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Genome-wide lethality screen identifies new PI4,5P_2 effectors that regulate the actin cytoskeleton

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To further understand the roles played by the essential phosphoinositide PI4,5P_2, we have used a synthetic lethal analysis, which systematically combined the *mss4*^{ts} mutation, partially defective in PI4P 5-kinase activity, with each of approximately 4700 deletion mutations. This genomic screening technique uncovered numerous new candidate effectors and regulators of PI4,5P_2 in yeast. In particular, we identified Slm1 (Yil105c), a previously uncharacterized PI4,5P_2 binding protein. Like Mss4, Slm1 and its homolog Slm2 (Ynl047c) were required for actin cytoskeleton polarization and viability. Co-immunoprecipitation experiments revealed that Slm1 interacts with a component of TORC2, a Tor2 kinase-containing complex, which also regulates the actin cytoskeleton. Consistent with these findings, phosphorylation of Slm1 and Slm2 was dependent on TORC2 protein kinase activity, both *in vivo* and *in vitro*, and Slm1 localization required both PI4,5P_2 and functional TORC2. Together, these data suggest that Slm1 and Slm2 function downstream of PI4,5P_2 and the TORC2 kinase pathway to control actin cytoskeleton organization.

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Introduction

Over the past decade, derivatives of the lipid phosphatidylinositol, collectively known as phosphoinositides, have emerged as essential regulatory molecules involved in a diverse spectrum of cellular processes (Fruman *et al*, 1998; Takenawa and Itoh, 2001). Multiple characterized effectors of phosphoinositides harbor specific domains important for lipid recognition. Pleckstrin homology (PH) domains from several proteins have been shown to interact with phosphoinositides, sometimes showing a clear preference for a single lipid (Lemmon and Ferguson, 2000). The PH domain from phospholipase C-δ_1 (PLCδ_1) specifically binds PI4,5P_2 and its soluble head group IP_3 *in vitro* (Cifuentes *et al*, 1993), and when fused to GFP, can be used as an *in vivo* probe for this lipid (Stauffer *et al*, 1998). These data suggest that proteins containing PH domains or other phosphoinositide interacting motifs (i.e. FYVE, PX, ENTH, etc.) are recruited and/or activated by binding phosphoinositides on specific membrane compartments. Through the action of a set of well-conserved and tightly regulated phosphoinositide kinases, phosphatases, and lipases, phosphoinositides can efficiently modulate downstream events mediated by their effectors in response to a variety of stimuli. The yeast *Saccharomyces cerevisiae* has proven to be a useful model system for the study of phosphoinositides, showing essential roles for PI3P in endosomal membrane trafficking, PI4P in Golgi secretion, and PI4,5P_2 in endocytosis and actin cytoskeleton organization (Odorizzi *et al*, 2000).

Generation of PI4,5P_2 is catalyzed by a single phosphoinositide kinase in yeast, Mss4. Using temperature-sensitive alleles of *MSS4*, multiple groups have demonstrated that PI4,5P_2 generated at the plasma membrane is required for actin cytoskeleton organization in yeast (Desrivières *et al*, 1998; Homma *et al*, 1998). Correspondingly, studies using several different biological systems indicate the existence of numerous PI4,5P_2 effector proteins involved in cytoskeletal dynamics, including gelsolin, cofilin, the Arp2/3 activator WASP, ERM proteins, and exchange factors for small Rho-type GTPases (Takenawa and Itoh, 2001). In particular, Mss4-generated PI4,5P_2 has been shown to recruit/activate the Rho1 GTPase exchange factor Rom2, which is required for Rho1 activation and maintenance of actin polarization (Audhya and Emr, 2002). However, the mechanism(s) behind how PI4,5P_2 coordinates the recruitment/activation of its many effectors remains unclear.

In addition to Mss4, multiple other factors have been implicated in the regulation of actin cytoskeleton organization, several of which affect Rho1 activation. Previous studies have shown that Tor2 also functions upstream of Rho1, and *tor2*^{ts} mutant cells exhibit a defect in actin organization similar to *mss4*^{ts} cells (Schmidt *et al*, 1997). TOR, the target of rapamycin, is a conserved phosphatidylinositol

kinase-related protein kinase that controls cell growth in response to nutrient conditions. Yeast cells express two TOR homologs, Tor1 and Tor2, which can participate in two distinct complexes, TORC1 and TORC2 (Loewith *et al*, 2002). TORC1 is rapamycin sensitive, contains either Tor1 or Tor2, and controls translation initiation, ribosome biogenesis, transcription, and other growth-related readouts. In contrast, TORC2 is rapamycin insensitive, contains Tor2 but not Tor1, and controls polarization of the actin cytoskeleton (Loewith *et al*, 2002). In addition to Tor2, TORC2 also contains five additional subunits: Avo1, Avo2, Avo3, Bit61, and Lst8 (Loewith *et al*, 2002; Wedaman *et al*, 2003; Reinke *et al*, 2004). Interestingly, *tor2*^{ts} mutations can be suppressed by overexpression of Mss4, suggesting an interaction between the Tor2 protein kinase and the Mss4 lipid kinase (Helliwell *et al*, 1998).

To initiate an unbiased investigation of the physiological roles of PI4,5P$_2$, we conducted a genome-wide synthetic lethal screen using a strain expressing a functionally impaired, temperature-sensitive allele of *MSS4*. We identified two previously uncharacterized proteins, Slm1 and Slm2, which are downstream effectors of both Mss4 and TORC2 and are required for organization of the actin cytoskeleton. These findings suggest an integration of lipid kinase function with TORC2 protein kinase regulation, both necessary for spatial and temporal regulation of actin cytoskeleton dynamics.

Results

Synthetic genetic array analysis reveals an Mss4 network of genetic interactions

MSS4 encodes an essential PI4P 5-kinase in yeast. To extend our understanding of Mss4 regulation and activity, we utilized a method, termed synthetic genetic array (SGA) analysis, for systematic construction and characterization of double-mutant strains. An *mss4*^{ts} mutation was combined with each of approximately 4700 deletion mutations at the permissive growth temperature for *mss4*^{ts} cells. Under these conditions, *mss4*^{ts} cells generate approximately 40% reduced levels of PI4,5P$_2$ as compared to wild-type cells (Stefan *et al*, 2002), slightly compromising but not inactivating PI4,5P$_2$-dependent cellular functions. We reasoned that inviable or slow-growing double-mutant progeny could identify a functional interaction between Mss4 and the product of the wild-type version of the deleted gene. Such proteins could represent regulators of the PI4P 5-kinase or targets of its product, PI4,5P$_2$. Previous work indicated that mutations in *MSS4* are lethal in combination with mutations that affect activation of the Rho1 GTPase (Audhya and Emr, 2002). In agreement with these studies, the SGA analysis identified Rom2, the exchange factor for Rho1. Since Rom2 requires PI4,5P$_2$ synthesis for its appropriate localization (Audhya and Emr, 2002), the SGA analysis should identify potential novel PI4,5P$_2$ effectors.

In total, almost 80 double-deletion combinations were initially isolated in the screen that resulted in a synthetic growth defect. Of these, approximately half were confirmed using an alternative method (see Supplementary Table I). Multiple genes that were *S*ynthetic *L*ethal with *M*ss4 (*SLM*) encoded proteins that have been implicated in actin cytoskeleton organization and/or cell polarity including Cap1, Cap2, Avo2, Spa2, Bem4, Boi2, Myo5, Cyk3, Ilm1, Van1, and Api2. Additionally, multiple components of the GimC complex, a chaperone believed to be important for normal folding of actin monomers (Siegers *et al*, 1999), were isolated in this screen. The identification of genes encoding proteins involved in a number of other processes including cellular stress response, ribosomal biosynthesis, protein/lipid modification, vesicle trafficking, and mitochondrial function suggests that Mss4 is involved in multiple biological pathways. Taken together, the results from the SGA analysis confirmed an essential function for Mss4 in cytoskeletal organization, but also indicated that Mss4/PI4,5P$_2$ likely plays other roles in maintaining cell structure and viability (Figure 1A).

