TORC2 Signaling Is Antagonized by Protein Phosphatase 2A and the Far Complex in *Saccharomyces cerevisiae*

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ABSTRACT The target of rapamycin (TOR) kinase, a central regulator of eukaryotic cell growth, exists in two essential, yet distinct, TOR kinase complexes in the budding yeast *Saccharomyces cerevisiae*: rapamycin-sensitive TORC1 and rapamycin-insensitive TORC2. Lst8, a component of both TOR complexes, is essential for cell viability. However, it is unclear whether the essential function of Lst8 is linked to TORC1, TORC2, or both. To that end, we carried out a genetic screen to isolate *lst8* deletion suppressor mutants. Here we report that mutations in *SAC7* and *FAR11* suppress lethality of *lst8*Δ and TORC2-deficient (*tor2-21*) mutations but not TORC1 inactivation, suggesting that the essential function of Lst8 is linked only to TORC2. More importantly, characterization of *lst8*Δ bypass mutants reveals a role for protein phosphatase 2A (PP2A) in the regulation of TORC2 signaling. We show that Far11, a member of the Far3-7-8-9-10-11 complex involved in pheromone-induced cell cycle arrest, interacts with Tpd3 and Pph21, conserved components of PP2A, and deletions of components of the Far3-7-8-9-10-11 complex and PP2A rescue growth defects in *lst8*Δ and *tor2-21* mutants. In addition, loss of the regulatory B′ subunit of PP2A Rts1 or Far11 restores phosphorylation to the TORC2 substrate Slm1 in a *tor2-21* mutant. Mammalian Far11 orthologs FAM40A/B exist in a complex with PP2A known as STRIPAK, suggesting a conserved functional association of PP2A and Far11. Antagonism of TORC2 signaling by PP2A-Far11 represents a novel regulatory mechanism for controlling spatial cell growth of yeast.

THE target of rapamycin (TOR) kinase is a phosphatidylinositol kinase-related protein kinase that controls eukaryotic cell growth and proliferation in response to nutrient conditions (Inoki *et al.* 2005; Wullschleger *et al.* 2006; Zoncu *et al.* 2011). The TOR kinase is inhibited by the complex of rapamycin and Fpr1, a peptidyl-prolyl *cis–trans* isomerase. The TOR kinase is conserved in eukaryotes. Unlike fungal species, which may possess two TOR kinases, higher eukaryotes such as humans possess only one. The TOR kinase exists in multi-protein complexes, which have been purified from many different eukaryotic systems. There exist two distinct TOR kinase complexes. In yeast, rapamycin-sensitive TORC1 consists of Tor1 or Tor2, Lst8, Kog1, and Tco89, and rapamycin-insensitive TORC2 consists of Tor2, Lst8, Avo1, Avo2, Avo3, and Bit61 (Loewith *et al.* 2002; Wedaman *et al.* 2003; Reinke *et al.* 2004). Both complexes are partially conserved in mammals: mTORC1 contains the yeast Kog1 ortholog raptor, while mTORC2 contains mSin1 and rictor, orthologs of yeast Avo1 and Avo3, respectively; GβL, the ortholog of yeast Lst8, exists in both mTORC1 and mTORC2 (Zoncu *et al.* 2011).

TOR regulates cell growth by sensing and responding to changes in nutrient conditions (Schmelzle and Hall 2000). TORC1 has an essential function involving the regulation of cell growth that is carried out when either Tor1 or Tor2 is in the complex. Under favorable growth conditions, TORC1 promotes cell growth by maintaining robust ribosome biogenesis (Marion *et al.* 2004; Martin *et al.* 2004; Zurita-Martinez and Cardenas 2005). When TORC1 is inactive, there is a dramatic downregulation of general protein translation, an upregulation of autophagy, accumulation of the storage carbohydrate glycogen, increased sorting and turnover of amino acid permeases, and activation of stress-responsive transcription factors via nuclear translocation (Wullschleger *et al.* 2006). TOR inhibition via rapamycin treatment activates a subset of stress-responsive transcription factors (Beck and Hall 1999; Cardenas *et al.* 1999; Shamji *et al.* 2000; Cooper 2002; Chen and Kaiser 2003). Rapamycin treatment can also lead to

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reduced gene expression, including encoding ribosomal proteins (RP) (Wullschleger *et al.* 2006).

TORC2 has a separate essential function that is Tor2 specific, which involves cell cycle-dependent polarization of the actin cytoskeleton (Cybulski and Hall 2009). TORC2 mediates the organization of the actin cytoskeleton through the activation of a Rho1/2p GTPase switch, composed of the Rho GTPases Rho1 and Rho2, the Rho GDP-GTP exchange factor Rom2, and the Rho GTPase-activating protein Sac7. Activated GTP-bound Rho1 activates Pkc1, which activates the cell-wall integrity pathway MAP kinase cascade, Bck1-Mkk1/2-Mpk1. Activation of Rho1 and the cell-wall integrity pathway restores cell growth and actin polarization to *tor2* mutant cells. *sac7* mutations suppress TORC2 deficiency by increasing the levels of GTP-bound Rho1. How TORC2 mediates the organization of the actin cytoskeleton is unclear and might involve three TORC2 substrates: Slm1, Slm2, and Ypk2 (Audhya *et al.* 2004; Fadri *et al.* 2005; Kamada *et al.* 2005; Tabuchi *et al.* 2006; Aronova *et al.* 2008).

Lst8 is essential for cell viability in *Saccharomyces cerevisiae* (Roberg *et al.* 1997). It is unknown whether the essential function of Lst8 is linked to TORC1, TORC2, or both. In TORC2, Lst8 binds to the C-terminal kinase domain of Tor2, independently of Avo1/2/3, and Lst8 depletion destabilizes the interaction between Tor2 and Avo2 or Avo3 (Wullschleger *et al.* 2005). Lst8 is also required for full Tor2 kinase activity *in vitro*, and its depletion leads to a depolarized actin cytoskeleton similar to *tor2* mutations (Loewith *et al.* 2002; Wullschleger *et al.* 2005). Overexpression of *MSS4* encoding a phosphatidylinositol-4-phosphate 5-kinase, *RHO1/2*, *ROM2*, *PKC1*, *MKK1*, or *BCK1* suppresses *tor2* and *avo1* mutations (Helliwell *et al.* 1998a,b; Loewith *et al.* 2002); however, these suppressors were reported to be unable to suppress an *lst8* mutation (Loewith *et al.* 2002). The question remains whether the essential function of Lst8 is linked to TORC2, and the role of Lst8 in TORC1 is largely unknown.

Here, we provide evidence that the essential function of Lst8 is linked to TORC2, but not to TORC1. We show that components of the Far3-7-8-9-10-11 complex, which have been implicated in pheromone-induced cell cycle arrest, vacuolar protein sorting, and cell fitness, negatively regulate TORC2 signaling. We find that Far11 interacts with protein phosphatase 2A and that mutations in the PP2A-Rts1 subcomplex suppress TORC2 deficiency. We propose that the Far3-7-8-9-10-11 complex and PP2A-Rts1 antagonize TORC2 signaling by promoting dephosphorylation of TORC2 substrates.

Materials and Methods

Strains, plasmids, and growth media and growth conditions

Yeast strains and plasmids used in this study are listed in Table 1, Table 2, Table S2, and Table S3. Yeast cells were grown at 30° or 37° in SD (0.67% yeast nitrogen base plus 2% dextrose), YNBcasD (SD medium plus 1% casamino acids), Ura Leu dropout (SD plus Complete Supplement Mixture without uracil and leucine; Bio101), or YPD (1% yeast extract, 2% peptone, 2% dextrose) medium as indicated in the text and in the figure legends. For *lst8*Δ bypass assays, SD medium with or without 1 g/liter 5-fluroorotic acid was used to select for growth of cells that have lost *URA3* plasmids. When necessary, amino acids, adenine, and/or uracil were added to the growth medium at standard concentrations to cover auxotrophic requirements (Amberg *et al.* 2005).

Transposon mutagenesis

*lst8*Δ mutant bypass genetic screens were conducted using transposon mutagenesis as described (Liu *et al.* 2001). Briefly, *ade2*Δ lst8Δ cells carrying plasmid pRS412-LST8 were used for transposon mutagenesis; after mutagenesis, cells were plated on YPD medium to select for colonies that were red or sectoring, indicating loss of the pRS412-LST8 plasmid. Putative *lst8*Δ bypass mutants were confirmed to be recessive, single-gene mutations using standard yeast genetic techniques. Determination of transposon insertion sites were carried out as described (Burns *et al.* 1994). Briefly, a recovery plasmid encoding the ampicillin-resistance gene (Amp^R) and *URA3* was integrated into the transposon integration site of the *leb1* and *leb2* mutants by homologous recombination, and transformants were selected on uracil-dropout medium. Genomic DNA was then isolated, digested with *Eco*RI, and ligated with T4 DNA ligase. Ligation products were transformed into bacteria, and Amp^R transformants were selected for on LB broth supplemented with ampicillin. Transposon integration sites were determined by sequencing of the recovered plasmids.

Northern blotting

Total cellular RNAs were isolated using a hot phenol method as described (Giannattasio *et al.* 2005). Cells were grown in appropriate medium to $\sim OD_{600}$ 0.6 and collected for isolation of total cellular RNA. ^{32}P-labeled probes against *RPL3*, *RPS6A*, and *ACT1* were used to probe mRNA immobilized on nylon membranes. PhosphorImager was used to record signals of the RNA transcripts.

Cellular extract preparation, immunoblotting, and immunoprecipitation

Total cellular protein extracts were prepared by disrupting yeast cells in extraction buffer (1.85 N NaOH–7.5% β-mercaptoethanol) followed by precipitation with trichloroacetic acid as described (Yaffe and Schatz 1984). Phosphatase treatment of total cellular proteins was conducted as described (Liu *et al.* 2008). For co-immunoprecipitation experiments, cellular lysates were prepared in immunoprecipitation (IP) buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.5% Triton X-100, and protease inhibitors). Cell extracts (~3 mg proteins) were incubated at 4° for 1 hr with anti-myc antibody (9E10; Roche), after which 30 μl of a 50% slurry of protein G-Sepharose (Roche) was added to each sample, and the samples were further incubated at 4° for 2 hr. Washed immunoprecipitates bound to the sepharose beads were released by boiling in 1× SDS-PAGE loading buffer. The released immune

