAL1-ZDS1ΔN400* causes toxicity to the cells in a manner dependent on Cdc55 interaction. Since PP2A-Cdc55 is localized to the bud cortex and to the bud neck in a manner exclusively dependent on Zds1/Zds2,[16] cell polarity regulators are attractive candidate to be the targets of PP2A–Cdc55–Zds1/Zds2 complex in the cytoplasm. In agreement with this idea, Cdc55 and Zds1/Zds2 are known to regulate polarized growth and cell wall biogenesis.[35,36] Identification of the molecular targets and functions of Cdc55 in the cytoplasm requires further investigation.

Why Zds1-family proteins are unique to fungal cells?

One interesting question is why yeasts, but not animal cells, require Zds1-family proteins in addition to ENSA-family proteins to control PP2A-Cdc55. Because Zds1 homologs are only found in fungal species, they are likely dedicated for fungal cell-specific regulations or functions of PP2A-Cdc55. One attractive idea is that Zds1-dependent exclusion of PP2A-Cdc55 during mitosis is critical only in fungal cells whose nuclear envelope does not break down. If exclusion of Cdc55 from the nucleus is the important function of Zds1-family proteins, they are not necessary in animal cells, because there is no nuclear envelope during mitosis.

Another, but not mutually exclusive, idea is that Zds1-family proteins are used for fungi-specific functions of PP2A. One attractive candidate of fungi-specific target of PP2A is cell wall biogenesis machinery because PP2A-Cdc55 and Zds1/Zds2 are known to promote cell wall assembly.[36] Further investigation is required to fully understand the molecular mechanisms by which Zds1-family proteins and ENSA-family proteins regulate PP2A functions in vivo, but our analysis revealed that these PP2A regulators are functioning in a totally distinct fashion in the cell.

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*). Standard yeast genetic was used to generate the strains. Yeast strains are listed in the **Table 1**. PY3295 and SY strains were gifts from D Pellman (Dana-Farber Cancer Institute). Gene deletions or modifications were performed with PCR-mediated one-step gene replacement using pFA6a vectors provided by J Pringle (Stanford University)[38] and confirmed by PCR. The *ZDS1* and *IGO2* plasmids were obtained from the National Bio-Resource Project (NBRP). The *GAL1-CDC55* plasmid was a gift from Y Kikuchi (Gakushuin University). Nocodazole was used at 15 μg/ml. Benomyl was used at 12.5 μg/ml or 20 μg/ml as indicated in the legends. For galactose induction, 2% of galactose was added to the medium.