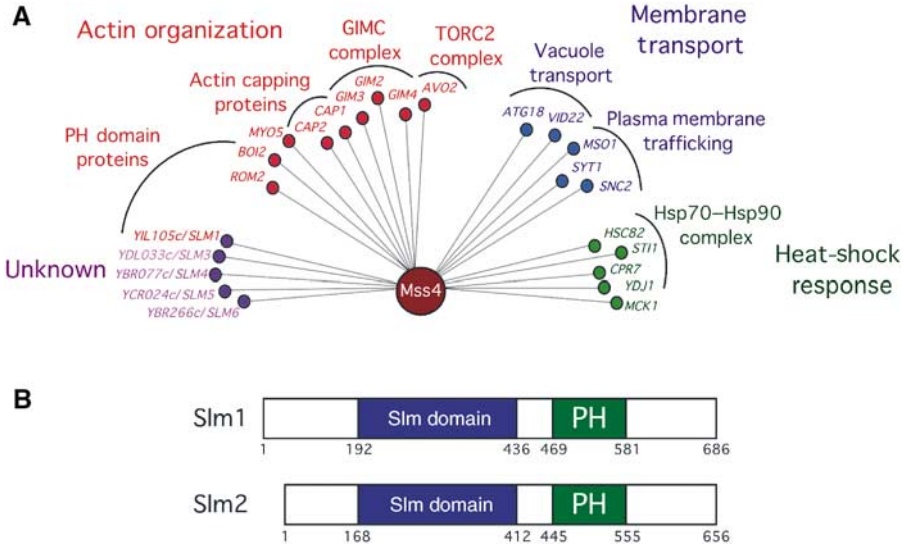

Figure 1 SGA analysis using the *mss4*^{ts} mutation indicates diverse roles for PI4,5P$_2$. (**A**) The genes that are required for normal growth of *mss4*^{ts} cells are represented as nodes. Each node is color coded based on its functional classification as defined by published literature. (**B**) Cartoon showing the domains of Slm1 and Slm2. Both proteins contain PH domains that interact with phosphoinositides. Additionally, Slm1 and Slm2 share a highly conserved domain, the Slm domain, which has been highlighted.

Of particular interest is the SGA analysis that revealed a genetic interaction between *MSS4* and *YIL105c*, a gene encoding a protein that contains a PH domain (Figure 1B), which has been shown to interact weakly with phosphoinositides *in vitro* (Yu *et al*, 2004). Moreover, plasma membrane localization of the PH domain from Yil105c requires Mss4-dependent PI4,5P$_2$ production (Yu *et al*, 2004). Consistent with studies of the Yil105c PH domain, full-length Yil105c also interacted with multiple phosphoinositides in liposome binding assays (Supplementary Figure 1).

The genetic interaction between the *mss4*^{ts} mutation and *yil105c*Δ was confirmed in the SEY6210 background, where *mss4*^{ts}*yil105c*Δ double-mutant cells exhibited a potent growth defect at 31.5°C in contrast to the near-normal growth of both single-mutant strains at the same temperature (Figure 2A). Analysis of phosphoinositide levels in an *mss4*^{ts} mutant and in *mss4*^{ts}*yil105c*Δ double-mutant cells at 31.5°C revealed comparable levels of phosphoinositides (data not shown), suggesting that Yil105c is not upstream of Mss4 and is consistent with the idea that PI4,5P$_2$ may play a role in the recruitment/activation of Yil105c. Given its genetic interaction with the *mss4*^{ts} mutation, we have named this gene *SLM1* for *S*ynthetic *L*ethal with *M*ss4.

Since cells deleted for *SLM1* alone failed to display any significant phenotype, we performed a database search to detect potential homologs that may serve a redundant function. This analysis led to the identification of *YNL047c*, which encodes a protein that is 53% identical to Slm1. Due to this strong similarity, we have named this gene *SLM2* (Figure 1B). Slm2 also contains a PH domain that binds phosphoinositides, with a preference for PI4,5P$_2$ when expressed as a GFP fusion in cells (Yu *et al*, 2004). Deletion of *SLM2* did not yield any noticeable effects on cell growth, but in combination with deletion of *SLM1*, cells were no longer viable (Figure 2B). Together, these data indicate that Slm1 and Slm2 serve an essential function in yeast, likely downstream of Mss4/PI4,5P$_2$.

Although Slm1 and Slm2 appear to be redundant in function, only *SLM1* was uncovered in the SGA analysis using the *mss4*^{ts} mutation. Consistent with this observation, *mss4*^{ts}*slm2*Δ double-mutant cells did not exhibit a significant synthetic growth defect in contrast to *mss4*^{ts}*slm1*Δ double-mutant cells. These data suggest that Slm1 may play a more dominant role in cells as compared to Slm2. To test directly the expression levels of Slm1 and Slm2, we measured the cellular levels of each protein by first constructing strains that expressed chromosomally GFP-tagged forms of each gene. Both fusion proteins were found to be functional, as determined by the normal growth of *slm1*Δ*SLM2-GFP* and *SLM1-GFPslm2*Δ cells. Western blot analysis revealed that levels of Slm1 protein were approximately 10-fold higher than those of Slm2 (Figure 2C), confirming that Slm1 is more abundant and likely plays a more significant role than Slm2 in cells. Consistent with these data, Slm1-GFP was clearly visible at endogenous levels, while Slm2-GFP fluorescence was more difficult to detect (see below).

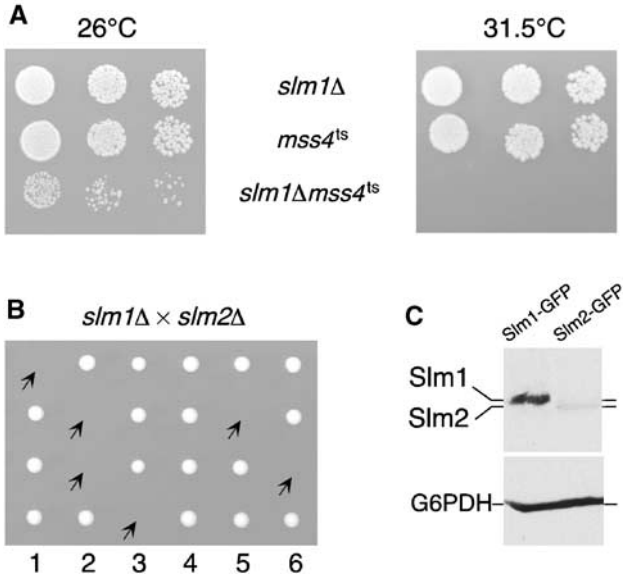

Figure 2 Slm1 and Slm2 perform an essential function downstream of Mss4. (**A**) *slm1*Δ, *mss4*^{ts} and *slm1*Δ*mss4*^{ts} cells were grown at 26 or 31.5°C for 60 h. (**B**) *slm1*Δ and *slm2*Δ cells were mated, and the resulting diploids were sporulated and dissected. Tetrads (1–6) are shown, yielding four viable spores (indicative of two *slm1*Δ colonies and two *slm2*Δ colonies), three viable spores (indicative of one wild-type colony, one *slm1*Δ colony, one *slm2*Δ colony, and one inviable *slm1*Δ*slm2*Δ colony), or two viable spores (indicative of two wild-type colonies and two inviable *slm1*Δ*slm2*Δ colonies). The arrows highlight inviable *slm1*Δ*slm2*Δ colonies. (**C**) TCA-precipitated extracts from cells expressing *SLM1-GFP* or *SLM2-GFP* were analyzed by SDS–PAGE followed by Western blot using α-GFP antibodies to detect Slm1 and Slm2 or α-G6PDH antibodies as a loading control.

Slm1 and Slm2 are new PI4,5P$_2$ effectors

In contrast to the confluent plasma membrane localization of the PH domains from both Slm1 and Slm2 (Yu *et al*, 2004), localization of each full-length protein showed they were enriched in punctate structures distributed at the cell periphery (Figure 3A and Supplementary Figure 2). The Slm1 patches were not polarized, as is the case with cortical actin patches. Time-lapse video microscopy indicated only

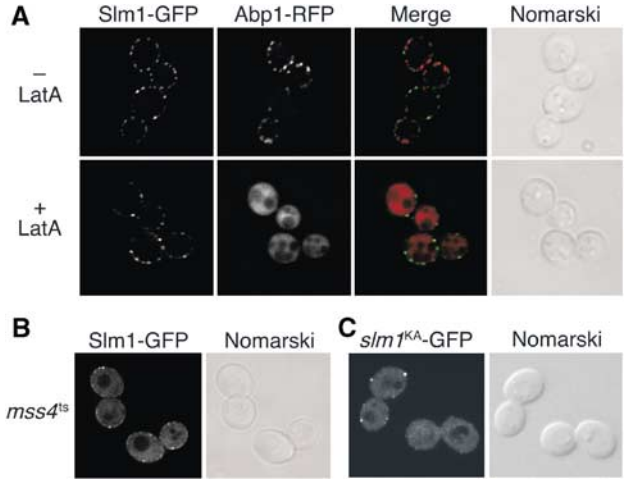

Figure 3 Localization of Slm1 is dependent on PI4,5P$_2$ but not F-actin. (**A**) Cells expressing *SLM1-GFP* and *ABP1-RFP* were visualized by fluorescence microscopy in the presence or absence of latrunculin A. (**B**) *mss4*^{ts} cells expressing *SLM1-GFP* were visualized by fluorescence microscopy following a 60 min shift to 37°C. (**C**) *slm1*Δ*slm2*Δ cells expressing *slm1*^{KA}-GFP were visualized by fluorescence microscopy at 26°C.

occasional movement of Slm1 on the mother cell cortex, while Slm1 patches were more dynamic on the daughter cell cortex (Supplementary Movie 1). Additionally, Slm1 patches were not affected by latrunculin A, a drug that causes actin depolymerization, indicating that Slm1 localization was independent of F-actin (Figure 3A). Since the PH domains from both proteins showed localizations dependent on Mss4 activity, localization of Slm1-GFP was analyzed in *mss4*^{ts} cells. Following shift to the nonpermissive temperature, Slm1 appeared more soluble in *mss4*^{ts} cells as compared to wild-type cells treated identically, although a portion of Slm1 could still associate with cortical punctate structures (Figure 3B). These data suggest that Slm1 is at least in part recruited to the plasma membrane by PI4,5P$_2$ and then potentially stabilized by other protein–protein or protein–lipid interactions.