Table 1 Yeast strains used in this study

Strain	Genotype	Source	Application
ZLY2254	*MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0* lst8::*kanMX4 ade2Δ::HIS3 [pRS412-LST8]*	This study	
ZLY3081 (WT)	*MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0* lst8::*kanMX4 ade2Δ::HIS3 [pRS412-LST8]*	This study	Figure 1
TPY104 (*leb1*)	*MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0* lst8::*kanMX4 ade2Δ::HIS3 leb1::Tn3::LEU2 [pRS412-LST8]*	This study	Figure 1
TPY103 (*leb2*)	*MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 lst8::kanMX4 ade2Δ::HIS3 leb2::Tn3::LEU2 [pRS412-LST8]*	This study	Figure 1
ZLY423 (WT)	*MAT***a** *ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 [pRS412-LST8]*	This study	Figure 2A
TPY115 (*far11 lst8*)	*MAT***a** *ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 far11::kanMX4*	This study	Figure 2A
TPY114 (*far11*)	*MAT***a** *ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 far11::kanMX4 [pRS412-LST8]*	This study	Figure 2A
TPY122 (*fpr1*)	*MAT***a** *ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 fpr1::kanMX4 [pRS412-LST8]*	This study	Figure 2A
ZLY2405 (*sac7 lst8*)	*MAT***a** *ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 sac7::kanMX4*	This study	Figure 2A
ZLY2404 (*sac7*)	*MAT***a** *ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 sac7::kanMX4 [pRS412-LST8]*	This study	Figure 2, A and B
ZLY2845 (*sac7 lst8*)	*MAT***a** *ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 sac7::kanMX4 [pRS412]*	This study	Figure 2B
TWY680	*MAT***a** *AVO3-GFP::kanR ura3 trp1 leu2 his3 ade2 can1-100*	Berchtold and Walther (2009)	
TWY696	*MAT***a** *BIT61-GFP::HIS ura3 trp1 leu2 his3 ade2 can1-100*		
TWY748	*MAT***a** *KOG1-GFP::HIS ura3 trp1 leu2 his3 ade2 can1-100*		
TPY1264 (WT, no GFP)	*MAT***a** *sac7::kanMX4 far11::TRP1 ura3 leu2 his3 ade2 trp1 can1-100*	This study	Figure 2C
TPY1266 (lst8, no GFP)	*MAT***a** *sac7::HIS3 far11::TRP1 lst8::LEU2 ura3 leu2 his3 ade2 trp1 can1-100*	This study	Figure 2C
TPY369 (*AVO3-GFP*)	*MAT***a** *AVO3-GFP::kanR ura3 trp1 leu2 his3 ade2 can1-100 sac7::HIS3 far11::TRP1*	This study	Figure 2
TPY407 (*lst8 AVO3-GFP*)	*MAT***a** *AVO3-GFP::kanR ura3 trp1 leu2 his3 ade2 can1-100 sac7::HIS3 far11::TRP1 lst8::LEU2*	This study	Figure 2
TPY358 (*BIT61-GFP*)	*MAT***a** *BIT61-GFP::HIS ura3 trp1 leu2 his3 ade2 can1-100 sac7::kanMX4 far11::TRP1*	This study	Figure 2C
TPY366 (*lst8 BIT61-GFP*)	*MAT***a** *BIT61-GFP::HIS ura3 trp1 leu2 his3 ade2 can1-100 sac7::kanMX4 far11::TRP1 lst8::LEU2*	This study	Figure 2C
TPY371 (*KOG1-GFP*)	*MAT***a** *KOG1-GFP::HIS ura3 trp1 leu2 his3 ade2 can1-100 sac7::kanMX4 far11::TRP1*	This study	Figure 2C
TPY413 (*lst8 KOG1-GFP*)	*MAT***a** *KOG1-GFP::HIS ura3 trp1 leu2 his3 ade2 can1-100 sac7::kanMX4 far11::TRP1 lst8::LEU2*	This study	Figure 2C
SY2227	*MAT***a** *ade1-1 leu2-2,113 trp1 ura3-52 bar1 HIS3::pFUS1::HIS3 mfa2-Δ1::FUS1-lacZ* rad16::*pGAL1::STE4*	Kemp and Sprague (2003)	
SY4078	SY2227 *FAR7-myc13-KAN <pSL2771>*		
SY4079	SY2227 *FAR8-myc13-KAN <pSL2771>*		
SY4080	SY2227 *FAR9-myc13-KAN <pSL2771>*		
SY4081	SY2227 *FAR10-myc13-KAN <pSL2771>*		
SY4082	SY2227 *FAR11-myc13-KAN <pSL2771>*		
TPY978	SY2227 *FAR7-myc13-KAN [pRS416-FAR11-HA]*	This study	Figure 3A
TPY981	SY2227 *FAR8-myc13-KAN [pRS416-FAR11-HA]*	This study	Figure 3A
TPY1001	SY2227 *FAR9-myc13-KAN [pRS416-FAR11-HA]*	This study	Figure 3A
TPY1002	SY2227 *FAR10-myc13-KAN [pRS416-FAR11-HA]*	This study	Figure 3A
TPY1003	SY2227 *FAR11-myc13-KAN [pRS416-FAR11-HA]*	This study	Figure 3A

(continued)

Table 1, *continued*

Strain	Genotype	Source	Application
RBY231 (WT)	*MAT*α *ura3 leu2 lys2 [pRS416-LST8]*	This study	Figure 3B, Figure 5B
RBY223 (*lst8*)	*MAT*α *ura3 leu2 lys2 lst8::LEU2 [pRS416-LST8]*	This study	Figure 3B, Figure 5B
MOY142 (*lst8 far3*)	*MAT*α *ura3 leu2 lys2 lst8::LEU2 far3::kanMX4 [pRS416-LST8]*	This study	Figure 3B
MOY145 (*lst8 far7*)	*MAT*α *ura3 leu2 lys2 lst8::LEU2 far7::kanMX4 [pRS416-LST8]*	This study	Figure 3B
MOY146 (*lst8 far8*)	*MAT*α *ura3 leu2 lys2 lst8::LEU2 far8::kanMX4 [pRS416-LST8]*	This study	Figure 3B
MOY169 (*lst8 far9*)	*MAT*α *ura3 leu2 lys2 lst8::LEU2 far9::kanMX4 [pRS416-LST8]*	This study	Figure 3B
MOY149 (*lst8 far10*)	*MAT*α *ura3 leu2 lys2 lst8::LEU2 far10::kanMX4 [pRS416-LST8]*	This study	Figure 3B
MOY150 (*lst8 far11*)	*MAT*α *ura3 leu2 lys2 lst8::LEU2 far11::kanMX4 [pRS416-LST8]*	This study	Figure 3B
SH100 (WT)	*MAT***a** *leu2-3,112 ura3-52 rme1 trp1 his4 HMLa ade2 tor2::ADE2 [YCplac111::TOR2]*	Helliwell *et al.* (1998a)	Figure 3C, Figure 4, Figure 5D, Figure 6A–C
SH121 (*tor2-21*)	*MAT***a** *leu2-3,112 ura3-52 rme1 trp1 his4 HMLa ade2 tor2::ADE2 [YCplac111::tor2-21]*		Figure 3C, Figure 4, Figure 5D, Figure 6, B and C
SH221 (*tor1 tor2-21*)	*MAT***a** *leu2-3,112 ura3-52 rme1 trp1 his3 HMLa ade2 tor1::HIS3 tor2::ADE2 [YCplac111::tor2-21]*		
TPY157 (*tor2-21 far3*)	SH121 *far3::kanMX4*	This study	Figure 3C
TPY147 (*tor2-21 far7*)	SH121 *far7::kanMX4*	This study	Figure 3C
TPY213 (*tor2-21 far8*)	SH121 *far8::kanMX4*	This study	Figure 3C
TPY207 (*tor2-21 far9*)	SH121 *far9::kanMX4*	This study	Figure 3C
TPY151 (*tor2-21 far10*)	SH121 *far10::kanMX4*	This study	Figure 3C
TPY116 (*tor2-21 far11*)	SH121 *far11::kanMX4*	This study	Figure 3C, Figure 4C, Figure 6B
TPY110 (*tor2-21 sac7*)	SH121 *sac7::kanMX4*	This study	Figure 4A, Figure 4C, Figure 6B
TPY311 (*tor2-21 far11*)	SH121 *far11::TRP1*	This study	Figure 4, C and D
TPY301 (*tor2-21 sac7 far11*)	SH121 *sac7::kanMX4 far11::TRP1*	This study	Figure 4C
TPY680 (*tor2-21 far11 rom2*)	SH121 *rom2::kanMX4 far11::TRP1*	This study	Figure 4D
BY4741	*MAT***a** *ura3 leu2 his3 met15*	Yeast Genome Deletion Project	
BY4741 *far11*	BY4741 *far11::kanMX4*		Figure 5A
TPY633	BY4741 *tpd3::kanMX4 far11::HIS3*	This study	Figure 5A
TPY632	BY4741 *pph21::kanMX4 pph22::kanMX4 far11::HIS3*	This study	Figure 5A
TPY625 (*lst8 tpd3*)	*MAT*α *ura3 leu2 lys2 lst8::LEU2 tpd3::kanMX4 [pRS416-LST8]*	This study	Figure 5B
TPY648 (*lst8 rts1*)	*MAT*α *ura3 leu2 lys2 lst8::LEU2 rts1::kanMX4 [pRS416-LST8]*	This study	Figure 5B
TPY732 (*lst8 cdc55*)	*MAT*α *ura3 leu2 lys2 lst8::LEU2 cdc55::kanMX4 [pRS416-LST8]*	This study	Figure 5B
BY4741 (WT)	*MAT***a** *ura3 leu2 his3 met15 [pRS416]*	This study	Figure 5C
BY4741 (*lst8*)	*MAT***a** *ura3 leu2 his3 met15 lst8::LEU2 [pRS416-LST8]*	This study	Figure 5C
BY4741 (*pph21/22*)	*MAT***a** *ura3 leu2 his3 met15 pph21::kanMX4 pph22::kanMX4[pRS416-LST8]*	This study	Figure 5C
TPY622 (*lst8 pph21/22*)	*MAT***a** *ura3 leu2 his3 met15 lst8::LEU2 pph21::kanMX4 pph22::kanMX4[pRS416-LST8]*	This study	Figure 5C
TPY665 (*rts1*)	*MAT***a** *leu2-3,112 ura3-52 rme1 trp1 his4 HMLa ade2 tor2::ADE2 rts1::kanMX4 [YCplac111::TOR2]*	This study	Figure 5D, Figure 6C

(continued)

Table 1, *continued*

Strain	Genotype	Source	Application
TPY601 (*tor2-21 rts1*)	*MAT***a** *leu2-3,112 ura3-52 rme1 trp1 his4 HMLa ade2 tor2::ADE2 rts1::kanMX4 [YCplac111::tor2-21]*	This study	Figure 5D, Figure 6C
TB50a	*MAT***a** *leu2–3,112 ura3–52 trp1 his3 rme1 HMLa*	Wullschleger *et al.* (2005)	Figure 7
SW70	*TB50a 3HA-TOR2*		Figure 7
TPY1246	*TB50a 3HA-TOR2 sac7::HIS3*	This study	Figure 7
TPY1249	*TB50a 3HA-TOR2 far11::TRP1*	This study	Figure 7

complexes were analyzed by Western blotting. myc- and HA-tagged proteins were probed with anti-myc antibody and anti-HA antibody (3F10; Roche), respectively. Chemiluminescence images of Western blots were captured using the Bio-Rad Chemi-Doc photo documentation system.

Actin staining and GFP fluorescence microscopy

The actin cytoskeleton was visualized in rhodamine phalloidin-stained, formaldehyde-fixed cells, as described (Amberg *et al.* 2005). Overnight cultures were diluted to $\sim OD_{600}$ 0.1 and allowed to grow at 30° for 2 hr and then switched to 37° for 3 hr before formaldehyde was added to a final concentration of 3.7%. After fixing for 1 hr, 1 ml of fixed cells were collected, washed in PBS buffer, and stained with rhodamine phalloidin conjugate (Invitrogen) and visualized by fluorescence microscopy. GFP fluorescence of GFP-tagged proteins was analyzed in cells grown to log phase. Fluorescence images of rhodamine phalloidin-labeled actin structures and GFP-tagged proteins were acquired with a Photometrics Coolsnap fx CCD camera and Metamorph Imaging Software and processed using ImageJ (National Institutes of Health) and Adobe Photoshop.

