Different polarisome components play distinct roles in Slt2p-regulated cortical ER inheritance in Saccharomyces cerevisiae

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INTRODUCTION

In eukaryotic cells, the endoplasmic reticulum (ER) forms a contiguous structure of tubules and sheets, all interconnected to form an extended polygonal network (Voeltz et al., 2002; Du et al., 2004). This network has connections to the outer membrane of the nuclear envelope and spreads throughout the cell, all the way to the cortex, where it is tethered to the inner surface of the plasma membrane. As an essential, single-copy number organelle that cannot be generated de novo, the ER must be actively segregated into daughter cells during cell division. The process of ER inheritance has been most extensively analyzed in the yeast Saccharomyces cerevisiae. The ER in yeast forms a polygonal network of sheets and tubules that lies just beneath the plasma membrane (cortical ER [cER]), with several tubular connections through the cytoplasm to the nuclear envelope. Soon after bud emergence a new ER tubule emerges from the nuclear envelope. This segregation tubule becomes aligned along the mother–bud axis and actively extends into the daughter cell. It forms a stable contact at the tip of the bud but then quickly spreads along the cortex of the bud (Fehrenbacher et al., 2002; Estrada et al., 2003). Once the bud has grown to about one-third the diameter of the mother cell, the ER in the daughter cell is present predominantly in the form of a cortical network, very similar in morphology to that of the mother cell (Voeltz et al., 2002; Du et al., 2004).

Genetic analysis in yeast has identified a number of components of the ER inheritance machinery and defined three stages in the inheritance pathway. In strains lacking the nonessential type V myosin, Myo4p, most cells fail to form an ER segregation tubule from the nuclear envelope at the start of the cell cycle, and, when a tubule is observed, it fails to align along the mother–bud axis and extend into the daughter cell (Estrada et al., 2003). Mutations in several of the exocyst genes, including sec3Δ, block ER inheritance at a later stage (Wiederkehr et al., 2003; Reinke et al., 2004). In these mutants, a tubule forms from the nuclear envelope and extends into the daughter cell but fails to form a stable attachment to the bud tip and often recedes back into the mother cell. A somewhat different phenotype...
is seen in mutants lacking the serine/threonine phosphatase, Ptc1p (Du et al., 2006). In ptc1Δ cells an ER tubule extends into the daughter cell and becomes stably attached to the bud tip, yet fails to spread along the cortex of the bud. Ptc1p controls ER inheritance by downregulating the cell wall integrity (CWI) mitogen-activated protein kinase (MAPK) pathway: loss of Ptc1p results in increased activation of Slt2p, the final kinase of the CWI MAPK cascade, and deletion of Slt2 suppresses the ER inheritance defect of ptc1Δ cells (Du et al., 2006). Slt2p normally undergoes cell cycle–dependent activation (Zarzov et al., 1996), with the time of peak activation correlating with the time at which the initial ER tubule becomes anchored to the bud tip (Li et al., 2010). The failure to inactivate Slt2p by Ptc1p-dependent dephosphorylation arrests ER inheritance at this stage. Although most Slt2p is present in the nucleus, a pool is concentrated at the tip of small buds and the neck of large-budded cells. Localization of Slt2p to the bud tip or neck requires the function of Spa2p, a component of the polarisome complex (van Drogen and Peter, 2002). Either loss of Spa2p function or mutation of a sequence within Slt2p that is needed for its bud tip localization results in suppression of the ptc1Δ ER inheritance defect (Li et al., 2010). These findings indicate that it is the activation of a pool of Slt2p at the bud tip that serves to block the progression of ER from the bud tip to the bud cortex in ptc1Δ cells.