To further investigate a role for PI4,5P$_2$ in the recruitment of Slm1 to the plasma membrane, we generated a mutant form of Slm1 (*slm1*^{KA}), which harbors point mutations in critical lysine resides (K483A, K487A) in the PH domain that were previously shown to be required for phosphoinositide binding (Yu *et al*, 2004). *slm1*Δ*slm2*Δ double-mutant cells expressing *slm1*^{KA} were viable but temperature sensitive for growth, indicating an essential requirement for Slm1 phosphoinositide binding under conditions of stress. Strikingly, even at permissive temperatures, *slm1*^{KA}-GFP was largely mislocalized, accumulating mostly in the cytoplasm, with only a weak ability to associate with the plasma membrane, similar to *mss4*^{ts} cells expressing wild-type Slm1-GFP (Figure 3C). Western blot analysis revealed that this form of Slm1 was stable, both at permissive and nonpermissive temperatures, indicating that cytoplasmic accumulation of GFP fluorescence was not due to clipping of the GFP fluorophore. Together, these data suggest a role for Mss4-generated PI4,5P$_2$ in the recruitment of Slm1 to the plasma membrane, and also indicate the existence of other factor(s) that contribute to Slm1 localization.

Slm1 and Slm2 are essential for normal actin cytoskeleton organization

To gain further insight into the function of Slm1 and Slm2, we constructed temperature-sensitive forms of *SLM1* using error-prone PCR and plasmid shuffle techniques. Western blot analysis indicated that each form of Slm1 was stable, but three different patterns of Slm1 localization emerged from the screen (Table I). One group of temperature-sensitive mutants localized similarly to wild-type Slm1 at both permissive and nonpermissive temperatures (class A). In contrast, a second group of mutants showed normal localization at 26°C, but accumulated in the cytoplasm following shift to nonpermissive temperature (class B). Finally, a third group of mutants showed strong cytoplasmic fluorescence independent of temperature, similar to *slm1*^{KA} described above (class C). Representative members from each class of mutants were chosen for sequencing. Both class A and B mutants harbored mutations between amino acids 192 and 436, a region we refer to as the Slm domain, due to its high conservation between Slm1 and Slm2 (Figure 1B). However, only class C mutants contained mutations in the PH domain (amino acids 469–581). Although mutations in the Slm domain could affect the localization of Slm1 (class B), its association with PI4,5P$_2$ as determined by liposome binding assays was not significantly perturbed (Supplementary Figure 1). These data further indicate the importance of the Slm1 PH domain for localization, and also highlight an additional region of Slm1 as being important for both function and maintenance of Slm1 at the plasma membrane.

Previous studies have indicated a role for PI4,5P$_2$ in the maintenance of actin cytoskeleton organization and cell wall integrity, at least in part through activation of the Rho1–Pkc1 pathway. To determine if Slm1 and Slm2 also function as downstream effectors of Mss4 in the maintenance of actin polarization, we examined actin localization in all three classes of *slm1*^{ts}*slm2*Δ cells. At the permissive temperature, both wild-type and *slm1*^{ts}*slm2*Δ cells with relatively small buds showed normal polarization of the actin cytoskeleton, with cortical actin patches restricted to daughter cells and actin cables extending through mother cells. In contrast to wild-type cells, at the nonpermissive temperature *slm1*^{ts}*slm2*Δ cells exhibited a complete depolarization of the actin cytoskeleton, with actin patches randomly distributed throughout both mother and daughter cells and an apparent loss of actin cables (Figure 4A). Additionally, extended incubation of *slm1*^{ts}*slm2*Δ cells at 37°C (greater than 3 h) resulted in cell lysis. These phenotypes were identical to those observed in *mss4*^{ts} cells placed at the nonpermissive temperature.

Since actin cables were rather poorly preserved following fixation, we directly examined actin cable organization in living cells by visualizing a GFP fusion to the actin cable binding protein Abp140 (Yang and Pon, 2002). Although long actin cables oriented along the mother–bud axis were clearly

Table I Three classes of *slm1*^{ts} alleles each show different localizations

Class	Strain	Mutation(s)	Growth		Localization	
			26°C	37°C	26°C	37°C
A	AAY1622	F236S, K254R, N436S	+ +	—	PM	PM
B	AAY1623	I207N, N371T, V392A	+ + +	—	PM	C
	AAY1624	L192P	+ +	—	PM	C
C	AAY1625	N134S, D351A, K471T	+ + +	—	C	C
	AAY1626	K480Q, H498R	+ +	—	C	C

Cells were grown at 26 or 37°C for 2 days on synthetic growth media. For localization studies, cells were shifted to 37°C for 1 h. Observations were based on visualization of >100 cells/condition.
PM: plasma membrane; C: cytoplasm.

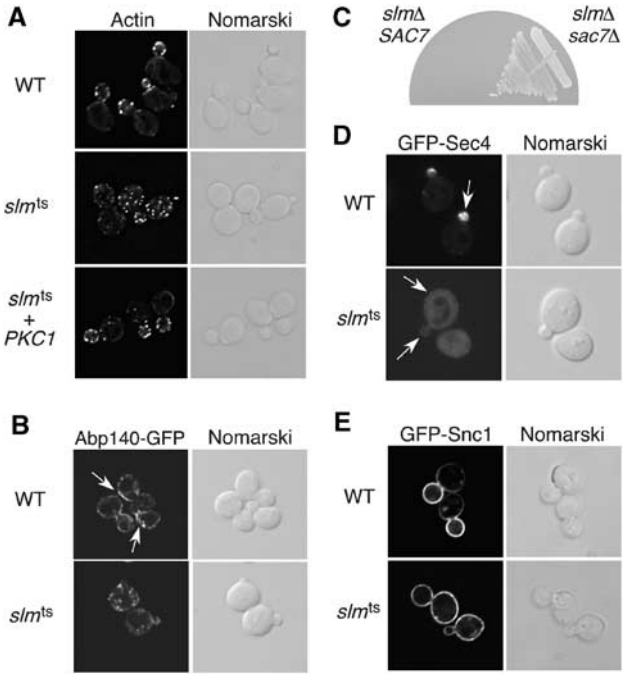

Figure 4 Slm1 and Slm2 control actin cytoskeleton organization and polarized secretion. (**A**) Wild-type and *slm1*^{ts}*slm2*Δ cells expressing either an activated allele of *PKC1* or an empty vector were shifted to 37°C for 2 h, fixed and stained with rhodamine–phalloidin, and visualized by fluorescence microscopy. (**B**) Wild-type and *slm1*^{ts}*slm2*Δ cells expressing *ABP140-GFP* were shifted to 37°C for 1 h and visualized by fluorescence microscopy. The arrows indicate long actin cables found in wild-type cells. (**C**) *slm1*Δ*slm2*Δ double-mutant and *slm1*Δ*slm2*Δ*sac7*Δ triple-mutant cells carrying pRS416*SLM1* were grown on 5-FOA for 4 days at 26°C. (**D**) Wild-type and *slm1*^{ts}*slm2*Δ cells expressing *GFP-SEC4* were shifted to 37°C for 1 h and visualized by fluorescence microscopy. Arrows indicate Sec4 localization to the bud or mislocalization to the mother cell cortex. (**E**) Wild-type and *slm1*^{ts}*slm2*Δ cells expressing *GFP-SNC1* were shifted to 37°C for 1 h and visualized by fluorescence microscopy.

visible in *slm1*^{ts}*slm2*Δ cells at 26°C, they were absent following a 60-min shift to the nonpermissive temperature (Figure 4B). Under identical conditions, wild-type cells maintained actin cable orientation independent of temperature. Together, these data indicate a requirement for Slm1 and Slm2 in both actin patch polarization and actin cable stability/orientation.