Preparation of recombinant 6×His-tagged Slm1

PCR-amplified *SLM1* coding sequence was cloned into the *Sac*I and *Xho*I sites of pET24a vector (Novagen). The resultant plasmid was transformed into BL21(DE3)-competent cells (Novagen), and expression of 6×His-tagged Slm1 was induced by adding 1 mM IPTG to bacterial cultures grown at 20° for 16 hr. Recombinant C-terminal 6xHis-tagged Slm1 was purified under native conditions using Ni-NTA agarose beads (Qiagen) as described in the product instruction manual. Slm1-His6 (14 mg) was obtained from 1 liter of induced culture.

In vitro kinase assay of Slm1 phosphorylation by Tor2-HA

Yeast strains expressing 3×HA-tagged Tor2 were grown overnight to mid-log phase, and cellular lysates were prepared in lysis buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, and protease inhibitors) by vortexing with glass

Table 2 Plasmids used in this study

Plasmid	Description	Reference	Application
pZL2422	pRS416-SAC7	This study	Figure 1
pZL2550	pRS416-FAR11	This study	Figure 1
pZL1255	pRS412-LST8	This study	Figure 2, A and B
pZL2762	pRS416-FAR11-HA, expressing Far11 from its own promoter with a 3×HA tag at the C terminus	This study	Figure 3A, Figure 5A
pTP242	pRS415-ADH1-TPD3-*myc*, expressing Tpd3 from the ADH1 promoter with a 3×myc tag at the C terminus	This study	Figure 5A
pTP244	pRS415-PPH21-*myc*, expressing Pph21 from its own promoter with a 3×*myc* tag at the C terminus	This study	Figure 5A
pZL339	pRS416-LST8	Liu *et al.* (2001)	Figure 3B, Figure 5B
pTP311	pRS416-SLM1-HA, expressing Slm1 from its own promoter with a 3×HA tag at the C terminus	This study	Figure 6A
pTP377	pRS416-SLM2-HA, expressing Slm2 from its own promoter with a 3×HA tag at the C terminus	This study	Figure 6
pTP271	pRS416-YPK2-HA, expressing Ypk2 from its own promoter with a 3×HA tag at the C terminus	This study	Figure 6
pZL3031	pET24a-SLM1, expressing Slm1 with a C-terminal 6×His tag under the control of an IPTG-inducible promoter	This study	Figure 7

beads. Cell extracts (~3 mg proteins) were incubated with 100 μl protein A-agarose beads (Roche) at 4° for 1 hr to remove nonspecific binding proteins. Precleared cell lysates were then incubated with 16 μg anti-HA antibody (12CA5, Roche) for 1 hr, after which 40 μl of a 50% slurry of protein A-agarose beads was added to each sample, and the samples were further incubated at 4° for 1 hr. Precipitates were washed twice with 1 ml lysis buffer, twice with 1 ml wash buffer (50 mM Tris–HCl, pH 7.6, 300 mM NaCl, and protease inhibitors), and once with 1 ml kinase buffer (20 mM Tris–HCl, pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF). After the final wash, beads were resuspended in 25 μl of kinase buffer plus 1 mM dithiothreitol (DTT). The reaction was initiated by adding 25 μl reaction mixture [kinase buffer with 1 mM DTT, 0.4 mM ATP, 5 μCi of [γ-^{32}P]ATP (PerkinElmer), 5 μg recombinant Slm1-His6]. After incubation for 30 min at 30°, the reaction was terminated by adding 25 μl 3× SDS gel-loading buffer (150 mM Tris–HCl, pH 6.8, 6% SDS, 30% glycerol, 0.3% bromophenol blue) and 8.5 μl 1 M DTT and boiling for 4 min. Samples (20 μl) were fractionated by SDS–PAGE on 7.5% polyacrylamide gels in triplicate, with one dried for detecting ^{32}P-incorporation in Slm1 by autoradiography (Molecular PhosphorImager FX, Bio-Rad), one stained by Coomassie Blue for detecting 6×His-tagged Slm1, and one processed for Western blotting for detecting HA-Tor2. A mock treatment experiment was conducted using anti-HA immunoprecipitates from cells expressing nontagged Tor2.

Results

Mutations in SAC7 and FAR11 suppress lethality due to an lst8Δ mutation

To gain insights into the essential function of Lst8, we conducted a genetic screen to search for mutations that allow cells to survive without Lst8 by employing an *ade2* colony sectoring assay. This assay takes advantage of a build-up of purine precursors in the vacuole, which results in colonies that appear red in *ade2* mutant cells. We utilized a previously constructed *Tn3*::*lacZ*::*LEU2* mutagenesis library (Burns *et al.* 1994) to introduce mutations in an *lst8*Δ ade2Δ mutant carrying plasmid pRS412-LST8 (*CEN LST8 ADE2*) and screened for red or sectoring **l**s*t8*Δ **b**ypass (*leb*) mutant cells on YPD medium, which had lost or were in the process of losing the pRS412-LST8 plasmid. Of ~30,000 *Leu*$^+$ transformants, 49 viable solid red or sectoring colonies were selected for further analysis. Of the 49, 11 colonies were pure red. Crossing to an *ade1*Δ strain resulted in noncomplementation of the red phenotype in 1 of the 11 pure red colonies, indicating that the red phenotype was due to a mutation in *ADE1* and not to the loss of the pRS412-LST8 plasmid. Alternatively, crossing to an *ade2*Δ strain resulted in noncomplementation of the red phenotype in the remaining 10 pure red colonies, indicating that the red phenotype is due to a mutation in *ADE2* or to loss of the pRS412-LST8 plasmid. However, wild-type *LST8* was found by PCR to be present in all 11 pure red colonies, which were deemed false positives. The remaining 38 sectoring colonies were analyzed similarly, and the red sectoring phenotype of one was due to an *ADE1* mutation while 35 were due to mutations in *ADE2*. PCR genotyping confirmed the absence of pRS412-*LST8* in the remaining two mutants, which we termed *leb1* and *leb2*. In these two *leb* mutants, the *lst8*Δ bypass phenotype was found to cosegregate with *Leu*$^+$ after crossing to an *lst8*Δ *leu2 ade2*Δ *pRS412-LST8* strain of the opposite mating type. Tetrads were dissected, indicating that the transposon insertion had produced the mutant phenotype. Figure 1A shows that, in contrast to wild-type cells, *leb1* and *leb2* mutants form both red and sectoring colonies, indicating loss of the pRS412-LST8 plasmid.

We identified the transposon insertion site in the *leb1* and *leb2* mutants by plasmid rescue and sequencing of the recovered plasmids as described by Burns *et al.* (1994). The transposon insertion sites in the *leb1* and *leb2* mutants were found to be in the open reading frames of *SAC7* and *FAR11*, respectively. Consistently, Figure 1, B and C, shows that wild-type *SAC7* and *FAR11* on a centromeric plasmid can complement *leb1* and *leb2* mutations, respectively. Furthermore, a *sac7*Δ or *far11*Δ mutation in an *lst8*Δ *ade2*Δ *pRS412-LST8* strain also led to *lst8*Δ bypass (loss of pRS412-LST8 is indicated by the red colony phenotype in Figure 2A).

sac7Δ and far11Δ mutants are sensitive to rapamycin

Whether the essential function of Lst8 is linked to TORC1, TORC2, or both has yet to be determined. We tested the ability of *sac7*Δ and *far11*Δ mutations to suppress TORC1 inactivation by rapamycin treatment and TORC2 deficiency due to a temperature-sensitive *tor2-21* mutation. An *fpr1*Δ mutation enables cells to grow in the presence of rapamycin; however, *far11*Δ *lst8*Δ, *far11*Δ (*far11*Δ *lst8*Δ pRS412-LST8), *sac7*Δ *lst8*Δ, and *sac7*Δ (*sac7*Δ *lst8*Δ pRS412-LST8) cells were unable to grow on YPD medium supplemented with 200 nM rapamycin (Figure 2A), demonstrating that these two *lst8*Δ bypass mutations do not suppress a severe or complete loss of TORC1 activity. Consistently, *sac7*Δ and far11Δ mutations failed to restore cell growth to *tor1*Δ *tor2-21* double mutants grown at 37°, which have defects in the function of both TORC1 and TORC2 (Supporting Information, Figure S1) (Schmidt *et al.* 1997). In contrast, *sac7*Δ and *far11*Δ mutations restored cell growth to a *tor2-21* mutant grown at 37°, which causes a specific defect in only TORC2 (Figure 3C and Figure 4C), consistent with previous findings that *sac7*Δ suppresses a *tor2-21* mutation (Schmidt *et al.* 1997). These data suggest that the essential function of Lst8 may be linked to TORC2, but not to TORC1.

It is conceivable that *lst8*Δ may result in a partial loss of TORC1 activity, which is not sufficient to support cell growth but is not severe enough to prevent *sac7*Δ and *far11*Δ mutations from restoring partial cell growth to *lst8*Δ mutant cells. To test this possibility, we tested the sensitivity of wild-type, *fpr1*Δ, *sac7*Δ, and *far11*Δ mutant cells to lower concentrations of rapamycin (Figure S2). It has been reported that *sac7*Δ and *far11*Δ mutant strains in the BY4741 background

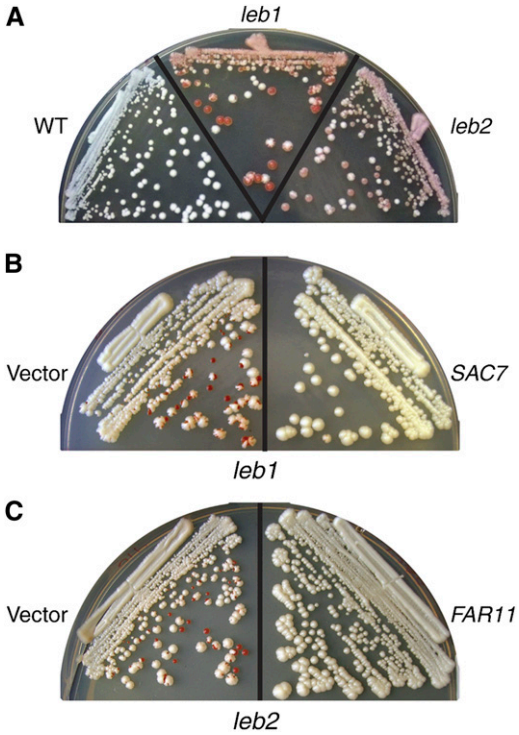

Figure 1 Mutations in *SAC7* and *FAR11* bypass *lst8*Δ. (A) Isolation of *lst8*Δ bypass (*leb*) mutants using a *ade2*-based colony-sectoring assay. Wild-type (*lst8*Δ *ade2*Δ) (ZLY3081) and isogenic *leb1* (TPY104) and *leb2* (TPY103) mutant cells carrying a centromeric plasmid encoding *ADE2* and *LST8* (pZL1255) were streaked onto YPD medium. (B) *SAC7* complements a *leb1* mutation. *leb1* mutant cells (*lst8*Δ *ade2*Δ *leb1*) carrying pRS412-LST8 and either pRS416 empty vector (Vector) or pRS416-SAC7 (*SAC7*, pZL2422) were grown on YNBcasD medium supplemented with adenine. (C) *FAR11* complements a *leb2* mutation. *leb2* (*lst8*Δ *ade2*Δ *leb2*) mutant cells carrying pRS412-LST8 and either pRS416 empty vector (Vector) or pRS416-FAR11 (*FAR11*, pZL2550) were grown on YNBcasD medium supplemented with adenine.

are hypersensitive to treatment with 10 nM rapamycin (Xie *et al.* 2005). In the presence of <10 nM rapamycin, a *far11*Δ mutant in the BY4741 background has been reported to grow better than wild-type cells (Huber *et al.* 2009). We analyzed cell growth in the presence of 2–20 nM rapamycin and found that *sac7*Δ resulted in hypersensitivity to rapamycin treatment. A *far11*Δ mutant, in contrast, grew slightly better than wild-type cells when treated with 3 and 5 nM rapamycin. In the presence of 7–20 nM rapamycin, however, *far11*Δ cells no longer grew better than wild-type cells. In our strain background, treatment of wild-type cells with 10 nM rapamycin likely reduces TORC1 activity to just below the threshold that supports cell growth. Our observations that *sac7*Δ and *far11*Δ bypass *lst8*Δ but not treatment with 10 nM rapamycin strongly suggest that the essential function of lst8 is not linked to TORC1.