ER inheritance is also blocked in response to ER stress, such as during growth in the presence of the reducing agent dithiothreitol or the glycosylation inhibitor tunicamycin, and this response similarly requires the activation of Slt2p (Babour et al., 2010). The inhibition of ER inheritance in response to stress serves to delay the segregation of nonfunctional ER into the daughter cell until the misfolded proteins accumulated within this organelle can be refolded or degraded. Thus some of the same signaling components used to control the normal cell cycle–dependent timing of ER inheritance are also used to delay inheritance in response to stress.

Ptc1p has also been implicated in the inheritance of mitochondria (Roeder et al., 1998), vacuoles (Du et al., 2006), and peroxisomes (Jin et al., 2009). In the case of mitochondrial inheritance, as in ER inheritance, Ptc1p is needed to down-regulate Slt2p, as loss of Slt2p function suppresses the mitochondrial inheritance defect of ptc1Δ cells (Li et al., 2010). Of interest, loss of Spa2p does not suppress the ptc1Δ mitochondrial inheritance defect, suggesting that it is not the bud tip–associated pool of Slt2p that controls the inheritance of this organelle (Li et al., 2010).

The polarisome comprises a number of different components that associate with each other at sites of polarized cell growth, including Spa2p, Sph1p, Bni1p, Bud6p, Pea2p, Msb3p, and Msb4p. Spa2p serves as a scaffold for the other components (Sheu et al., 1998), and Sph1p is a Spa2p homologue (Roemer et al., 1998). The formin protein, Bni1p, catalyzes the assembly of filamentous actin cables, and its activity is promoted by Bud6p, an actin monomer–binding protein (Moseley et al., 2004). The function of Pea2p is not well defined. Msb3p and Msb4p are redundant proteins that serve as GTPase-activating proteins for the rab protein, Sec4p, although they have an additional function in the regulation of the polarity establishment GTPase, Cdc42p (Tcheperegine et al., 2005). Here we systematically explore the role of each of these components in the Slt2p–dependent regulation of ER inheritance.

RESULTS

Polarisome components are involved in Ptc1p-regulated cER inheritance

We previously showed that, in ptc1Δ mutant cells, it is the failure to inactivate the MAPK Slt2p that underlies the observed delay in the propagation of ER segregation tubules from their docking site at the bud tip to the cortex of the growing bud (Du et al., 2006). The pool of Slt2p that controls this process requires a component of the polarisome complex, Spa2p, for its retention at the bud tip (Li et al., 2010). In yeast the polarisome complex has seven identified components: Bni1p, Spa2p, Pea2p, Bud6p, Sph1p, Msb3p, and Msb4p. They are all localized to the tip of small buds and the neck of large-budded cells. We systematically examined polarisome deletions to determine whether they display genetic interactions with ptc1Δ. As shown in Supplemental Figure S1, deletion of each individual polarisome component gene had no effect on cell growth in a wild-type (wt) background but displayed varying synthetic growth phenotypes in a ptc1Δ background. The bni1Δ, spa2Δ, msb3Δ, msb4Δ, pea2Δ, spa2Δ, and spa2Δ, ptc1Δ—displayed no significant change in growth relative to the ptc1Δ single mutant. Because Msb3p and Msb4p are functionally redundant proteins, we also constructed a msb3Δ msb4Δ double-deletion strain, which grows somewhat more slowly than wt, and found that these mutations, together, were synthetically lethal in combination with ptc1Δ. The varying synthetic interactions suggest that different polarisome components might play distinct roles in Ptc1p-regulated cellular processes.