Slm1 and Slm2 are important for polarization of the secretory pathway

To further investigate the functions of Slm1 and Slm2, we performed a gene dosage-dependent suppression screen using class A *slm1*^{ts}*slm2*Δ cells to identify proteins that when overexpressed could suppress the temperature sensitivity of the mutant. Analysis of 40 000 transformants allowed the isolation of five genes, each of which could reproducibly suppress the growth defect of *slm1*^{ts}*slm2*Δ cells: *SLM1*, *SLM2*, *PKC1*, *MSS4*, and *SEC4*, a Rab-type GTPase essential for protein secretion. Moreover, overexpression of these genes also suppressed the actin defects characteristic of *slm1*^{ts}*slm2*Δ cells at 37°C, suggesting that increased signaling through the Rho1–Pkc1 cell integrity pathway could bypass the requirement for Slm1 and Slm2 (Figure 4A and Table II). Consistent with this idea, expression of *PKC1*^{R398P}, encoding a constitutively activated form of Pkc1, allowed growth of cells completely lacking *SLM1* and *SLM2* (*slm1*Δ*slm2*Δ) at 26°C. However, at elevated temperatures, *slm1*Δ*slm2*Δ cells harboring *PKC1*^{R398P} were no longer viable, indicating the existence of other factors necessary for bypass of Slm1 and Slm2. In contrast to the rescue of *slm1*Δ*slm2*Δ cells by *PKC1*^{R398P}, expression of constitutively activated alleles of *BCK1* or *MKK1*, both downstream effectors of Pkc1, failed to suppress the phenotypes of *slm1*^{ts}*slm2*Δ or *slm1*Δ*slm2*Δ cells (Table II).

Since Pkc1 is an effector of Rho1, we tested whether elevated Rho1-GTP levels were sufficient to bypass the requirement for Slm1 and Slm2 for cell viability at elevated temperature. Deletion of *SAC7*, a gene that encodes a Rho1 guanine nucleotide activating protein, has been shown to elevate levels of cellular Rho1-GTP (Schmidt *et al*, 1997). Strikingly, in contrast to *slm1*Δ*slm2*Δ double-mutant cells, *slm1*Δ*slm2*Δ*sac7*Δ triple-mutant cells were viable both at 26 and 38°C, indicating that Slm1 and Slm2 are dispensable under conditions where Rho1 is independently activated (Figure 4C).

Previous work has demonstrated a requirement for the Sec4-dependent secretory pathway in transport of Rho1 (Abe *et al*, 2003). Interestingly, inactivation of Sec4 causes a defect in actin cytoskeleton organization (Mulholland *et al*, 1997), possibly due to a defect in transport of Rho1 (or other machinery necessary for actin organization) to sites of polarized growth. Since overexpression of Sec4 suppressed the growth defect of *slm1*^{ts}*slm2*Δ cells, we examined whether Sec4 localization was perturbed in the absence of Slm1 and

Table II Genetic interactions in *slm1*^{ts}*slm2*Δ cells

Plasmid or mutation in *slm1*^{ts}*slm2*Δ cells	Growth		Actin polarization		Snc1 polarization	
	28°C	37°C	26°C	37°C	26°C	37°C
None	+ + +	—	+ + +	—	+ + +	—
2μ *PKC1*	+ + +	+ +	+ + +	+ +	+ + +	+ +
2μ *MSS4*	+ + +	+ +	+ + +	+ +	+ + +	+ +
2μ *SEC4*	+ + +	+ +	+ + +	+ +	+ + +	+ +
PKC1^{R398P}	+ + +	+ +	+ + +	+ +	+ + +	+ +
BCK1-20	+ + +	—	+ + +	—	+ + +	—
2μ *MKK1*^{S386P}	+ + +	—	+ + +	—	+ + +	—
*sac7*Δ	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
sec4-8	—	—	ND	ND	ND	ND

For growth studies, cells were grown at 28 or 37°C for 2 days on synthetic growth media. For actin and Snc1 polarization studies, cells were shifted to 37°C for 2 h. Observations were based on visualization of >100 small budded cells/condition. ND: not determined.

Slm2 activity. At 26°C, Sec4 localized to sites of polarized growth in *slm1*^{ts}*slm2*Δ cells, similar to that observed in wild-type cells. However, following a 60-min shift to the nonpermissive temperature, Sec4 was mislocalized in greater than 75% of *slm1*^{ts}*slm2*Δ cells, accumulating in the cytoplasm as well as in unpolarized patches decorating the entire cell periphery (Figure 4D). Since Sec4 transport requires polarized actin cables (Pruyne *et al*, 1998), these data are consistent with a primary defect in actin cytoskeleton organization when Slm1 and Slm2 function is compromised. Similar to the defect in Sec4 localization, examination of another secretory vesicle marker, the v-SNARE Snc1, showed that it also became unpolarized in *slm1*^{ts}*slm2*Δ cells at elevated temperature (Figure 4E). Increased signaling through the Rho1–Pkc1 pathway or overexpression of Sec4, which may elevate Rho1–Pkc1 signaling through enhanced delivery of Rho1 to the cortex, returned Snc1 to sites of polarized growth in *slm1*^{ts}*slm2*Δ cells at elevated temperature (Table II). Together, these data indicate that appropriate Sec4 localization to sites of polarized growth becomes limiting in the absence of Slm1 and Slm2 activity, likely due to a primary defect in actin organization. Furthermore, Sec4 mislocalization potentially exacerbates the defect in actin polarization observed in *slm1*^{ts}*slm2*Δ cells by virtue of its role in actin organization. Consistent with this idea, combining the temperature-sensitive *sec4-8* mutation with the *slm1*^{ts}*slm2*Δ mutations resulted in synthetic lethality at 28°C, a normally permissive temperature for *sec4-8* or *slm1*^{ts}*slm2*Δ cells individually (Table II).

Phosphorylation of Slm1 and Slm2 requires TORC2 protein kinase activity

High-throughput two-hybrid analysis has previously suggested that Slm1 and Slm2 have a common set of interactors (Uetz *et al*, 2000; Ito *et al*, 2001). To test directly whether Slm1 functions as part of a complex, we used a tandem affinity purification (TAP) method to isolate Slm1 and any tightly associated proteins. From this analysis, we found only one other copurifying protein by mass spectrometry following stringent washing conditions, Slm2 (Figure 5A). Interestingly, we identified several electrophoretic species of both Slm1 and Slm2 in our purification. Western blot analysis directed against the S-tag epitope fused to Slm1 (and used during its purification) revealed that only two of the four bands identified as being Slm1 by mass spectrometry contained the S tag. These data indicate that Slm1 can self-associate *in vivo*. The existence of multiple forms of Slm1 and Slm2 suggested that both proteins undergo post-translational modification. To test this directly, we immunoprecipitated newly synthesized Slm1-GFP and Slm2-GFP after briefly labeling cells with ^{35}S and chasing with unlabeled cysteine and methionine. After 10 min of labeling, we detected single bands corresponding to Slm1 and Slm2, but following a brief period of chase, we observed a shift in the molecular weight of Slm1, which migrated as two distinct, higher molecular weight species, and Slm2, which migrated as a single, higher molecular weight species (Figure 5B). Due to its higher level of expression, we focused on Slm1 to determine if the gel shift corresponded to phosphorylation.

Cells expressing Slm1-GFP were labeled with ^{32}P, and extracts were immunoprecipitated with GFP antibodies. This analysis indicated that Slm1 was phosphorylated on at least two sites *in vivo* (Figure 5C). Additionally, treatment of ^{35}S-labeled cell extracts with phosphatase eliminated the gel mobility shift, confirming that the shift in Slm1 gel mobility was specifically due to phosphorylation (Figure 5D). Similar studies with Slm2 confirmed that it is also phosphorylated (data not shown).

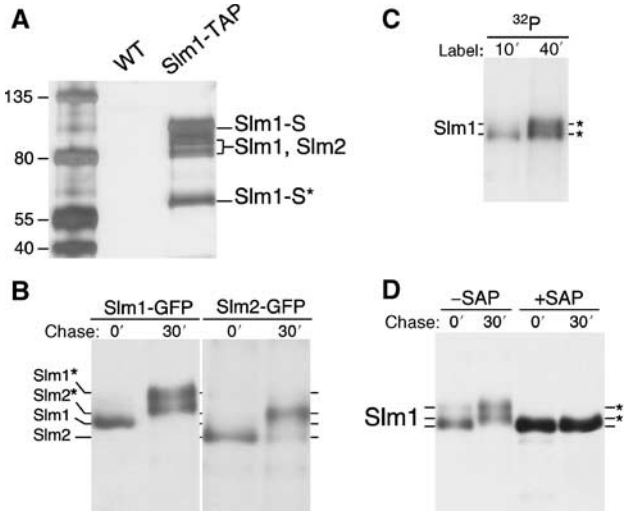

Figure 5 Slm1 and Slm2 are phosphoproteins that form a tightly associated complex *in vivo*. (**A**) Silver-stained SDS–PAGE gel showing the Slm1/Slm2 complex following TAP. Slm1-S refers to Slm1 fused to the S tag used during purification. Slm1-S* is a breakdown product of Slm1-S as detected by Western blot analysis using antibodies directed against the S tag. (**B, D**) Cells expressing *SLM1-GFP* or *SLM2-GFP* were metabolically labeled for 10 min with ^{35}S-labeled cysteine and methionine and chased for the indicated time. Extracts were immunoprecipitated with anti-GFP antibodies, incubated in the presence or absence of shrimp alkaline phosphatase (SAP), and subjected to SDS–PAGE analysis followed by autoradiography. The asterisks indicate the phosphorylated forms of Slm1. (**C**) Cells expressing *SLM1-GFP* were metabolically labeled for the indicated time with ^{32}P-labeled orthophosphate. Extracts were immunoprecipitated with anti-GFP antibodies, followed by SDS–PAGE analysis and autoradiography.