To further corroborate our hypothesis that Lst8 is not essential for TORC1 activity, we examined the effect of *lst8*Δ on the expression of genes encoding RPs, which are positively regulated by TORC1. Utilizing *sac7*Δ to obtain a viable *lst8*Δ mutant, we compared the expression of two RP genes, *RPL3* and *RPS6A*, encoding the L3 protein of the large (60S) ribosomal subunit and the S6 protein of the small (40S) ribosomal subunit, respectively, in *LST8 sac7*Δ *vs.* *lst8*Δ *sac7*Δ mutant cells by Northern blot analysis. As expected, TORC1 inactivation due to rapamycin treatment inhibited expression of both *RPL3* and *RPS6A* (Figure 2B). In contrast, *lst8*Δ only mildly reduced the expression of *RPL3* and *RPS6A*, suggesting that *lst8*Δ does not lead to severe loss of TORC1 activity.

*lst8*Δ causes mislocalization of Bit61 and Avo3, but not of Kog1

Recent research has demonstrated that the TORC1 components are located on intracellular membranes with a concentration on the vacuolar membrane while TORC2 components appear as punctate spots at the plasma membrane (Wedaman *et al.* 2003; Araki *et al.* 2005; Sturgill *et al.* 2008; Berchtold and Walther 2009; Binda *et al.* 2009). It has been proposed that plasma membrane localization of TORC2 is essential for cell viability (Berchtold and Walther 2009). Isolation of *lst8*Δ mutant suppressors allowed us to examine the role of Lst8 in the cellular localization of TOR complex components. GFP fluorescence was analyzed in *sac7*Δ *far11*Δ double mutants and *sac7*Δ *far11*Δ *lst8*Δ triple mutants expressing the GFP-tagged TORC1 component Kog1 and TORC2 components Bit61 and Avo3. As previously reported, Bit61 and Avo3 localized to the plasma membrane as punctate spots in wild-type *LST8* cells (Figure 2C) (Berchtold and Walther 2009). An *lst8*Δ mutation, however, largely abolished punctate plasma membrane localization of Bit61 and Avo3. In contrast, *lst8*Δ did not affect localization of Kog1 to the vacuolar membrane (Figure 2C). These findings are consistent with our hypothesis that the essential function of Lst8 is linked to TORC2, but not to TORC1, and further suggest that Lst8 is required for proper cellular localization of TORC2.

Mutations of the Far3-7-8-9-10-11 complex bypass *lst8*Δ and *tor2-21* mutations

Far11 has been shown to be involved in cell cycle arrest in response to a mating pheromone in a multi-protein complex with Far3, Far7, Far8, Far9, and Far10 (Horecka and Sprague 1996; Kemp and Sprague 2003). Interactions among these six Far proteins are based mostly on yeast two-hybrid assays (Kemp and Sprague 2003; Lai *et al.* 2011); however, Far3 has been reported to interact with Far11 by co-immunoprecipitation. We generated strains coexpressing HA-tagged Far11 and *myc*-tagged Far7, Far8, Far9, Far10, and Far11 under the control of their respective endogenous promoters to test whether Far11 interacts with Far7, Far8, Far9, Far10, or itself by co-immunoprecipitation. *FAR11-HA* was found to be functional by its ability to complement a *far11*Δ mutation using the colony-sectoring assay described for Figure 1C (Figure S3). *myc*-tagged Far proteins are functional as described previously (Kemp and Sprague 2003). Far11-HA in cell lysates prepared for co-immunoprecipitation exists as two bands on Western blots (Figure 3A). The faster mobility form of Far11-HA is likely to be a proteolytically truncated form of Far11 since Far11-HA exists as a single band on Western blots when the

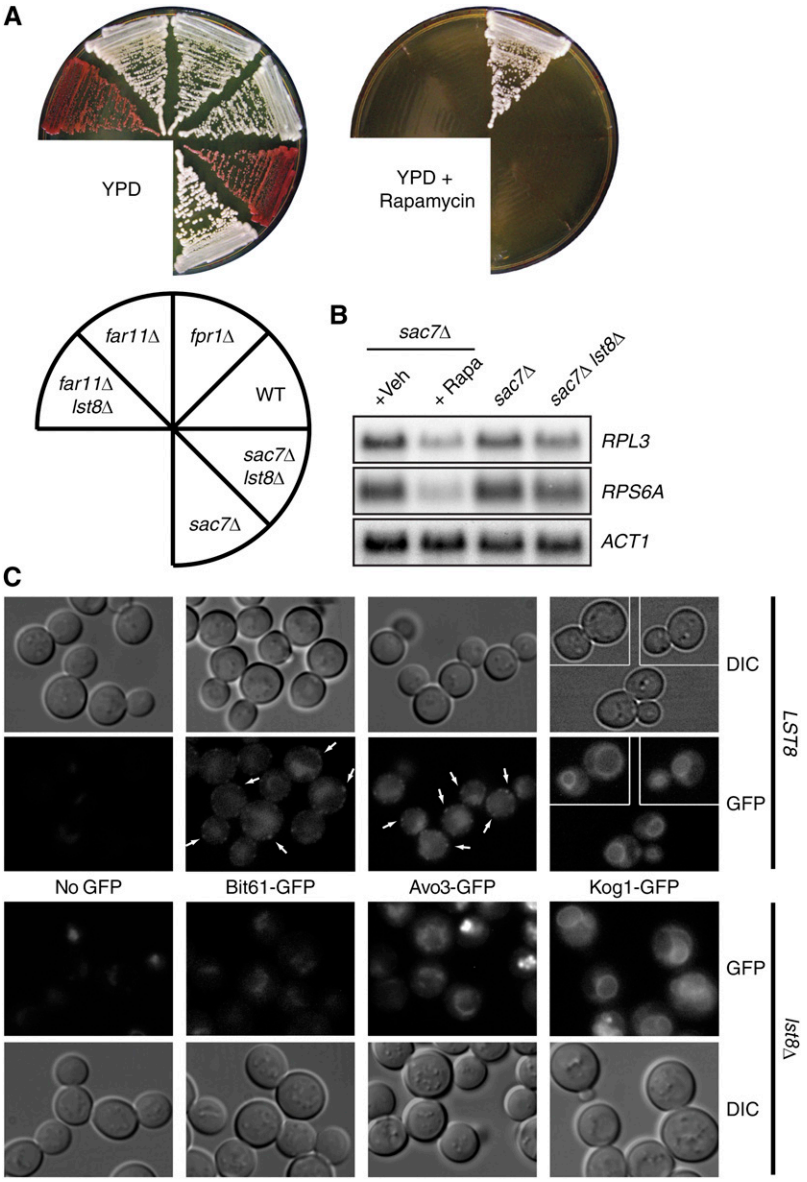

Figure 2 TORC1 function is not grossly affected in an *lst8Δ* mutant. (A) *sac7*Δ and *far11*Δ mutations do not confer resistance to rapamycin. Wild type (ZLY423) and isogenic mutant cells as indicated (*far11*Δ *lst8*Δ, TPY115; *far11*Δ, TPY114; *fpr1*Δ, TPY122; *sac7*Δ *lst8*Δ, ZLY2405; *sac7*Δ, ZLY2404) were grown on YPD medium with or without 200 nM rapamycin. The lack of a pRS412-LST8 plasmid results in the *far11*Δ *lst8*Δ and *sac7*Δ *lst8*Δ double-mutant cells having a red phenotype. (B) *lst8*Δ has little effect on the expression of *RPL3* and *RPS6A*. Expression of *RPL3*, *RPS6A*, and *ACT1* in *sac7*Δ mutant cells (ZLY2404) treated with drug vehicle (+Veh) or 200 nM rapamycin (+Rapa), and *sac7*Δ single-mutant (ZLY2404) and *sac7*Δ *lst8*Δ double-mutant (ZLY2845) cells were analyzed by Northern blotting as described in *Materials and Methods*. (C) *lst8*Δ causes mislocalization of Bit61 and Avo3, but not of Kog1. Wild-type *LST8* (*LST8 sac7*Δ *far11*Δ) and *lst8*Δ mutant (*lst8*Δ *sac7*Δ *far11*Δ) cells expressing no GFP-tagged proteins (TPY1264, TPY1266), Bit61-GFP (TPY358, TPY366), Avo3-GFP (TPY369, TPY407), or Kog1-GFP (TPY371, TPY413) were grown in SD medium and observed by bright field (DIC) and GFP fluorescence microscopy. GFP fluorescence images were captured and processed using the same parameters. Arrows indicate punctate plasma membrane localization of Bit61 and Avo3. Background signals in cells expressing no GFP-tagged proteins, especially in the *lst8*Δ mutant strain, are due to autofluorescence caused by an *ade2* mutation.

total cellular proteins are prepared by disrupting cells in the presence of 1.85 N NaOH–7.5% β-mercaptoethanol followed by precipitation with trichloroacetic acid (Figure S4). *myc*-tagged proteins were precipitated from cell lysates using anti-*myc* antibody. Figure 3A shows that Far11-HA co-immunoprecipitates with *myc*-tagged Far7, Far8, Far9, Far10, but not Far11, demonstrating that Far11 interacts with other Far proteins *in vivo*.

Since Far11 is part of a multi-protein complex, it is possible that the entire complex is involved in TORC2 signaling. We examined whether mutations of the Far complex bypass *lst8*Δ and *tor2-21* as well. *lst8*Δ *far*Δ double mutants, each carrying a centromeric plasmid encoding *LST8* and *URA3* (*[CEN URA LST8]*), were grown on SD medium without or with 5-fluoroorotic acid (5-FOA), which selects for cells that have lost the *URA3* plasmid. On the basis of their relative growth in the presence of 5-FOA, mutations in *FAR3*, *FAR7*, *FAR8*, *FAR9*, *FAR10*, and *FAR11* bypass *lst8*Δ to varying degrees: *far11* > *far8/9* > *far3/7* > *far10* (Figure 3B). *far*Δ mutations were subsequently found to suppress a *tor2-21* mutation at 37° (Figure 3C). The *tor2-21* suppression phenotypes of *far3*, *far7*, *far8*, *far9*, *far10*, and *far11* mutations largely mirror that of their respective *lst8*Δ bypass (Figure 3, B and C), indicating that the function of Lst8 is tightly linked to TORC2.

Far9 and Far10 are homologous proteins with 31% sequence identity and 47% sequence similarity, yet they have different *lst8*Δ and *tor2-21* suppression phenotypes. We generated a *tor2-21 far9*Δ *far10*Δ triple mutant and found that its growth was only marginally better than that of the *tor2-21 far9*Δ double mutant (Figure S5), indicating that, of the two, Far9 plays the primary role in TORC2 signaling.