We next determined whether polarisome mutations, alone or in combination with ptc1Δ, have any effects on ER inheritance. In wt cells, ER tubules move into newly forming buds along the mother–bud axis but then rapidly disperse to form an evenly distributed cortical network in small buds. Therefore, at any one time, only a minor fraction of small-budded cells display ER tubules along the mother–bud axis, whereas most buds (~80%) exhibit cortical ER. In ptc1Δ cells, >80% of small buds contain only cytoplasmic ER tubules oriented along the mother–bud axis, with no detectable cortical ER in the bud (Du et al., 2006). As we previously showed, deletion of SPA2 significantly suppresses the cortical ER inheritance defect of ptc1Δ cells (Li et al., 2010). We found that most of the other polarisome mutations—bud6Δ, bni1Δ, pea2Δ, and sph1Δ—also significantly suppressed the cortical ER inheritance defect of ptc1Δ cells. As shown in Figure 1 and Supplemental Figure S2, in bud6Δ ptc1Δ, bni1Δ ptc1Δ, pea2Δ ptc1Δ, sph1Δ ptc1Δ, and spa2Δ ptc1Δ double-mutant cells, only ~10–30% of small buds exhibited ER tubules along the mother–bud axis, and ~70–90% of small buds possessed a well-distributed cortical ER network. Two polarisome component mutations, msb3Δ and msb4Δ, had no suppression activity, possibly because these two gene products are redundant. Owing to the inviability of the msb3Δ msb4Δ ptc1Δ triple mutant, we were unable to assess the suppression activity of simultaneously deleting both MSB genes.

Polarisome components play distinct roles in Slt2p function, affecting either its localization or its activity

Activation of Slt2p at the bud tip leads to a delay in the distribution of ER tubules to the bud cortex in ptc1Δ mutant cells (Li et al., 2010). Polarisome mutations could suppress the ER inheritance defect in ptc1Δ cells either by regulating Slt2p localization at the bud tip, as shown for Spa2p (Li et al., 2010), or controlling the level of Slt2p activation. If a polarisome mutation disrupts the localization of Slt2p to the bud tip, even though Slt2p is still activated in ptc1Δ cells, it would not be able to phosphorylate the relevant downstream substrate at the bud tip to block cER spreading. Alternatively, if the polarisome mutation decreases the level of Slt2p activation without affecting localization, it would antagonize Slt2p function and thereby restore normal cER inheritance.
To address these two possible mechanisms, we assessed Slt2p localization and activation in the various polarisome deletion mutants. We used a plasmid expressing an Slt2p–green fluorescent protein (GFP) fusion from the endogenous SLT2 promoter. Slt2p-GFP is predominantly nuclear at all stages of the cell cycle, yet also localizes to the tip of small buds and to the bud neck late in mitosis (large buds). Approximately 70% of small buds exhibit a concentration of Slt2p-GFP at the bud tip in a wt strain. Deletion of the polarisome gene PEA2 reduced Slt2p-GFP localization at bud tips (Figure 2, A and B), as shown for the deletion of SPA2 (van Drogen and Peter, 2002). In contrast, the deletion of other polarisome component genes did not significantly affect Slt2p-GFP localization at bud tips (Figure 2 and Supplemental Figure S3).

Slt2p is the final kinase of the CWI MAPK cascade (Levin, 2005). It is activated by a series of upstream kinases: MEKK1 (Bck1p) and the MEks (Mkk1p and Mkk2p). Mkk1p and Mkk2p are two redundant kinases that, like Slt2p, are localized in part at sites of polarized growth. We explored the possibility that one or more polarisome components are required for localization of Mkk1p or Mkk2p at bud tips. A previous study indicated a role for Spa2p in Mkk1p localization (van Drogen and Peter, 2002). We used a GFP fusion protein, Mkk1p-GFP, expressed from the ADH1 promoter and followed its localization in wt and polarisome mutant cells. Mkk1p-GFP is localized at the tips of small buds and at the mother–bud neck in large-budded cells. The deletion of polarisome gene BUD6, BN1T, or SPH1 did not affect Mkk1p-GFP localization at bud tips or bud necks (Supplemental Figure S4), whereas spa2Δ decreased Mkk1p localization, as shown for spa2Δ (van Drogen and Peter, 2002).