Since the Rho1–Pkc1 pathway responds to heat stress by first causing depolarization of the actin cytoskeleton followed by a period of repolarization (Delley and Hall, 1999), we also analyzed the effect of elevated temperature on the phosphorylation of Slm1. Heat shock significantly reduced the phosphorylation of Slm1 normally observed at low temperature. However, Slm1 phosphorylation recovered at later time points (Figure 6A), which correlated with the timing of actin cytoskeleton repolarization, again suggesting a link between Slm1 activity and organization of the actin cytoskeleton.

To determine which kinase(s) phosphorylated Slm1, we first analyzed results from high-throughput two-hybrid analyses, which suggested that both Slm1 and Slm2 may interact with Avo2, a component of the Tor2 protein kinase-containing TORC2 complex, required for proper actin cytoskeleton organization (Loewith *et al*, 2002). Although TAP of Slm1 failed to indicate an interaction with Avo2, immunoprecipitation of Slm1-GFP from cells expressing Avo2-Myc showed that Avo2 could associate with Slm1. This interaction was extremely sensitive to salt conditions used during TAP, suggesting that Slm1 and Avo2 only weakly associate (Figure 6B). While mutations in the Slm domain of Slm1 failed to perturb

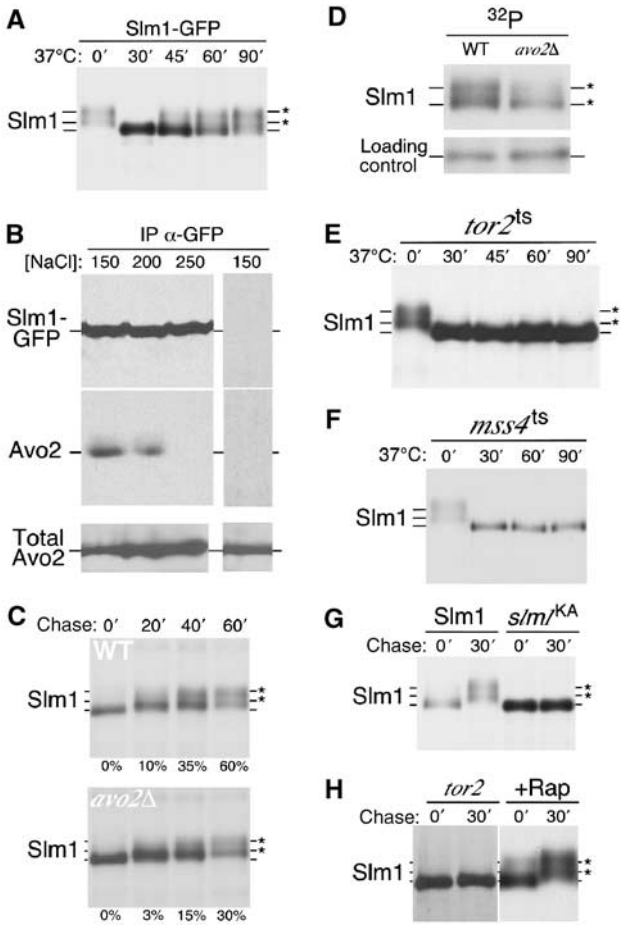

Figure 6 Slm1 associates with the TORC2 component Avo2 and depends on TORC2 for appropriate phosphorylation. (**A**) Wild-type cells expressing *SLM1-GFP* were metabolically labeled for 10 min with ^{35}S-labeled cysteine and methionine, chased for 30 min and shifted to 37°C for the indicated time. Extracts were immunoprecipitated with anti-GFP antibodies and subjected to SDS–PAGE analysis followed by autoradiography. (**B**) Extracts from cells expressing either *AVO2-MYC* alone or *AVO2-MYC* and *SLM1-GFP* were immunoprecipitated using α-GFP antibodies under varying salt conditions. Total Avo2 protein in the extract is shown below. (**C**) Wild-type or *avo2*Δ cells expressing *SLM1-GFP* were metabolically labeled as in Figure 5A. Below each gel is the percentage of dually phosphorylated Slm1-GFP as determined by densitometry. (**D**) Wild-type or *avo2*Δ cells expressing *SLM1-GFP* were metabolically labeled as in Figure 5C. (**E, F**) *tor2*^{ts} and *mss4*^{ts} cells expressing *SLM1-GFP* were metabolically labeled as in (A). (**G**) *slm1*Δ*slm2*Δ cells expressing *SLM1-GFP* or *slm1*^{KA}-GFP were treated as in Figure 5B. (**H**) Wild-type cells or cells grown for 16 h in glucose-containing media and expressing *TOR2* under the control of an integrated *GAL1-10* promoter (to repress expression of genomic *TOR2*) were metabolically labeled for 10 min with ^{35}S-labeled cysteine and methionine and chased for the indicated time in the presence or absence of rapamycin (200 ng/ml). Extracts were treated as in Figure 5A.

this interaction, mutations in the PH domain of Slm1 resulted in a reduced association between Slm1^{KA} and Avo2 (Supplementary Figure 3). However, the weakened interaction may be an indirect consequence of Slm1^{KA} mislocalization. Additionally, SGA analysis of the *mss4*^{ts} mutation revealed a genetic interaction with *AVO2*, which was confirmed in the SEY6210 background (Figure 1A and Supplementary Table I). Consistent with a role for TORC2 in phosphorylation of Slm1, *avo2*Δ cells exhibited a kinetic defect in Slm1 phosphorylation (Figure 6C and D). Moreover, *tor2*^{ts} cells failed to show recovery of Slm1 phosphorylation at the nonpermissive temperature, indicating that Tor2 function is required for this process (Figure 6E). Since PI4,5P$_2$ also plays a role in Slm1 recruitment/activation, we determined the effect of Mss4 inactivation on Slm1 phosphorylation. As in *tor2*^{ts} cells, Slm1 phosphorylation failed to recover in the absence of Mss4 activity, suggesting that PI4,5P$_2$ and Tor2 are both required for localization and phosphorylation of Slm1 (Figure 6F). Consistent with these data, the mutant form of Slm1 harboring mutations in its PH domain was not normally phosphorylated, likely due to its inability to target efficiently to the plasma membrane (Figure 6G).

Tor2 has previously been demonstrated to function in two different protein complexes, one required for the response to nutrient availability (TORC1), while the other regulates actin cytoskeleton organization (TORC2). Studies have also indicated that TORC1 is uniquely sensitive to the effects of the drug rapamycin (Loewith *et al*, 2002). To determine if Slm1 phosphorylation requires TORC1, we treated cells with rapamycin and examined phosphorylation of Slm1. Following a 30 min pretreatment with rapamycin, we found that Slm1 phosphorylation was unperturbed, suggesting that TORC2, but not TORC1, multiply phosphorylates Slm1 (Figure 6H). Consistent with these data, cells specifically repressed for *TOR2* expression showed a defect in Slm1 phosphorylation (Figure 6H).

TORC2 phosphorylates Slm1 and Slm2 in vitro and is partially required for Slm1 localization

To investigate further a role for TORC2 in phosphorylation of Slm1 and Slm2, recombinant forms of Slm1 and Slm2 were purified from bacteria and subjected to an *in vitro* kinase assay. In the presence of purified TORC2, both GST-Slm1 and GST-Slm2, but not GST alone, were phosphorylated, similar to a previously characterized mammalian TOR substrate, 4EBP1 (Figure 7A). However, purified TORC2 containing a kinase-dead form of Tor2 failed to phosphorylate Slm1 or Slm2, suggesting that a contaminant from the purification was not responsible for the observed kinase activity.

Finally, to investigate a function for Slm1 phosphorylation by TORC2, we tested whether the localization of Slm1 depends on Tor2 activity. Since our studies have shown a partial role for PI4,5P$_2$ in the localization of Slm1, we examined whether the remaining plasma membrane association of Slm1 was dependent on Tor2. Therefore, wild-type and *tor2*^{ts} cells expressing a mutant form of Slm1 that cannot interact with PI4,5P$_2$ (*slm1*^{KA}-GFP) were visualized following a 90 min shift to 37°C. In contrast to wild-type cells, *tor2*^{ts} cells failed to exhibit any localization of *slm1*^{KA}-GFP to the cell periphery. Cells lacking other plasma membrane-associated protein kinase activities, including *pkc1*^{ts}, *bck1*Δ, *slt2*Δ, *tpk1*Δ*tpk2*^{ts}*tpk3*Δ, *ypk1*^{ts}*ypk2*Δ, and *pkh1*^{ts}*pkh2*Δ cells, did not affect *slm1*^{KA}-GFP localization (Figure 7B and data not shown). Together, these data suggest that Slm1 and Slm2 function downstream of TORC2 in signaling to the actin cytoskeleton, and represent the first substrates of TORC2 protein kinase activity.