It is unclear whether an *lst8*Δ or *tor2-21* mutation leads to complete loss of TORC2 activity. Deletion of *TOR2*, encoding the only TOR kinase in TORC2, abolishes TORC2 activity. To test whether *far11*Δ bypasses *tor2*Δ, spores of tetrads from *FAR11/far11 TOR2/tor2*Δ::*kanMX4* diploid cells were assessed for viability. All tetrads produced at most two viable

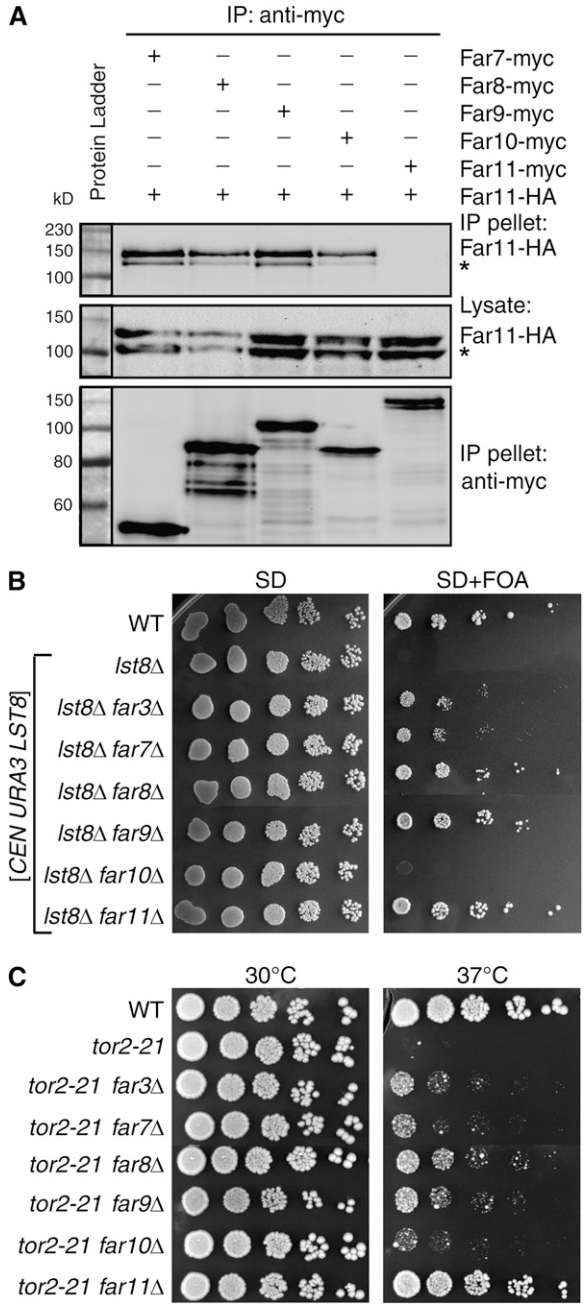

Figure 3 Mutations in *FAR3*, *FAR7*, *FAR8*, *FAR9*, *FAR10*, and *FAR11* bypass *lst8*Δ and *tor2-21*. (A) Far11-HA interacts with *myc*-tagged Far7, Far8, Far9, and Far10. Cell lysates of strains TPY978 (Far7-*myc*), TPY981 (Far8-*myc*), TPY1001 (Far9-*myc*), TPY1002 (Far10-*myc*), and TPY1003 (Far11-*myc*) coexpressing Far11-HA (pZL2762) were subjected to immunoprecipitation with anti-*myc* antibody. HA- and *myc*-tagged proteins were detected by Western blotting. An asterisk denotes a likely truncation product of Far11-HA. (B) Mutations in *FAR3*, *FAR7*, *FAR8*, *FAR9*, *FAR10*, and *FAR11* bypass *lst8*Δ. Serial dilutions of wild-type (WT) (RBY231) and isogenic mutant cells (*lst8*Δ, RBY223; *lst8*Δ *far3*Δ, MOY142; *lst8*Δ *far7*Δ, MOY145; *lst8*Δ *far8*Δ, MOY146; *lst8*Δ *far9*Δ, MOY169; *lst8*Δ *far10*Δ, MOY149; *lst8*Δ *far11*Δ, MOY150) carrying a centromeric plasmid encoding *URA3* and *LST8* (pZL339) as indicated were grown on SD medium without or with 5-FOA. (C) Mutations in *FAR3*, *FAR7*, *FAR8*, *FAR9*, *FAR10*, and *FAR11* suppress *tor2-21* at 37 °C. Serial dilutions of indicated cells (WT, SH100; *tor2-21*, SH121; *tor2-21 far3*Δ, TPY157; *tor2-21 far7*Δ, TPY147; *tor2-21 far8*Δ, TPY213; *tor2-21 far9*Δ, TPY207; *tor2-21 far10*Δ, TPY151; *tor2-21 far11*Δ, TPY116) were grown on YPD medium at 30 °C and 37 °C for 3–4 days.

spores, none of which were geneticin resistant (*kanXM4* confers geneticin resistance) (Figure S6). Since *FAR11* and *TOR2* are not located on the same chromosome, 25% of spores should have the genotype *tor2*Δ::*kanMX4 far11*Δ. Failure to obtain viable geneticin-resistant spores indicates that *far11*Δ is unable to bypass *tor2*Δ. Similarly, we found that *far11*Δ failed to bypass *avo1*Δ or *avo3*Δ (Figure S6) . Since *far11*Δ is able to suppress *lst8*Δ and *tor2-21* mutations, it is likely that *lst8*Δ and *tor2-21* mutations do not completely abolish TORC2 activity and that *far11*Δ can restore growth to cells with a severe loss, but not a total loss, of TORC2 activity.

*sac7*Δ *and far11*Δ *additively suppress tor2-21*

TORC2 is involved in the organization of the actin cytoskeleton. A *tor2-21* mutant shows depolarization of the actin cytoskeleton, and several *tor2-21* suppressors can restore actin polarization to *tor2-21* mutant cells (Schmidt *et al.* 1997). We compared actin structures in wild-type, *tor2-21*, *tor2-21 sac7*Δ, and *tor2-21 far11*Δ mutant cells to establish whether actin polarization defects caused by a *tor2-21* mutation could be restored by a *far11*Δ mutation. As expected, a *sac7*Δ mutation restored polarization of the actin cytoskeleton in *tor2-21* mutant cells grown at 37° (Figure 4A). Similarly, a *far11*Δ mutation restored polarization of actin structures in *tor2-21* mutant cells grown at 37° (Figure 4B). A recent genome-wide study of genetic interactions in yeast showed that *far11*Δ restored actin polarization to *tsc11-1* (*avo3-1*) mutant cells (Baryshnikova *et al.* 2010). Together, these data establish that Far11 negatively regulates TORC2-mediated polarization of the actin cytoskeleton.

Similar phenotypes of *far11*Δ and *sac7*Δ mutations prompted us to determine whether they act through the same molecular mechanism. Accordingly, we compared the growth of a *tor2-21 sac7*Δ *far11*Δ triple mutant to wild-type, *tor2-21*, *tor2-21 sac7*Δ, and *tor2-21 far11*Δ mutants at 30° *vs.* 37°. Figure 4C shows that, while all strains grew equally well at 30°, the *tor2-21 sac7*Δ *far11*Δ mutant grew better than either the *tor2-21 far11*Δ or the *tor2-21 sac7*Δ mutant at 37°, indicating that *far11*Δ and *sac7*Δ have additive effects in suppressing *tor2-21*. To examine whether *sac7*Δ and *far11*Δ have an additive effect in suppressing an actin depolarization defect in *tor2-21* mutant cells, we determined the percentage of cells with a polarized actin cytoskeleton in wild-type and isogenic *tor2-21*, *tor2-21 sac7*Δ, *tor2-21 far11*Δ, and *tor2-21 sac7*Δ *far11*Δ mutant cells. Table S1 shows that the effect of *sac7*Δ and *far11*Δ on the restoration of actin structure polarization is additive. Sac7 and Rom2 have opposing roles in mediating TORC2 function, and it has been proposed that Tor2 activates Rho1 via Rom2 (Schmidt *et al.* 1997). Therefore, to determine whether *tor2-21* suppression by *far11*Δ is Rom2 dependent, we introduced a *rom2*Δ mutation into the *tor2-21 far11*Δ mutant. Figure 4D shows that a *rom2*Δ mutation greatly reduced the *tor2-21* suppression phenotype of a *far11*Δ mutation at 37° but did not abolish it, suggesting that *far11*Δ suppression of TORC2 deficiency is not entirely dependent on the Rom2-mediated Rho1/2 GTPase switch.

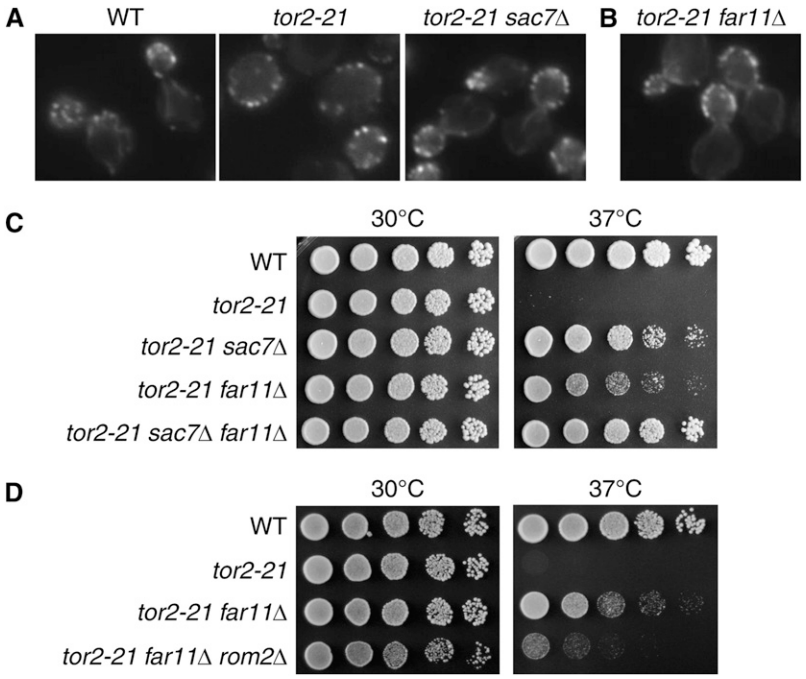

Figure 4 *sac7*Δ and *far11*Δ additively suppress *tor2-21*. (A and B) Mutations in *SAC7* and *FAR11* restore polarization of the actin cytoskeleton to *tor2-21* mutant cells grown at 37°. Actin structures in indicated cells (WT, SH100; *tor2-21*, SH121; *tor2-21 sac7*Δ, TPY110; *tor2-21 far11*Δ, TPY116) were detected by staining with rhodamine phalloidin as described in *Materials and Methods*. (C) *sac7*Δ and *far11*Δ have an additive effect in suppressing *tor2-21*. Serial dilutions of indicated cells (WT, SH100; *tor2-21*, SH121; *tor2-21 sac7*Δ, TPY110; *tor2-21 far11*Δ, TPY311; *tor2-21 sac7*Δ *far11*Δ, TPY301) were grown on YPD medium at 30 °C and 37 °C for 2–3 days. (D) *far11*Δ suppression of *tor2-21* is partially dependent on *ROM2*. Serial dilutions of indicated cells (WT, SH100; *tor2-21*, SH121; *tor2-21 far11*Δ, TPY311; *tor2-211 far11*Δ *rom2*Δ, TPY680) were grown on YPD medium at 30° and 37°.