We next determined whether the activation of Slt2p by phosphorylation is affected by loss of any of the polarisome components. Whole-cell lysates from the indicated strains were blotted using an antibody that specifically recognizes only the active, phosphorylated form of Slt2p or an antibody that recognizes the total pool of Slt2p. The results are shown separately for convenience (Figure 3A), with phosphorylation of Slt2p blocked, leading to a large increase of
level of Slt2p phosphorylation (Figure 3). This is consistent with their failure to suppress the cER inheritance defect of ptc1Δ cells. Slt2p-GFP localization also appeared to be unaffected in an msb3Δ msb4Δ double-mutant strain (Supplemental Figure S3). The msb3Δ msb4Δ double mutant displayed only a low level of phospho-Slt2p; due to the inviability of msb3Δ msb4Δ ptc1Δ triple mutants; however, we were unable to test the effects of the combined loss of Msb3 and Msb4 on Slt2p activation. Taken together, all components of the polarisome, with the possible exception of Msb3p and Msb4p, affect the function of Slt2p in the regulation of cortical ER inheritance. Pea2p and Spa2p are needed to recruit Slt2p to bud tips, and Bni1p, Bud6p, and Sph1p affect the level of Slt2p activation.

Depolymerization of the actin cytoskeleton bypasses Ptc1p and Sec3p function in ER inheritance

Ptc1p promotes vacuolar inheritance by facilitating the interaction of the type V myosin, Myo2p, with Vac1p, a receptor on the surface of the vacuole, and it has been suggested that Ptc1p might control the inheritance of other organelles by related mechanisms (Jin et al., 2009). In ptc1Δ mutant cells, ER tubules migrate into the bud but fail to spread along the bud cortex (Du et al., 2006). Because Myo4p drives the vectorial movement of ER segregation tubules into the bud (Estrada et al., 2003), it appears that the connection between the ER and this motor must be functional in the absence of Ptc1p. Nonetheless, one possible explanation for the ER inheritance phenotype of ptc1Δ cells is that the connection between Myo4p and the ER persists even after the tubules have been delivered to the bud, such that they are not free to propagate along the cortex but instead remain trapped on actin cables. To explore this possibility, we treated ptc1Δ cells with the actin depolymerization reagent latrunculin A. Remarkably, after only 10 min of latrunculin A treatment the cytoplasmic ER tubules in the buds of ptc1Δ cells were replaced with normal cortical ER (Figure 4). Time-lapse microscopy suggests that the ER tubules present in the bud of ptc1Δ cells spreads along the bud cortex in response to the addition of latrunculin A (Supplemental Figure S5). Therefore we propose that in ptc1Δ cells, the ER fails to spread to the cortex of the bud because it is bound to the actin cytoskeleton. This result also implies that the propagation of ER along the cortex of the bud is not an actin-dependent process.

In sec3Δ cells, as in ptc1Δ cells, ER tubules extend into the bud but fail to propagate along the cortex (Wiederkehr et al., 2003). Addition of latrunculin A to sec3Δ cells reversed this ER phenotype (Figure 4). The ER tubules extending into the bud, typical of sec3Δ
cells, were no longer observed and were replaced by normal, cortical ER in the bud. Time-lapse microscopy suggests that the ER tubule in the bud spreads along the bud cortex in response to latrunculin A (Supplemental Figure S5). The myo4Δ mutant is partially defective in the formation of ER segregation tubules. In contrast to ptc1Δ and sec3Δ cells, the ER phenotype of myo4Δ cells was not suppressed by addition of latrunculin A (Figure 4).