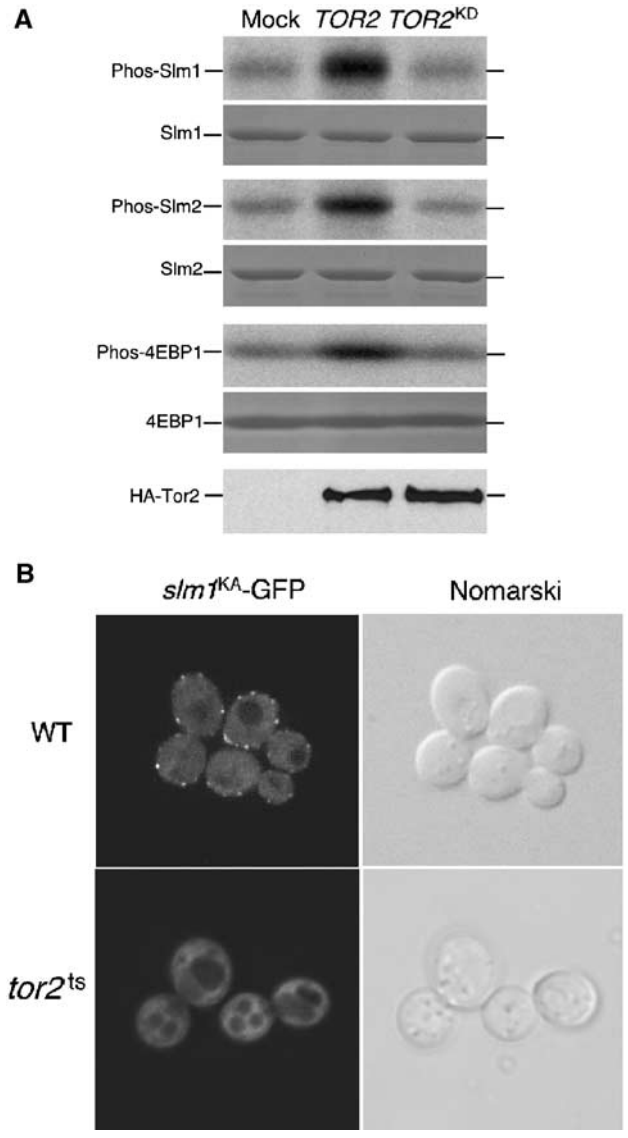

Figure 7 Slm1 phosphorylation is TORC2 dependent *in vitro*. (**A**) SW80-1D cells expressing *TOR2* under the control of an integrated *GAL1-10* promoter and TAP-tagged *AVO2* were transformed with either vector, (HA)$_3$*TOR2* (*TOR2*) or (HA)$_3$*TOR2*^{D2998E} (*TOR2*^{KD}). After growth for 16 h in glucose-containing media (to repress expression of genomic *TOR2*), TORC2 (via Avo2-TAP) was precipitated with IgG-Sepharose beads and tested for Slm1, Slm2, and 4EBP1 kinase activity. Shown are autoradiographs (Phos-Slm1, Phos-Slm2, Phos-4EBP1) and the corresponding Coomassie blue-stained region of the SDS–PAGE gel (Slm1, Slm2, 4EBP1). The bottom panel is a Western blot showing the amount of HA-tagged TOR2/TOR2^{KD} added to the respective kinase assays. (**B**) Wild-type or *tor2*^{ts} cells expressing *slm1*^{KA}-GFP were visualized by fluorescence microscopy following a 90 min shift to 37°C.

Discussion

We and others have previously shown that regulation of PI4,5P$_2$ synthesis at the plasma membrane is critical for regulation of the actin cytoskeleton, at least in part through activation of the Rho1 GTPase (Desrivières *et al* 1998; Homma *et al*, 1998; Audhya and Emr, 2002). Using an unbiased approach to identify additional factors involved in PI4,5P$_2$ signaling, we have identified two new PI4,5P$_2$ effectors, Slm1 and Slm2, which also appear to be critical for regulation of actin cytoskeleton organization. Furthermore, our screen showed that deletion of *AVO2*, which encodes a nonessential component of the Tor2 protein kinase-containing TOR complex 2, is lethal in combination with the *mss4*^{ts} mutation, suggesting that TORC2 and Mss4 function together in regulating actin organization. Consistent with this hypothesis, overexpression of *MSS4* suppresses a *tor2* mutation (Helliwell *et al*, 1998), and both Slm1 and Slm2 are substrates of the TORC2 kinase. Together, our data suggest the existence of multiple PI4,5P$_2$ effectors in yeast, whose actions can be coordinated through additional signaling events to regulate polarity of the actin cytoskeleton. Importantly, Slm1 and Slm2 are the first effectors identified that require both TORC2-dependent phosphorylation and Mss4-dependent recruitment to control the actin cytoskeleton, possibly through activation of Rho1. We speculate that Mss4 functions as a sensor for cell stress, such as heat shock, generating variable levels of PI4,5P$_2$ to alter actin polarization and cell wall integrity, while other inputs, such as TORC2 kinase regulation, further modulate the magnitude/duration of these changes.

Multiple roles for PI4,5P$_2$ revealed by synthetic genetic array analysis

Previous studies have established that PI4,5P$_2$ plays essential roles in both actin cytoskeleton organization and endocytosis, and several effectors of PI4,5P$_2$ have been identified that are important for these processes (Takenawa and Itoh, 2001). Using an unbiased, genome-wide-based approach in yeast, we have identified several new genes that may be involved in regulating PI4,5P$_2$ synthesis or that function downstream of PI4,5P$_2$ (Figure 8). When combined with the *mss4*^{ts} mutation, several components of two protein chaperone complexes, the Hsp70–Hsp90 complex and the GIMC complex (Siegers *et al*,

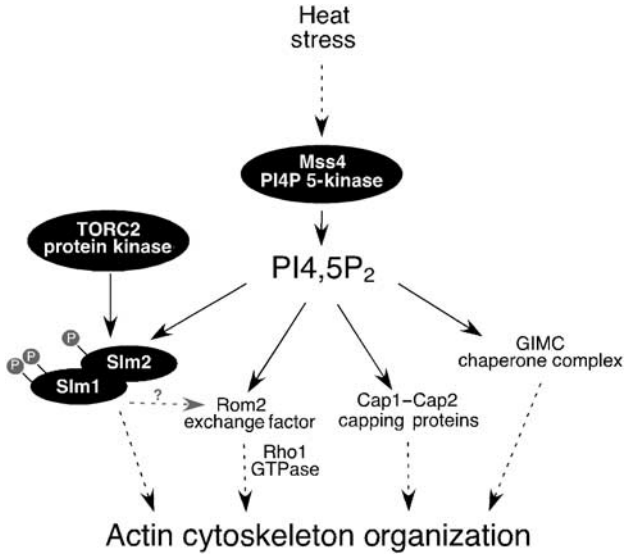

Figure 8 Regulation of actin cytoskeleton organization by PI4,5P$_2$. Genetic interactions identified in the Mss4 SGA analysis indicate multiple roles for Mss4-mediated PI4,5P$_2$ production in actin organization. In particular, PI4,5P$_2$ recruitment/activation of the Slm1–Slm2 complex is required for maintenance of actin cytoskeleton polarity. Additional factors including TORC2 protein kinase activity further modulate and specify Slm1–Slm2 function in actin organization.

1999), became essential for cell viability, suggesting a role for PI4,5P$_2$ in the response to heat shock. Consistent with this interpretation, studies have indicated that PI4,5P$_2$ levels transiently increase following heat shock, potentially required for preventing protein misfolding through activation of a chaperone complex (Desrivières *et al*, 1998; Audhya *et al*, 2000). However, further studies are required to determine whether PI4,5P$_2$ influences chaperone function during heat stress.

Additionally, the SGA analysis uncovered a number of proteins directly involved in actin organization and cell polarity. Of particular interest was the identification of three proteins that harbor PH domains, Rom2, Boi2, and Slm1. Rom2, an exchange factor for the Rho1 GTPase, has been shown to bind PI4,5P$_2$ and function downstream of Mss4 in activation of the Rho1–Pkc1 pathway (Audhya and Emr, 2002). Similarly, the PH domain of Boi2, a protein important for polarized growth and bud formation, has been shown to interact with phosphoinositides *in vitro* (Yu *et al*, 2004). Additionally, a functionally redundant homolog of Boi2, Boi1, has been shown to interact with PI4,5P$_2$ via its PH domain, and this lipid–protein interaction in part mediates its normal localization and is essential in the absence of Boi2 (Hallett *et al*, 2002).