Far11 interacts with Tpd3 and Pph21, components of PP2A

Our data so far raise the question: How does the Far3-7-8-9-10-11 complex mediate TORC2 signaling? The human ortholog of yeast Far11 exists in the human STRIPAK complex, which also contains components of PP2A (Goudreault *et al.* 2009). The Far11 ortholog in *Drosophila* has also been reported to interact with PP2A in the dSTRIPAK complex (Ribeiro *et al.* 2010). Therefore, we tested whether Far11 in yeast also exists in a complex with PP2A. In yeast, the heterotrimeric PP2A phosphatase consists of the regulatory A subunit Tpd3, the regulatory B subunit Cdc55 or B′ subunit Rts1, and one of the two homologous and functionally redundant catalytic C subunits, Pph21 or Pph22 (Duvel and Broach 2004). Among ∼75 proteins that genetically or biochemically interact with Far11 in various genome-wide gene/protein interaction studies, Tpd3 has been found to interact with Far11 by yeast two-hybrid analysis (Uetz *et al.* 2000). The significance of this interaction remains unknown.

To establish the interaction between Far11 and PP2A in yeast, lysates from cells coexpressing 3× HA-tagged Far11 and 3× *myc*-tagged Tpd3 or Pph21 were subjected to immunoprecipitation using anti-*myc* antibody. *TPD3-myc* and *PPH21-myc* constructs were found to be functional by their ability to rescue growth defects of *tpd3*Δ and *pph21/22*Δ mutants, respectively (Figure S7). No Far11-HA was detected in the IP pellet from cells expressing Far11-HA alone. In contrast, Far11-HA was recovered in the IP pellet from cells coexpressing Tpd3-*myc* and, to a lesser extent, from cells coexpressing Pph21-*myc* (Figure 5A) likely because C-terminal tagging of Pph21 perturbs methylation at its C terminus required for PP2A complex stability (Wei *et al.* 2001). These findings establish that Far11 interacts with PP2A phosphatase.

*Defects in PP2A-Rts1 bypass lst8*Δ *and tor2-21 mutations*

To investigate whether PP2A is involved in TORC2 signaling, we examined whether mutations in PP2A components bypass *lst8*Δ. We analyzed the growth of an *lst8*Δ *tpd3*Δ double mutant, an *lst8*Δ *rts1*Δ double mutant, an *lst8*Δ *cdc55*Δ double mutant, and an *lst8*Δ *pph21*Δ *pph22*Δ triple mutant, each carrying a centromeric plasmid encoding *URA3* and *LST8* on SD medium without or with 5-FOA. *tpd3*Δ, *rts1*Δ, and *pph21/22*Δ mutations, but not a *cdc55*Δ mutation, were able to bypass *lst8*Δ (Figure 5, B and C), indicated by their ability or inability to grow in the presence of 5-FOA, suggesting that reduced activity in the PP2A-Rts1 subcomplex results in *lst8*Δ bypass. We then tested whether *rts1*Δ and *tpd3*Δ mutations suppress *tor2-21*. Figure 5D shows that *rts1*Δ suppresses a *tor2-21* mutation by restoring cell growth at 37°. A *tpd3*Δ mutation led to temperature-sensitive growth defects in the *TOR2* wild-type strain used in our study; therefore, we could not assay *tor2-21* suppression by *tpd3*Δ. These findings indicate that mutations in genes encoding components of the PP2A-Rts1 subcomplex suppress TORC2 deficiency.

*far11*Δ *and rts1*Δ *restore phosphorylation of Slm1 to a tor2-21 mutant*

One known function of TORC2 is its role in the organization of the actin cytoskeleton possibly by phosphorylating Slm1, Slm2, and Ypk2. Our data above show that *far11*Δ, *sac7*Δ, and *rts1*Δ mutations suppress a *tor2-21* mutation and that Far11 interacts with PP2A. Therefore, we tested the possibility that Far11 may mediate dephosphorylation of Slm1, Slm2, and/or Ypk2 via PP2A by evaluating the phosphorylation states of Slm1, Slm2, and Ypk2 in wild-type, *tor2-21*, *tor2-21 far11*Δ, *tor2-21 sac7*Δ, and *tor2-21 rts1*Δ mutant

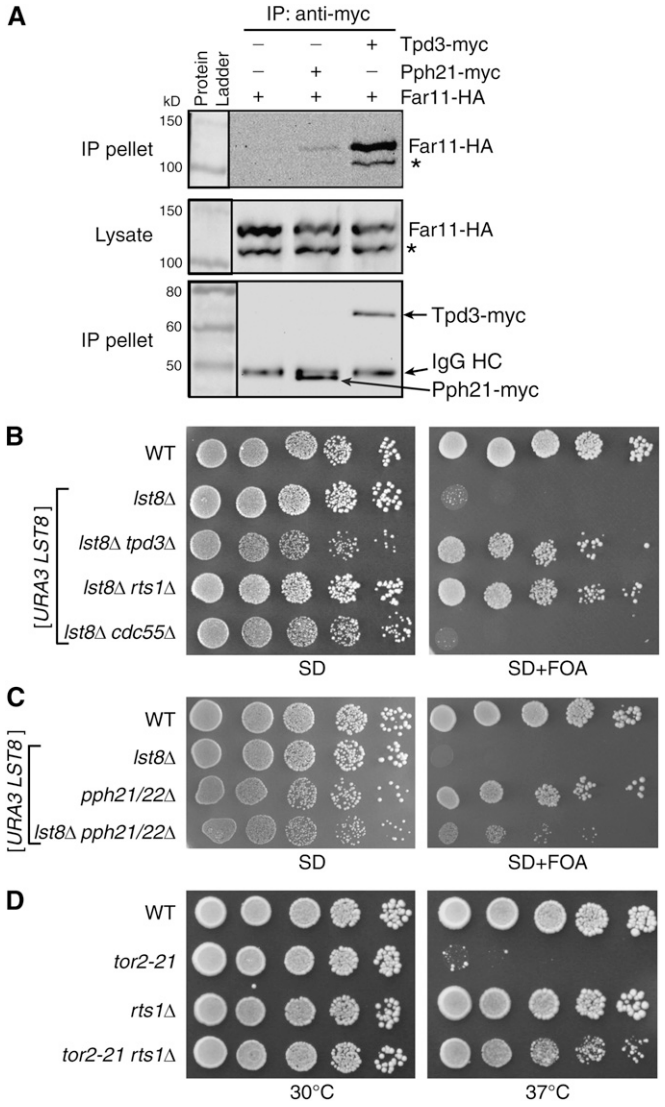

Figure 5 Mutations in *TPD3*, *RTS1*, and *PPH21/22* suppress TORC2 deficiency. (A) Far11-HA interacts with Tpd3-*myc* and Pph21-*myc*. Cell lysates of *far11*Δ mutant cells (BY4741 *far11*) expressing Far11-HA only (pZL2762), *tpd3*Δ *far11*Δ double-mutant cells (TPY633) coexpressing Far11-HA and Tpd3-*myc* (pTP242), and *pph21/22*Δ *far11*Δ triple-mutant cells coexpressing Far11-HA and Pph21-*myc* (pTP244) were subject to immunoprecipitation with anti-*myc* antibody. "IgG HC" indicates the heavy chain of the anti-*myc* antibody used for co-immunoprecipitation. An asterisk denotes a likely truncation product of Far11. (B) Mutations in *TPD3* and *RTS1* bypass *lst8*Δ. Serial dilutions of wild-type (WT, RBY231), *lst8*Δ (RBY223), *lst8*Δ *tpd3*Δ (TPY625), *lst8*Δ *rts1*Δ (TPY648), and *lst8*Δ *cdc55*Δ mutant (TPY732) cells carrying a centromeric plasmid encoding *LST8* (*[URA3 LST8]*, pZL339) were grown on SD medium without or with 5-FOA. (C) A *pph21/22*Δ double mutation bypasses *lst8*Δ. Serial dilutions of wild-type cells (WT, BY4741) carrying the empty vector pRS416 and *lst8*Δ single-mutant (BY4741 *lst8*), *pph21/22*Δ double-mutant (BY4741 *pph21/22*), and *lst8*Δ *pph21/22*Δ triple-mutant (TPY622) cells carrying a plasmid encoding *LST8* (pZL339) were tested for growth as described for B. (D) An *rts1*Δ mutation suppresses *tor2-21* at 37 °C. Serial dilutions of wild-type (SH100), *tor2-21* (SH121), *rts1*Δ (TPY665), and *tor2-21 rts1*Δ (TPY601) cells were grown on YPD medium at 30 °C and 37 °C.

cells, each expressing Slm1-HA, Slm2-HA, or Ypk2-HA from their respective endogenous promoters. The phosphorylation states of Ypk2 and Slm2 did not differ between the wild-type and the *tor2-21* mutant grown at 37° (Figure S8). Therefore, Ypk2 and Slm2 were not studied further. Consistent with previous reports on the phosphorylation state of GFP-tagged Slm1 (Audhya *et al.* 2004), Figure 6A shows that Slm1-HA is phosphorylated: lambda protein phosphatase (λPPase) treatment resulted in increased levels of the faster mobility forms of Slm1-HA with concomitant reduced levels of the slower mobility forms of Slm1-HA; phosphatase inhibitors largely abolished the effect of λPPase treatment. As reported previously, Figure 6B shows that Slm1 is dephosphorylated in *tor2-21* mutant cells grown at 37° (Audhya *et al.* 2004). Remarkably, *far11*Δ, but not *sac7*Δ, restored Slm1-HA phosphorylation to *tor2-21* mutant cells grown at 37° (Figure 6B), implicating Far11 in Slm1 dephosphorylation and suggesting that suppression of TORC2 deficiency by a *sac7*Δ mutation takes place downstream of Slm1. Furthermore, Slm1-HA was phosphorylated in *tor2-21 rts1*Δ mutant cells grown at 37° (Figure 6C). These data suggest that Far11-PP2A-Rts1 may antagonize TORC2 activity and decrease the levels of the phosphorylated form of the TORC2 substrate Slm1.

Effects of sac7Δ and far11Δ mutations on Tor2 kinase activity

Sac7 has been proposed to function downstream of TORC2. Our genetic data suggest that Far11-PP2A-Rts1 antagonizes TORC2 signaling. It is possible that Far11-PP2A-Rts1 may function downstream of TORC2 and promote dephosphorylation of TORC2 substrates. It is also likely that Far11-PP2A-Rts1 may function upstream of TORC2 and negatively impact TORC2 activity. To differentiate between these two possibilities, the activity of immunopurified Tor2 with a N-terminal 3×HA tag from wild-type and isogenic *far11*Δ mutant cells was determined in an *in vitro* kinase assay using Slm1 as a substrate. Slm1 has been reported to be a TORC2 substrate in *in vitro* kinase assays (Audhya *et al.* 2004; Fadri *et al.* 2005). For our assays, recombinant 6×His-tagged Slm1 was added to kinase reactions with immunopurified HA-Tor2 and [γ-^{32}P]ATP. Figure 7 shows that *far11*Δ has no significant effect on kinase activity of immunopurified HA-Tor2, suggesting that Far11 functions at a site downstream of TORC2.

Similarly, we performed an *in vitro* kinase assay using HA-Tor2 from *sac7*Δ mutant cells. Surprisingly, *sac7*Δ slightly increases kinase activity of immunopurified HA-Tor2 (Figure 7). Mutations in *SAC7* have been proposed to activate Rho1, which in turn activates Pkc1 and the cell-wall integrity MAP kinase cascade. Increased activity of Tor2 in *sac7*Δ mutant cells suggests positive feedback regulation in TORC2 signaling. Differential effects of *sac7*Δ and *far11*Δ mutations on Tor2 kinase activity further support the notion that Sac7 and Far11 mediate TORC2 signaling through different mechanisms.