sec3Δ cells have elevated levels of phospho-Slt2p, and their ER phenotype is suppressed by loss of bud6
The restoration of cER in small buds of both ptc1Δ and sec3Δ cells upon addition of latrunculin A suggested the possibility of a common underlying mechanism in their ER inheritance defects. We therefore measured the level of Slt2p phosphorylation in sec3Δ cells and found it to be elevated to a level similar to that of ptc1Δ cells (Figure 5, A and B, lanes 1–4). To determine whether the increased activation of Slt2p in sec3Δ cells was responsible for the observed ER inheritance defect, we attempted to construct a sec3Δ slt2Δ double mutant; however, these mutations proved to be synthetically lethal in combination (Figure 5C). Therefore we constructed double mutants that combined sec3Δ with deletions of the various polisome components, since these are required for either Slt2p localization or activation. The pea2Δ sec3Δ and spa2Δ sec3Δ double mutants were viable and grew somewhat more rapidly than the sec3Δ single mutant, whereas the bni1Δ sec3Δ single mutant was inviable. Although some of the bud6Δ sec3Δ double-mutant spores were inviable, others grew, albeit more slowly than the sec3Δ single mutant (Figure 5C).

We found that deletion of either pea2 or spa2 had no effect on the ER inheritance phenotype or the elevated Slt2 activation of sec3Δ cells, whereas loss of bud6 largely restored normal cortical ER morphology to sec3Δ cells (Figure 6, A and B) and reduced the level of phospho-Slt2p (Figure 5, A, lanes 5–9, and B). Thus an interesting pattern emerged: loss of either of the two polisome components needed for Slt2p localization had no effect on the ER inheritance phenotype of sec3Δ cells, whereas loss of one of the components needed for Slt2p activation did suppress the ER inheritance phenotype. One interpretation of these results is that loss of Sec3p triggers the activation of a pool of Slt2p, which in turn affects cER inheritance. This pool of Slt2p requires Bud6p for activation, yet, unlike the situation in ptc1Δ cells, it does not depend on Spa2p or Pea2p and therefore may act at a site other than the bud tip. This difference is consistent with the somewhat different ER inheritance phenotypes of sec3Δ and ptc1Δ cells. Because bud6Δ cells exhibit a modest defect in actin structure (Amberg et al., 1997), it is also possible that this, rather than the loss of Slt2p activation, is the basis of the suppression of the sec3Δ ER phenotype.

Polarisome components are not involved in Ptc1p-regulated mitochondrial inheritance
Mitochondrial inheritance, like ER inheritance, requires Ptc1p function (Roeder et al., 1998), and, as in the case of ER inheritance, the ptc1Δ mitochondrial inheritance phenotype is suppressed by deletion of SLT2 (Li et al., 2010). We nevertheless noted an important distinction: the mitochondrial inheritance phenotype of ptc1Δ is not suppressed by deletion of SPA2, suggesting that the relevant pool of Slt2p is not at the bud tip (Li et al., 2010). We predicted that the other polarisome components would similarly not be involved in the regulation of mitochondrial inheritance. As shown in Figure 7, mitochondrial segregation structures migrate into the small buds of wt cells, whereas in ptc1Δ cells mitochondria remain entirely within the mother cell. Deletion of individual polarisome genes failed to suppress the mitochondrial inheritance defects in ptc1Δ mutant cells. The percentage of small buds containing mitochondrial segregation structures in bud6Δ ptc1Δ, bni1Δ ptc1Δ, pea2Δ ptc1Δ, spa2Δ ptc1Δ, msb3Δ ptc1Δ, and msb4Δ ptc1Δ double-mutant cells was ∼10–20%, similar to that in ptc1Δ cells. We also examined whether disruption of actin with latrunculin A would restore mitochondrial inheritance in ptc1Δ cells (Supplemental Figure S6); no restoration was seen. The difference in polarisome and actin involvement in cER inheritance versus mitochondrial inheritance again confirms the requirement for distinct pools of Slt2p in the inheritance of these two organelles. Most of the single-polarisome-gene deletions did not exhibit mitochondrial inheritance defects; however, bud6Δ and bni1Δ cells displayed partial defects, and a marginally significant inheritance defect was seen in pea2Δ cells (Figure 7).