Beyond the identification of known PI4,5P$_2$ binding proteins, SGA analysis also identified a new PI4,5P$_2$ effector, Slm1. Together with its homolog and binding partner Slm2, these proteins are essential for maintaining proper actin cytoskeleton organization and cell wall integrity, possibly through regulation of the Rho1 GTPase (Figure 8). However, we were unable to identify a direct interaction between Slm1 or Slm2 and known regulators of Rho1, including its exchange factors (Tus1, Rom1, and Rom2) or its activating proteins (Sac7, Bem2, and Bem3). In an alternative model, Slm1 and Slm2 may function in parallel to Rho1 in regulation of actin organization. Genome-wide two-hybrid analysis suggests that Slm2 interacts with both subunits of calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase required during environmental stress, which may function in actin organization (Uetz *et al*, 2000; Ito *et al*, 2001; Cyert, 2003). Further studies will be necessary to determine the extent to which calcineurin may function in Slm1/Slm2 signaling.

Coordination of Mss4 and the TORC2 protein kinase in actin organization

Previous studies in organisms ranging from insects to humans have indicated a relationship between growth factor-stimulated phosphoinositide lipid signaling and nutrient-dependent TOR protein kinase signaling in the regulation of cell growth (Jacinto and Hall, 2003). We have found that this interaction may also extend to yeast, where PI4,5P$_2$ and Tor2 both function to control the actin cytoskeleton. Yeast express two forms of TOR: TORC1, which contains either Tor1 or Tor2, mediates rapamycin-sensitive growth in response to nutrients, while TORC2, which contains Tor2 but not Tor1, controls polarization of the actin cytoskeleton. However, substrates of TORC2 that affect actin organization have remained elusive. Our findings support a role for Slm1 and Slm2 in this capacity. Additionally, the physical interaction between Slm1 and Avo2, the only nonessential component of either TORC complex, suggests a role for this component of TORC2 in the recruitment of effector proteins. In the absence of Avo2, PI4,5P$_2$ is likely to be sufficient for recruitment of Slm1 and Slm2 to sites of TORC2 activity. However, loss of multiple inputs important for TORC2-dependent phosphorylation cannot be tolerated, as demonstrated by the lethality exhibited by *mss4*^{ts}*avo2*Δ double-mutant cells. Interestingly, Rom2, an exchange factor for Rho1, has also been identified as an effector of PI4,5P$_2$ and potentially Tor2, suggesting that integration of phosphoinositide-dependent recruitment and regulation by TOR may not be limited to Slm1 and Slm2.

Our analysis has successfully identified two novel proteins that function as essential regulators of actin polarity downstream of PI4,5P$_2$. We speculate that under conditions of cell stress such as heat shock, elevated PI4,5P$_2$ levels result in the increased recruitment and/or activation of effector proteins including Slm1, Slm2, and Rom2, all necessary for regulation of actin cytoskeleton organization. Moreover, to ensure both rapid and specific responses to cell stress, additional factors are necessary. In the case of PI4,5P$_2$-dependent Rom2 recruitment/activation to the cell cortex, the integral plasma membrane cell surface sensors, Wsc1 and Mid2, appear to serve this function (Audhya and Emr, 2002). Correspondingly, we have found that PI4,5P$_2$-dependent Slm1 and Slm2 recruitment requires the parallel activity of the TORC2 protein kinase complex, potentially also dependent on conditions of nutrient availability. Interestingly, following heat shock, the levels of Slm1 and Slm2 phosphorylation were diminished, likely due to reduced TORC2 activity and/or elevated protein phosphatase activity. This decreased level of Slm1 and Slm2 phosphorylation may be important for appropriate depolarization of the actin cytoskeleton and secretion observed following cell stress. Strikingly, phosphorylation of Slm1 and Slm2 recovered in parallel to actin repolarization, suggesting a link between Slm1 and Slm2 activity and actin cytoskeleton organization. Collectively, these data indicate that PI4,5P$_2$ functions together with both positive and negative control elements in a cellular stress response system necessary for organizing the actin cytoskeleton.

Materials and methods

Strains and media

Enzymes used for recombinant DNA techniques were used as recommended by the manufacturer. Standard recombinant DNA techniques and yeast genetics methods were performed, and growth media used have been described elsewhere (Maniatis *et al*, 1982; Gaynor *et al*, 1994). *S. cerevisiae* strains used in this study are listed in Table III. All gene disruptions and temperature-sensitive mutant strains were generated similarly, as described previously (Audhya and Emr, 2002). Integrated epitope tagging was performed as described previously (Longtine *et al*, 1998).

Synthetic genetic array analysis and yeast plasmids

SGA analysis was performed as described previously (Tong *et al*, 2001). Strains expressing temperature conditional alleles of *slm1* were generated similarly as described using error-prone PCR (Audhya *et al*, 2000). All point mutations were created using site-directed mutagenesis.

Metabolic labeling and immunoprecipitation

Cell labeling and immunoprecipitations were performed as described previously (Gaynor *et al*, 1994; Audhya *et al*, 2000, 2002). For ^{32}P labeling, cells were grown in low phosphate media to early log phase and labeled with 1 mCi ^{32}P-labeled orthophosphate in the absence of phosphate. Treatment with shrimp alkaline phosphatase and immunoprecipitation with antiserum against GFP have been described previously (Audhya and Emr, 2003).

Table III *S. cerevisiae* strains used in this study

Strain	Genotype	Reference or source
SEY6210	MATα *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al* (1988)
SEY6210.1	MATa *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al* (1988)
AAY202	SEY6210; *mss4Δ::HIS3MX6* carrying Ycplac*mss4-102* (*LEU2 CEN6 mss4-102*)	Stefan *et al* (2002)
SW80-1D	MATa *leu2 ura3 rme1 trp1 his3Δ* [*KANMX*] GAL1p::*TOR2 AVO2-TAP:HISMX*	This study
AAY1602	SEY6210; *slm1Δ::HIS3*	This study
AAY1608	AAY202; *slm1Δ::HIS3*	This study
AAY1610	SEY6210; *slm2Δ::HIS3*	This study
AAY1622	SEY6120; *slm1Δ::HIS3 slm2Δ::HIS3* carrying pRS415*slm1-1* (*LEU2 CEN6 slm1-1*)	This study
AAY1623	SEY6120; *slm1Δ::HIS3 slm2Δ::HIS3* carrying pRS415*slm1-2* (*LEU2 CEN6 slm1-2*)	This study
AAY1624	SEY6120; *slm1Δ::HIS3 slm2Δ::HIS3* carrying pRS415*slm1-3* (*LEU2 CEN6 slm1-3*)	This study
AAY1625	SEY6120; *slm1Δ::HIS3 slm2Δ::HIS3* carrying pRS415*slm1-4* (*LEU2 CEN6 slm1-4*)	This study
AAY1626	SEY6120; *slm1Δ::HIS3 slm2Δ::HIS3* carrying pRS415*slm1-5* (*LEU2 CEN6 slm1-5*)	This study
AAY1627	SEY6210; *SLM1-GFP:HIS3MX6*	This study
AAY1628	SEY6210; *SLM2-GFP:HIS3MX6*	This study
AAY1629	AAY1602; *SLM2-GFP:HIS3MX6*	This study
AAY1630	AAY1610; *SLM1-GFP:HIS3MX6*	This study
AAY1633	SEY6120; *slm1Δ::HIS3 slm2Δ::HIS3* carrying pRS415*slm1*^{KA} (*LEU2 CEN6 slm1*^{KA})	This study
AAY1635	AAY202; *SLM1-GFP:HIS3MX6*	This study
AAY1651	SEY6210; *GFP-SNC1:URA3*	This study
AAY1652	AAY1622; *GFP-SNC1:URA3*	This study
AAY1661	AAY1622; *sac7Δ::HIS3*	This study
AAY1663	SEY6210; *slm1Δ::HIS3 slm2Δ::HIS3 sac7Δ::HIS3*	This study
AAY1671	SEY6210; *AVO2-13MYC:HIS3MX6*	This study
AAY1674	AAY1627; *AVO2-13MYC:HISMX6*	This study
AAY1681	SEY6210; *avo2Δ::HIS3*	This study
AAY1683	AAY202; *avo2Δ::HIS3*	This study
AAY1692	AAY1628; *slm1Δ::HIS3*	This study
AAY1701	SEY6210; *ABP1-RFP::HIS3*	This study
AAY1709	SEY6210; *ABP140-GFP::HIS3*	This study
AAY1804	AAY1622; *sec4-8*	This study

Fluorescence microscopy

All fluorescence images were observed using a Zeiss Axiovert S1002TV fluorescent microscope and subsequently processed using a Delta Vision deconvolution system. For actin localization, cells were fixed and stained with rhodamine–phalloidin (Molecular Probes) as described previously (Audhya *et al*, 2000). To depolymerize F-actin, latrunculin A was added from a 10 mM DMSO stock to a final concentration of 200 μM for 30 min.