Table 1. Yeast strains used

Strain	Mat	Genotype	Background	Source
PY 3295	a	*leu2Δ0 his3Δ1 met15Δ0 ura3Δ0*	BY4741	Rossio and Yoshida 2011
SY 1390	a	*swe1::LEU2*	BY4741	Rossio and Yoshida 2011
SY 069	a	*mih1::kanMX6*	BY4741	This study
MH 35	a	WT + [*URA3-GAL1-CDC55*, 2μm]	BY4741	This study
MH 44	a	SY1390+ [*URA3*, 2μm]	BY4741	This study
MH 45	a	SY1390 + [*URA3-GAL1-CDC55*, 2μm]	BY4741	This study
MH 46	a	SY069+ [*URA3*, 2μm]	BY4741	This study
MH 47	a	SY069 + [*URA3-GAL1-CDC55*, 2μm]	BY4741	This study
VR 1291	a	*igo1::kanMX6, igo2::kanMX6*	BY4741	Gift from N. Talarek
VR 1295	a	VR 1291+ [*URA3*, 2μm]	BY4741	This study
VR 1303	a	VR 1291+ [*URA3-GAL1-CDC55*, 2μm]	BY4741	This study
VR 36		*zds1Δ::kanMX6 zds2Δ::His3MX6*	BY4741	Rossio and Yoshida 2011
VR 146	a	*zds1Δ::kanMX6-GAL1–3xHA-ZDS1-GFP-His3MX6*	BY4741	Rossio et al., 2013
VR148	a	*zds1Δ::His3MX6-GAL1–3xHA-zds1Δc800-GFP-kanMX6*	BY4741	Rossio et al., 2013
VR 200	a	*zds1Δ::His3MX6-GAL1–3xHA-ZDS1-GFP-kanMX6* + [*URA3*, 2μm]	BY4741	This study
VR 196	a	*zds1Δ::His3MX6-GAL1–3xHA-ZDS1-GFP-kanMX6* + [*URA3-GAL1-CDC55*, 2μm]	BY4741	This study
VR 202	a	*zds1Δ::His3MX6-GAL1–3xHA- zds1Δc800-GFP-kanMX6* + [*URA3*, 2μm]	BY4741	This study
VR 198	a	*zds1Δ::HIS3-GAL1–3xHA-zds1Δc800-GFP-kanMX6* + [*URA3-GAL1-CDC55*, 2μm]	BY4741	This study
VR 1541	a	*igo1Δ::His3MX6-GAL1-IGO1*	BY4741	This study
VR 1542	a	*igo2Δ::His3MX6-GAL1-IGO2*	BY4741	This study
VR 1980	a	*zds1Δ::kanMX6 zds2Δ::His3MX6 igo1Δ::natNT2 igo2Δ::hphR*	BY4741	This study
VR 1981	α	*zds1Δ::kanMX6 zds2Δ::His3MX igo1Δ::natNT2 igo2Δ::hphR*	BY4741	This study
VR 1293	a	*igo1Δ::natNT2 igo2Δ::hphR*	BY4741	Gift from N. Talarek
VR 1957	a	*rim15Δ::natNT2*	w303	Juanes et al., 2013
VR 1995	a	*zds1Δ::TRP1, CDC55-GFP::His3MX*	w303	This study
VR 1996	α	*zds1Δ::TRP1, rim15Δ::natNT2 CDC55-GFP::HIS3MX6*	w303	This study
VR 1994	α	*rim15Δ::natNT2 CDC55-GFP::His3MX6*	w303	This study
VR 1958	a	*igo1Δ::natNT2 igo2Δ::hphR*	w303	Juanes et al., 2013
VR 1959	a	*zds1Δ::TRP1 zds2Δ::hphR*	w303	Juanes et al., 2013
VR 81	a	*lte1Δ::kanMX6*	BY4741	Rossio and Yoshida 2011
VR 1334	α	*lte1Δ::kanMX6 igo1Δ::natNT2 igo2Δ::hphR*	BY4741	This study
VR 1335	a	*lte1Δ::kanMX6 igo1Δ::natNT2 igo2Δ::hphR*	BY4741	This study
VR 1474	α	*lte1Δ::kanMX6 spo12Δ::His3MX6-GAL1-SPO12*	BY4741	This study
VR 31	a	*cdc55Δ::kanMX6*	BY4741	Rossio and Yoshida 2011
VR 40	a	*cdc55Δ::kanMX6 swe1Δ::kanMX6*	BY4741	Rossio and Yoshida 2011
K8029	a	*cdc20–3*	w303	Gift from Kikuchi
VR 1556	a	*cdc20–3* + [*URA3*, 2μm]	w303	Rossio et al. 2013
VR 1557	a	*cdc20–3* + [*ZDS1-URA3*, 2μm]	w303	Rossio et al. 2013
VR 1761	a	*tom1–10::His3MX6*	w303	Sasaki et al., 2000

Table 1. Yeast strains used (continued)

VR 1966	a	*IGO1-GFP::His3MX6 NUP159-mCherry-kanMX6*	BY4741	This study
VR 1363	a	*CDC55-GFP::His3MX6*	w303	This study
VR 1972	a	*CDC55-GFP::His3MX6 zds1Δ::TRP1 zds2Δ::hphR*	w303	This study
VR 1969	a	*igo1Δ::natR igo2Δ::kanMX6 CDC55-GFP::His3MX6*	w303	This study
An70	a	*zds1Δ:: His3MX6-GAL1–3xHA-ZDS1-GFP-kanMX6*	BY4741	This study
An16	a	*zds1Δ::kanMX6-GAL1–3xHA-ZDS1 ΔN400-GFP-His3MX6*	BY4741	This study
An27	a	*zds1Δ:: kanMX6-GAL1–3xHA-ZDS1 ΔN400ΔC800-GFP-His3MX6*	BY4741	This study
An56	a	*zds1Δ::kanMX6-GAL1–3xHA-ZDS1 ΔN400-GFP-His3MX6 cdc55:: kanMX6*	BY4741	This study

Spindle assembly checkpoint (SAC) assay

Yeast cells arrest as large budded cells in mitosis in response to SAC activation; however, the SAC mutants continue through the cell cycle and form a new bud (re-budding). Logarithmically growing cells were treated with 15 μg/ml nocodazole, and the cells were fixed in 70% ethanol at each pf the indicated time points at room temperature. The samples were then washed twice in phosphate-buffered saline (PBS), vortexed vigorously, and examined by bright-field microscopy.

Fluorescence microscopy

Fluorescence images were acquired with a fluorescence microscope (Eclipse E600; Nikon) equipped with a charge-coupled device camera (DC350F; Andor) and 100× (NA1.45) or 60× (NA1.4) oil objectives. The images were captured using appropriate filters and were analyzed with NIS-Elements software (Nikon).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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