DISCUSSION
We systematically explored the requirements for the components of the polisome in Slt2p-dependent regulation of cER inheritance.
anchors at the bud tip, but fails to spread along the cortex of the bud (Du et al., 2006). Phosphorylation by Slt2p of a key component at the bud tip may trap the ER segregation tubule at this site, stabilizing a normally transient step in the inheritance process. Of interest, Scs2p, a VAP homologue needed to tether the ER to the plasma membrane, concentrates at bud tips in a Spa2p-dependent manner, and loss of Scs2p leads to a reduction in cER (Loewen et al., 2007; Manford et al., 2012).

Ptc1p has been proposed to regulate organelle inheritance by controlling the association of myosin motors with receptors on the surface of the affected organelles (Jin et al., 2009). The restoration of cortical ER in ptc1Δ cells in response to the depolymerization of actin is broadly consistent with this model. Activation of Slt2p at the bud tip might stabilize the interaction between the Myo4p motor and a component on the surface of the ER (Figure 8). This would lead to the persistence of ER segregation tubules bound to actin fibers oriented along the mother–bud axis. Depolymerization of actin fibers by addition of latrunculin A would then free those ER tubules to distribute along the cortex of the bud. This is somewhat different from the proposed role of phosphorylation in vacuole inheritance. In that case, increased phosphorylation resulting from the loss of the Ptc1p phosphatase inhibits the interaction of Myo2p with the vacuole, blocking an early stage in the vacuole inheritance pathway (Jin et al., 2009).

Three other components of the polarisome—Bni1p, Spf1p, and Bud6p—are also required for the Slt2p-dependent block in cER inheritance. In contrast to Spa2p and Pea2p, however, these components are not required for the localization of Slt2p to the bud tip. Instead, these components are needed for full activation of Slt2p in response to loss of Ptc1p function (Figure 8). Further studies will be needed to define the mechanism by which these components affect Slt2p activation. Nonetheless, our results extend the evidence for inhibition of cER inheritance by a pool of active Slt2p at the bud tip.

The remaining components of the polarisome—Msb3p and Msb4p—are functionally redundant. Therefore it is not surprising that the loss of either one alone has no effect on the ptc1Δ cER inheritance defect. These results are also consistent with the observation that neither of these mutations, alone, affects Slt2p localization or activation. Although we found no effect of an msb3Δ msb4Δ double mutant on Slt2p localization, the synthetic lethality of ptc1Δ in this double mutant precluded us from testing the effects of a simultaneous loss of both MSB genes on Slt2p activation.

The ER inheritance defect of sec3Δ cells, like that of ptc1Δ cells, is reversed by addition of latrunculin A, and the level of Slt2p activation...
in sec3Δ cells is similar to that of ptc1Δ cells, suggesting a common underlying mechanism. Nonetheless, the ER defect of sec3Δ cells is not suppressed by loss of either Pea2p or Spa2p, although it is suppressed by loss of Bud6p, which reduces the level of Slt2p activation. Taken together, these results suggest that, in sec3Δ cells, Slt2p is activated by a Bud6p-dependent pathway, yet the relevant pool of Slt2p is not localized to the bud tip by Pea2p and Spa2p. This is consistent with the observation that the ER tubules that migrate into the bud in sec3Δ cells are not anchored at the bud tip, as they are in ptc1Δ cells.

Although we previously demonstrated that the mitochondrial inheritance defect of ptc1Δ cells is suppressed by the disruption of SLT2 (Li et al., 2010), this defect was not suppressed by the loss of any of the polarisome components. Thus the inhibition of mitochondrial inheritance in ptc1Δ cells relies on a pool of Slt2p that functions independently of the polarisome. The relevant pool is therefore not at the bud tip, and its activation does not require Bni1p, Sph1p, or Bud6p. These findings are consistent with the observation that in ptc1Δ cells, mitochondrial inheritance is blocked before tubules enter the daughter. This represents an earlier stage in organelle delivery than the cER inheritance defect of ptc1Δ cells.