Tandem affinity purification and mass spectrometric analysis

TAP of Slm1 was performed as described previously (Cheeseman *et al*, 2002). Proteins in the SDS gel were stained by silver and protein bands were excised and digested by trypsin overnight. Peptides were extracted and analyzed by microcapillary liquid chromotography and tandem mass spectrometry. An in-house MS system consisting of an HPLC and LCQ-ion trap mass spectrometer (Thermo Finnigan) was used. All identified peptides were further manually inspected and verified.

TORC2 purification and in vitro kinase assay

SW80-1D cells (5 l) expressing either vector alone, *HA-TOR2*, or a kinase-inactive form of *HA-TOR* denoted *HA-TOR2*^{KD} (Jiang and Broach, 1999) pregrown to saturation in synthetic raffinose/glycerol medium were additionally grown at 30°C for 16 h in media containing glucose. Cells were lysed as described previously (Loewith *et al*, 2002). Pooled lysates were cleared, normalized to ~50 ml and ~375 mg protein and passed over 125 μl of Sepharose CL-4B (Sigma) previously equilibrated in lysis buffer. To the flow through was added 150 μl IgG-Sepharose (Amersham Bioscience) for 2 h. IgG beads were then incubated with either ~5 μg GST, ~2 μg GST-Slm2, ~2 μg GST-Slm1, or 2 μg PHAS-I (Stratagene) in 50 μl of lysis buffer with 20% glycerol. In all, 6 μl of 10× buffer (40 mM $MnCl_2$, 100 mM dithiothreitol, 10× Roche protease inhibitor cocktail–EDTA, 100 mM NaN_3, 100 mM NaF, 100 mM *p*-nitrophenylphosphate, 100 mM β-glycerophosphate) was added before the reaction was started with the addition of 4 μl of ATP mix (1.2 mM ATP, 2.5 μCi/μl [γ^{32}P]ATP (3000 Ci/mmol) in kinase assay buffer). Samples were then subjected to SDS–PAGE.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

Abe M, Qadota H, Hirata A, Ohya Y (2003) Lack of GTP-bound Rho1p in secretory vesicles of *Saccharomyces cerevisiae*. *J Cell Biol* **162:** 85–97

Audhya A, Foti M, Emr SD (2000) Distinct roles for the yeast phosphatidylinositol 4-kinases, Stt4p and Pik1p, in secretion, cell growth, and organelle membrane dynamics. *Mol Biol Cell* **11:** 2673–2689

Audhya A, Emr SD (2002) Stt4 PI 4-kinase localizes to the plasma membrane and functions in the Pkc1-mediated MAP kinase cascade. *Dev Cell* **2:** 593–605

Audhya A, Emr SD (2003) Regulation of PI4,5p$_2$ synthesis by nuclear-cytoplasmic shuttling of the Mss4 lipid kinase. *EMBO J* **22:** 4223–4236

Cheeseman IM, Anderson S, Jwa M, Green EM, Kang J, Yates III JR, Chan CS, Drubin DG, Barnes G (2002) Phospho-regulation of kinetochore–microtubule attachments by the Aurora Kinase Ipl1. *Cell* **111:** 163–172

Cifuentes ME, Honkanen L, Rebecchi MJ (1993) Proteolytic fragments of phosphoinositide-specific phospholipase C-delta 1. Catalytic and membrane binding properties. *J Biol Chem* **268:** 11586–11593

Cyert MS (2003) Calcineurin signaling in *Saccharomyces cerevisiae*: how yeast go crazy in response to stress. *Biochem Biophys Res Commun* **411:** 1143–1150

Delley PA, Hall MN (1999) Cell wall stress depolarizes cell growth via hyperactivation of RHO1. *J Cell Biol* **147:** 163–174

Desrivières S, Cooke FT, Parker PJ, Hall MN (1998) MSS4, a phosphatidylinositol-4-phosphate 5-kinase required for organization of the actin cytoskeleton in *Saccharomyces cerevisiae*. *J Biol Chem* **273:** 15787–15793

Fruman DA, Meyers RE, Cantley LC (1998) Phosphoinositide kinases. *Annu Rev Biochem* **67:** 481–507

Gaynor EC, Heesen ST, Graham TR, Aebi M, Emr SD (1994) Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. *J Cell Biol* **127:** 653–665

Hallett MA, Lo HS, Bender A (2002) Probing the importance of potential roles of the binding of the PH-domain protein Boi1 to acidic phospholipids. *BMC Cell Biol* **3:** 16

Helliwell SB, Howald I, Barbet N, Hall MN (1998) TOR2 is part of two related signaling pathways coordinating cell growth in *Saccharomyces cerevisiae*. *Genetics* **148:** 99–112

Homma K, Terui S, Minemura M, Qadota H, Anraku Y, Kanaho Y, Ohya Y (1998) Phosphatidylinositol-4-phosphate 5-kinase localized on the plasma membrane is essential for yeast cell morphogenesis. *J Biol Chem* **273:** 15779–15786

Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M, Sakaki Y (2001) A comprehensive two-hybrid analysis to explore the yeast protein ineractome. *Proc Natl Acad Sci USA* **98:** 4569–4574

Jacinto E, Hall MN (2003) Tor signaling in bugs, brain and brawn. *Nat Rev Mol Cell Biol* **4:** 117–126

Jiang Y, Broach JR (1999) Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast. *EMBO J* **18:** 2782–2792

Lemmon MA, Ferguson KM (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains. *Biochem J* **350:** 1–18

Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* **10:** 457–468

Longtine MS, McKenzie A, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* **14:** 953–961

Maniatis T, Fritsch EF, Sambrook J (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Press

Mulholland J, Wesp A, Riezman H, Botstein D (1997) Yeast actin cytoskeleton mutants accumulate a new class of Golgi-derived secretary vesicle. *Mol Biol Cell* **8:** 1481–1499

Odorizzi G, Babst M, Emr SD (2000) Phosphoinositide signaling and the regulation of membrane trafficking in yeast. *TIBS* **25:** 229–235

Pruyne DW, Schott DH, Bretscher A (1998) Tropomyosin-containing actin cables direct the Myo2p-dependent polarized delivery of secretory vesicles in budding yeast. *J Cell Biol* **143:** 1931–1945

Reinke A, Anderson S, McCaffery JM, Yates III JR, Aronova S, Chu S, Fairclough S, Iverson C, Wedaman KP, Powers T (2004) TOR complex 1 includes a novel component, Tco89p (YPL180w), and cooperates with Ssd1p to maintain cellular integrity in *Saccharomyces cerevisiae*. *J Biol Chem* **279:** 14752–14762

Schmidt A, Bickle M, Beck T, Hall MN (1997) The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell* **88:** 531–542

Siegers K, Waldmann T, Leroux MR, Grein K, Shevchenko A, Schiebel E, Hartl FU (1999) Compartmentation of protein folding *in vivo*: sequestration of non-native polypeptide by the chaperonin–GimC system. *EMBO J* **18:** 75–84

Stauffer TP, Ahn S, Meyer T (1998) Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P$_2$ concentration monitored in living cells. *Curr Biol* **8:** 343–346

Stefan CJ, Audhya A, Emr SD (2002) The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-bisphosphate. *Mol Biol Cell* **13:** 542–557

Takenawa T, Itoh T (2001) Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. *Biochim Biophys Acta* **1533:** 190–206

Tong AH, Evangelista M, Parsons AB, Xu H, Bader GD, Page N, Robinson M, Raghibizadeh S, Hogue CW, Bussey H, Andrews B, Tyers M, Boone C (2001) Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science* **294:** 2364–2368

Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, Lockshon D, Narayan V, Srinivasan M, Pochart P, Qureshi-Emili A, Li Y, Godwin B, Conover D, Kalbfleisch T, Vijayadamodar G, Yang M, Johnston M, Fields S, Rothberg JM (2000) A comprehensive analysis of protein–protein interactions in *Saccharomyces cerevisiae*. *Nature* **403:** 623–627

Wedaman KP, Reinke A, Anderson S, Yates III JR, McCaffery JM, Powers T (2003) Tor kinases are in distinct membrane-associated protein complexes in *Saccharomyces cerevisiae*. *Mol Biol Cell* **14:** 1204–1220

Yang HC, Pon LA (2002) Actin cable dynamics in budding yeast. *Proc Natl Acad Sci USA* **99:** 751–756

Yu JW, Mendrola JM, Audhya A, Singh S, Keleti D, DeWald DB, Murray D, Emr SD, Lemmon MA (2004) Genome-wide analysis of membrane targeting by *S. cerevisiae* pleckstrin homology (PH) domains. *Mol Cell* **13:** 677–688