Discussion

Lst8 is an essential protein that exists in both TOR kinase complexes. We found that mutations in genes encoding the

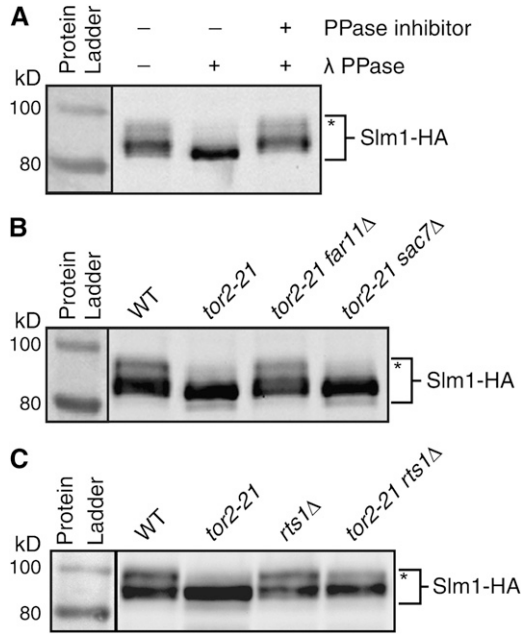

Figure 6 Mutations in *RTS1* and *FAR11*, but not in *SAC7*, restore Slm1 phosphorylation in *tor2-21* mutant cells. (A) Slm1-HA is phosphorylated. Cell lysates from wild-type cells (SH100) expressing Slm1-HA (pTP311) were prepared and treated with λPPase with or without phosphatase inhibitors as described in *Materials and Methods*. Slm1-HA was detected by Western blotting. An asterisk indicates phosphorylated Slm1. (B) A mutation in *FAR11*, but not in *SAC7*, restores Slm1 phosphorylation in *tor2-21* mutant cells grown at 37 °C. Indicated cells (WT, SH100; *tor2-21*, SH121; *tor2-21 far11*Δ, TPY116; *tor2-21 sac7*Δ, TPY110) expressing Slm1-HA were grown in YNBcasD medium at 30 °C to mid-log phase and switched to 37 °C for 3 hr before cellular proteins were processed for Western blotting. (C) An *rts1*Δ mutation restores Slm1 phosphorylation in *tor2-21* mutant cells grown at 37 °C. Indicated cells (WT, SH100; *tor2-21*, SH121; *rts1*Δ, TPY665; *tor2-21 rts1*Δ, TPY601) expressing Slm1-HA were analyzed for Slm1-HA phosphorylation as described for B.

PP2A-Rts1 subcomplex and the Far3-7-8-9-10-11 complex bypass *lst8*Δ and TORC2 deficiency. Analysis of these mutants led us to propose that the essential function of Lst8 is linked to TORC2. The Far3-7-8-9-10-11 complex components are partially conserved in *Drosophila* and mammals and have been reported to interact with PP2A phosphatase in the STRIPAK complex. We showed that the Far3-7-8-9-10-11 complex and PP2A negatively regulate TORC2 signaling possibly by mediating dephosphorylation of the TORC2 substrate Slm1, depicted by our proposed model in Figure 8. Our results not only demonstrate that the essential function of Lst8 is linked only to TORC2, but also, more importantly, reveal a novel link between the two major signaling protein complexes PP2A and TORC2.

Essential function of Lst8 is linked to TORC2, but not to TORC1

Yeast Lst8 has been reported to be important for TORC2 complex integrity *in vivo* and for Tor2 kinase activity in *vitro* (Wullschleger *et al.* 2005). Underlying its importance in TORC2 activity, the presence of Lst8 in TORC2 has been reported in multiple organisms including yeast, slime mold, worms, flies, and mammals (Cybulski and Hall 2009). Consistently, our data demonstrate that the essential function of Lst8 is linked to TORC2. Delocalization of Bit61 and Avo3 from punctate structures at the plasma membrane in *lst8*Δ mutant cells likely results from compromised TORC2 integrity in the absence of Lst8, indicating that Lst8 is also required for proper localization of the TORC2 complex.

Lst8 interacts with the kinase domain of Tor2 in yeast TORC2 (Wullschleger *et al.* 2005). Since *far11*Δ bypasses *lst8*Δ and *tor2-21*, but not *tor2*Δ, *avo1*Δ, and *avo3*Δ, our data suggest that neither *lst8*Δ nor the temperature-sensitive *tor2-21* mutation leads to total loss of TORC2 activity. This possibility helps explain our observation that *far11*Δ and *rts1*Δ can restore Slm1 phosphorylation to *tor2-21* mutant cells at the restrictive temperature: If *tor2-21* led to the total loss of TORC2 activity and consequent loss of Slm1 phosphorylation, PP2A inactivation could not restore Slm1 phosphorylation unless other protein kinases also phosphorylate Slm1. In this scenario, TORC2 would share a redundant function with the other putative kinase in phosphorylating Slm1 and may have another essential function separate from its kinase activity, for example, by maintaining interactions with other proteins to conduct downstream signaling.

Three TORC2 substrates in yeast are Slm1, Slm2, and Ypk2. A constitutively active Ypk2 mutant can restore growth to *tor2*Δ mutant cells, leading to the proposal that the essential function of TORC2 is linked mainly to Ypk2 phosphorylation (Kamada *et al.* 2005; Cybulski and Hall 2009). There is strong evidence that functionally redundant Slm1 and Slm2 are essential substrates of TORC2. First, Slm1 has been reported to interact with TORC2 and to be phosphorylated by TORC2 (Audhya *et al.* 2004; Fadri *et al.* 2005). Second, TORC2-dependent phosphorylation of Slm1 seems to correlate with its plasma membrane association (Audhya *et al.* 2004). Slm1 contains a PH domain that binds to multiply phosphorylated phosphoinositides and is required for Slm1's plasma membrane localization (Fadri *et al.* 2005), and putative loss of plasma membrane association of Slm1/2 leads to cell death. Third, a *sac7*Δ mutation suppresses both a *tor2-21* mutation and a *slm1*Δ *slm2*Δ double mutation, and the actin cytoskeleton is depolarized in both *slm1/2* and *tor2-21* mutant cells. Although we could not test whether phosphorylation of Slm2 and Ypk2 in a *tor2-21* mutant is restored by *far11* and *rts1* mutations, it is likely that, due to mutations in Far11-PP2A, increased phosphorylation of Slm1, Slm2, and/or Ypk2 leads to cell viability in TORC2-deficient cells.

The presence of Lst8 in TORC1 is conserved from yeast to mammals. In yeast, Lst8 localizes not only to the TORC1 compartment at the vacuolar membrane, but also to the TORC2 compartment as punctate structures at the plasma membrane (Berchtold and Walther 2009). Similar to its interaction with Tor2 in TORC2, Lst8 interacts with the Tor1 kinase domain in the TORC1 complex in yeast and humans (Kim *et al.* 2003; Adami *et al.* 2007). Therefore, our observation that Lst8 is not required for TORC1-dependent expression of genes encoding ribosomal proteins and Kog1 localization at the vacuolar membrane is surprising and raises the question: Is Lst8

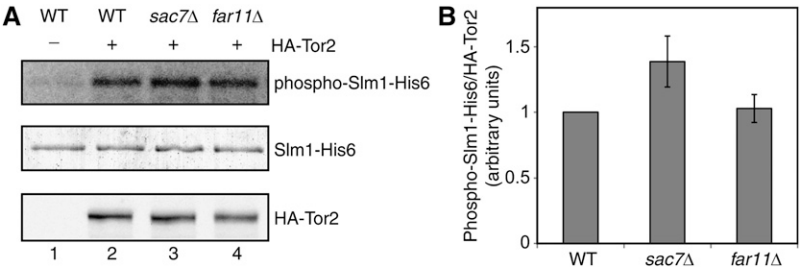

Figure 7 *In vitro* phosphorylation of Slm1 by immunopurified HA-Tor2. (A) Wild-type (SW70) and isogenic *sac7*Δ (TPY1246) and *far11*Δ (TPY1249) cells expressing N-terminal 3×HA-tagged Tor2 from the *TOR2* genomic locus were grown in YPD medium. Slm1-His6 phosphorylation assays by HA-Tor2 were conducted as described in *Materials and Methods*. Phospho-Slm1-His6 was detected by autoradiography. Total Slm1-His6 and HA-Tor2 in the assays were detected by Coomassie Blue staining and immunoblotting, respectively. The result of a mock kinase assay using cell lysates from wild-type cells expressing native, nontagged Tor2 (TB50a) was included in lane 1. (B) The amount of phospho-Slm1-His6 was normalized to that of HA-Tor2 and graphed. Kinase assays were performed with HA-Tor2 from two independent cell lysates, and the error bar indicates the standard deviation.

required for TORC1 activity at all? Missense mutations in *LST8* increase the expression of a subset of TORC1-target genes (Chen and Kaiser 2003; Giannattasio *et al.* 2005). Therefore, Lst8 is likely to be required for optimal TORC1 activity, but an *lst8*Δ mutation may not reduce TORC1 activity severely enough to lead to cell death. In mammals, the role of Lst8 in mTORC1 is unclear. mLst8 knockdown in human immortalized cell lines suggested that mLst8 is important for mTORC1 activity (Kim *et al.* 2003). Later in mice, mLst8 was found to be important for mTORC2, but not for mTORC1 function during mouse development (Guertin *et al.* 2006). The discrepancy could be attributed to the differences between a developing mouse embryo and an immortalized human cell line. However, the conclusions concerning the role of Lst8 in both yeast and mice are similar: the essential function of Lst8 is linked to TORC2 but not to TORC1.

Far3-7-8-9-10-11-PP2A as a negative regulator of TORC2 signaling

One of the important findings presented here is a negative regulatory role of the Far3-7-8-9-10-11 complex and PP2A in TORC2 signaling. Considering the extensive studies on PP2A and TORC2, it is surprising that, to our knowledge, our study may represent the first to present a direct genetic interaction between TORC2 and PP2A. More importantly, our data mirror a recent study in *Drosophila* demonstrating that the *Drosophila* Far complex works in concert with PP2A in the regulation of a different kinase pathway, the Hippo signaling pathway (Ribeiro *et al.* 2010). Thus, PP2A regulation by the Far protein complex appears to be evolutionarily conserved. In a proteomics study, the yeast Far11 orthologs Fam40A and Fam40B (STRIP1/2) were isolated in the STRIPAK complex, which also contains components of PP2A. Interestingly, *far11*Δ leads to the strongest suppression of TORC2 deficiency, and among the six Far proteins, Far11 is the most conserved. Far9 and Far10 are homologous proteins, and their *Drosophila* and mammalian orthologs show limited sequence homology, mostly in an FHA domain, which is known to interact with phosphothreonine epitopes on target proteins (Durocher and Jackson 2002). Far8 shows very limited sequence homology to striatin (Goudreault *et al.* 2009). The question remains: How does the Far3-7-8-9-10-11 complex affect PP2A activity? Interaction between Far11 and Tpd3, the scaffolding subunit of PP2A, suggests that the Far complex may directly regulate PP2A by either targeting the TORC2 substrate Slm1 and/or mediating PP2A activity. Unlike slow cell growth phenotypes due to a *tpd3*Δ single mutation or a *pph21/22*Δ double mutation, mutations in *FAR3*, *FAR7*, *FAR8*, *FAR9*, *FAR10*, and *FAR11* have little or no growth defects, suggesting that the Far complex is not integral to PP2A activity. In both the *Drosophila* study and ours, mutations in PP2A and/or Far components of the STRIPAK complex lead to increased phosphorylation of target proteins. Thus, it is possible that the Far complex might target certain substrates to PP2A.