The regulation of cER inheritance during the cell cycle and the comparison to mitochondrial inheritance highlight the flexibility,
subtlety, and complexity of the signaling pathways involved. Although in both cases regulation of inheritance uses the Slt2p kinase as a key component, different pools are used in different situations. Identification of the relevant substrates of Slt2p in each situation will be critical.

### MATERIALS AND METHODS

#### Strain construction

Table 1 lists the yeast strains used in this study. NY2959 (MATα, ptc1Δ::his5) and NY2965 (MATα, ptc1Δ::KanMX4) were generated by replacing the PTC1 coding sequence in BY4742 and SEY6210.1

| Strain | Genotype | Source | Strain | Genotype | Source |
|--------|----------|--------|--------|----------|--------|
| NY1210 | MATα his3-200 leu2-3, 112 ura3-52 | Novick lab collection | NY2975 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY1211 | MATα a his3-200 leu2-3, 112 ura3-52 | Novick lab collection | NY2976 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY2920 | MATα his3-200 leu2-3, 112 ura3-52 ptc1Δ::his5 | Novick lab collection | NY2977 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| SFNY1683 | MATα his3-200 leu2-3, 112 ura3-52::(URA3, HMG1-GFP) | Ferro-Novick lab collection | NY2978 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| SFNY1684 | MATα his3-200 leu2-3, 112 ura3-52::(URA3, HMG1-GFP) ptc1Δ::his5 | Ferro-Novick lab collection | NY2979 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| SFNY1235 | MATα his3-200 leu2-3, 112 ura3-52 myo4Δ::his5 | Ferro-Novick lab collection | NY2980 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| SFNY1153 | MATα his3-200 leu2-3, 112 ura3-52::(URA3, HMG1-GFP) sec3Δ::kanMX4 | Ferro-Novick lab collection | NY2981 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| SFNY1154 | MATα his3-200 leu2-3, 112 ura3-52::(URA3, HMG1-GFP) sec3Δ::kanMX4 | Ferro-Novick lab collection | NY2982 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY3020 | MATα his3-200 leu2-3, 112 ura3-52 slt2Δ::his5 | This study | NY2983 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY3022 | MATα his3-200 leu2-3, 112 ura3-52 spa2Δ::his5 | This study | NY2984 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY3023 | MATα his3-200 leu2-3, 112 ura3-52 pea2Δ::his5 | This study | NY2985 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY3025 | MATα his3-200 leu2-3, 112 ura3-52 bni1Δ::his5 | This study | NY2986 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY3024 | MATα his3-200 leu2-3, 112 ura3-52 bud6Δ::his5 | This study | NY2987 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY3028 | MATα his3-200 leu2-3, 112 ura3-52::(URA3, HMG1-GFP) bud6Δ::his5 sec3Δ::kanMX4 | This study | NY2988 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY3027 | MATα his3-200 leu2-3, 112 ura3-52::(URA3, HMG1-GFP) pea2Δ::his5 sec3Δ::kanMX4 | This study | NY2989 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY3026 | MATα his3-200 leu2-3, 112 ura3-52::(URA3, HMG1-GFP) spa2::his5 sec3Δ::kanMX4 | This study | NY2990 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY2968 | MATα his3-200 leu2-3, 112 ura3-52 bud6Δ::KanMX4 | This study | NY2991 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY2969 | MATα his3-200 leu2-3, 112 ura3-52 bud6Δ::KanMX4 ptc1Δ::his5 | This study | NY2992 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |
| NY2974 | MATα his3-200 leu2-3112 ura3-52 spa2Δ::KanMX4 | This study | NY2993 | MATα his3-200 leu2-3112 ura3-52 ptc1Δ::his5 | This study |

**Table 1:** Yeast strains used in this work.
(wild-type strains generously provided by Scott Emr, Cornell University, Ithaca, NY), respectively, with the Schizosaccharomyces pombe his5α gene or the KanMX4 module using the PCR-mediated gene deletion method (Longtine et al., 1998). To construct individual polarisome component mutant strains pea2Δ (NY2963), bni1Δ (NY2966), mbs1Δ (NY2970), mbs4Δ (NY2971), bud6Δ (NY2968), and spa2Δ (NY2974) or another set of strains in different background spa2Δ (NY3022), pea2Δ (NY3023), bud6Δ (NY3024), and spa2Δ (NY3025), the same strategy was used to replace the entire coding sequences of each gene with the S. pombe his5α gene or the KanMX4 module in SEY6210, NY1211, or NY1210 yeast strain.

The double mutants pea2Δ ptc1Δ (NY2964), bni1Δ ptc1Δ (NY2967), mbs1Δ ptc1Δ (NY2972), mbs4Δ ptc1Δ (NY2973), and bud6Δ ptc1Δ (NY2969), spa2Δ ptc1Δ (NY2975) were made from a cross between the corresponding single-deletion strain and NY2965 or NY2920. The double mutants spa2Δ sec3Δ (NY3026), pea2Δ sec3Δ (NY3027), and bud6Δ sec3Δ (NY3028) were made from a cross between the corresponding single-deletion strains and SFNY1153. sph1Δ (NY3030) is from the yeast genome-wide gene-deletion collection and was crossed to NY2959 to generate sph1Δ ptc1Δ (NY2960). NY2976 was generated by replacing the MSB4 coding sequence in NY2970 with TRP1 module by the PCR-mediated gene deletion method (Longtine et al., 1998). NY3020 was generated by replacing the SLT2 coding sequence in NY1210 with the S. pombe his5α module similarly. To visualize CER or mitochondria in living cells by microscopy, Stul-digested pRH475 integrating plasmid (Conrin et al., 2000) or plasmid pYDY104 encoding a mitochondria-targeting sequence fused to the NH2 terminus of the red fluorescent protein (RFP, Du et al., 2001) was transformed into each strain. To determine the subcellular localization of Slt2p or Mkk1p, plasmid pFD299 or pFD273 containing all the sequences of each gene with the CER or mitochondria targeting sequence were deconvolved with Openlab software (ImproVision, PerkinElmer, Waltham, MA).

Time-lapse microscopy of SFNY1684 (ptc1Δ) and SFNY1154 (sec3Δ) was performed on cells grown to early log phase in SC medium at 25°C. Cells were observed with an Axio Observer Z1 microscope (Zeiss) immediately after Lat-A treatment. Images were captured at 10-s intervals for 5 min using AxioVision 4.8 software (Zeiss).

Cell lysate extracts and immunoblotting

Yeast cells were grown to mid-exponential phase in yeast extract/peptone/dextrose or SC medium. For each strain an equal amount of cells was harvested, immediately frozen in liquid N2, and then lysed by a rapid alkaline lysis procedure essentially as described in Westfall et al. (2008). The activated Slt2p was monitored with a rabbit phospho-p44/p42 MAPK antibody (Cell Signaling Technology, Beverly, MA) that specifically detects dually phosphorylated Slt2p. The total Slt2p was detected with a mouse monoclonal Mpk1(E-9) antibody (sc-133189 from Santa Cruz Biotechnology, Santa Cruz, CA). Mouse phosphoxygenase kinase (Pgk1) monoclonal antibody was used to detect Pgk1p as a loading control. The density of gel blots was scanned using ImageJ software (National Institutes of Health, Bethesda, MD). To quantify the relative Slt2p activation, the density of the phospho-Slt2 band was divided by the density of Pgk1p, and the normalized phospho-Slt2p value of each strain was divided by the maximum value among the group.

ACKNOWLEDGMENTS

We thank Christina Howard for technical assistance. This study was supported by National Institutes of Health Grants GM073892 to PN. and S.F.N. and GM 35370 to P.N. Salary support for S.F.N. was provided by the Howard Hughes Medical Institute.

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