Our study demonstrates that Far11-PP2A-Rts1 modulates Slm1 phosphorylation by counteracting the kinase activity of TORC2, providing a molecular mechanism to explain how mutations in *FAR11* and genes encoding PP2A-Rts1 components might suppress TORC2 deficiency. The calcineurin phosphatase also mediates Slm1 dephosphorylation and counteracts TORC2 signaling (Bultynck *et al.* 2006; Mulet

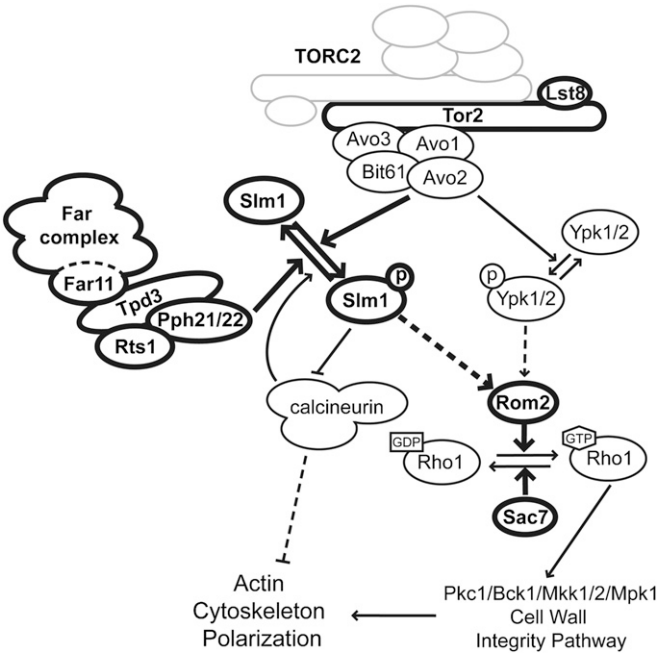

Figure 8 A model for the regulation of TORC2 signaling by the Far3-7-8-9-10-11 complex and PP2A-Rts1. TORC2 regulates the organization of the actin cytoskeleton via phosphorylation of Slm1 and Ypk1/2. Far3-7-8-9-10-11-PP2A-Rts1 antagonizes TORC2 signaling by promoting Slm1 dephosphorylation. Proteins in boldface type were analyzed in this study.

et al. 2006; Daquinag *et al.* 2007). It was further shown that mutations in *cnb1*, encoding the regulatory subunit of calcineurin, suppress an *avo3* temperature-sensitive mutation (Aronova *et al.* 2008). It remains to be determined whether a *cnb1* mutation restores phosphorylation of Slm1 to TORC2-deficient cells. Ypk2 is another essential effector of TORC2. Mutations in calcineurin could potentially restore phosphorylation of Ypk2 in *avo3* mutant cells, thereby suppressing the *avo3* temperature-sensitive growth phenotype. Furthermore, in a genome-wide study on genetic interactions in yeast, mutations in *PPG1*, encoding a PP2A-like phosphatase, also suppress TORC2 deficiency (Baryshnikova *et al.* 2010). Therefore, it is likely that these phosphatases may work together to mediate TORC2 signaling.

Various genetic screens in fungi have isolated mutations in the Far complex. In *Neurospora crassa*, a mutation in *ham-2*, the ortholog of yeast *FAR11*, leads to defects in hyphal fusion (Xiang *et al.* 2002). In *Sordaria macrospora*, mutations in *PRO22*, encoding the yeast Far11 ortholog, generate a novel type of sterile mutant with a defect in ascogonial septum formation (Bloemendal *et al.* 2010). In yeast, mutations in *FAR9/VPS64* and *FAR11/YNL127w* result in vacuolar sorting defects (Bonangelino *et al.* 2002), and mutations in *FAR3*, *FAR7*, *FAR8*, *FAR10*, and *FAR11* create long-lived mutants (Fabrizio *et al.* 2010). In all of these studies, the underlying mechanisms are unknown. In light of our findings, it is possible that these disparate phenotypes are due to the perturbation of PP2A activity and/or TORC2 signaling in these mutants.

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TORC2 Signaling Is Antagonized by Protein Phosphatase 2A and the Far Complex in *Saccharomyces cerevisiae*

Tammy Pracheil, Janet Thornton, and Zhengchang Liu

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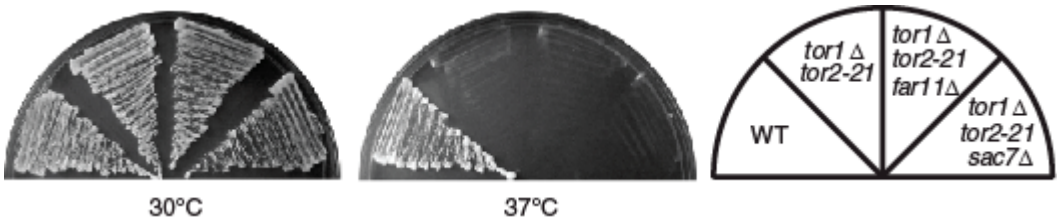


Figure S1 *sac7Δ* and *far11Δ* do not suppress the temperature-sensitive growth phenotype of a *tor1Δ tor2-21* double mutant. Wild-type (SH100), *tor1Δ tor2-21* (SH221), *tor1Δ tor2-21 sac7Δ* (TPY112), and *tor1Δ tor2-21 far11Δ* (TPY118) cells were grown on YPD plates at 30 °C and 37 °C.

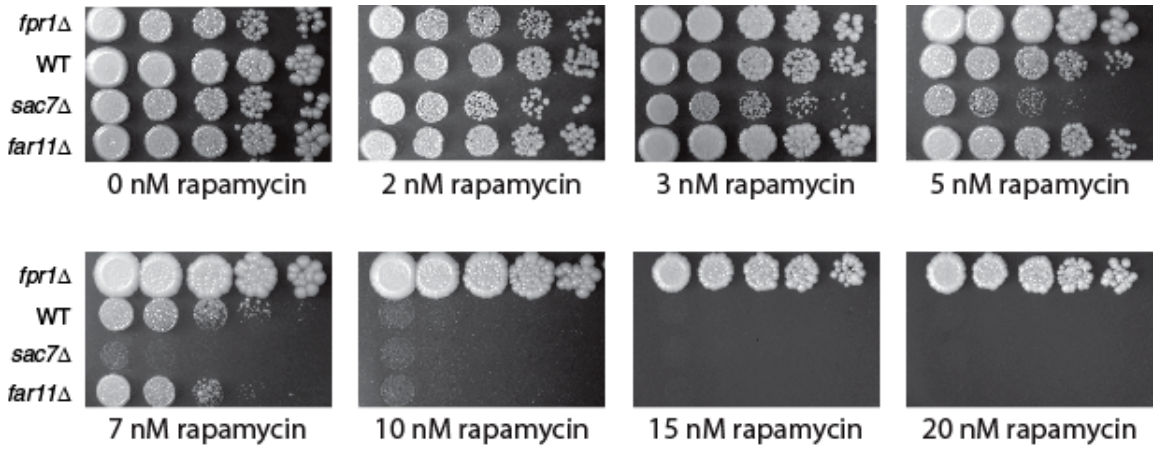
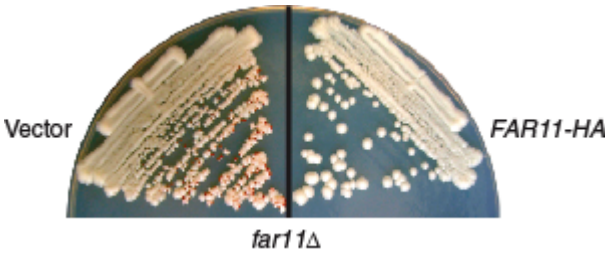


Figure S2 The effect of rapamycin on the growth of *sac7Δ* and *far11Δ* mutant cells. Cultures of wild-type (ZLY423), *fpr1Δ* (TPY122), *sac7Δ* (ZLY2404), and *far11Δ* mutant (TPY114) cells were serially diluted and spotted on YPD plates supplemented with different concentrations of rapamycin.



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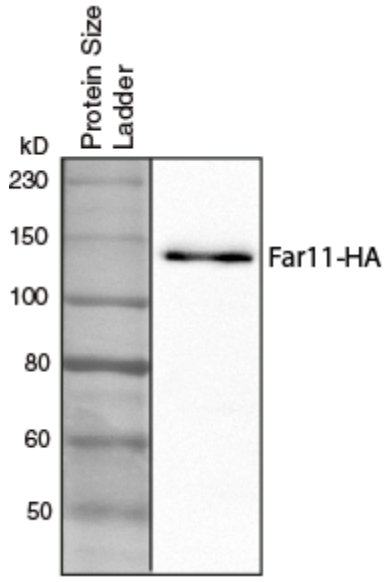

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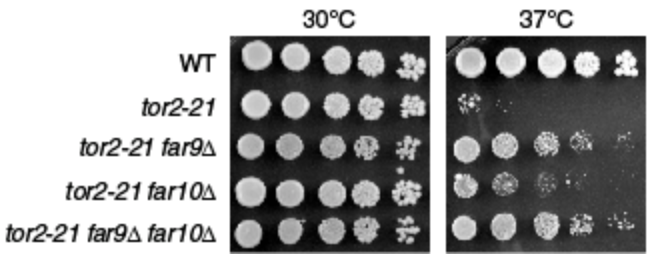
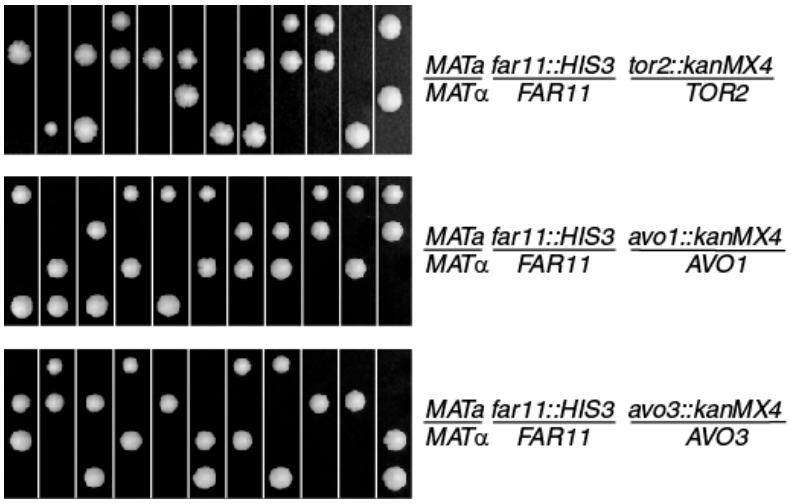


Figure S5 The effect of *far9Δ* and *far10Δ* on suppressing the temperature-sensitive growth phenotype of a *tor2-21* mutant. Wild-type (SH100), *tor2-21* (SH121), *tor2-21 far9Δ* (TPY207), *tor2-21 far10Δ* (TPY264) and *tor2-21 far9Δ far10Δ* mutant (TPY220) cells were serially diluted and spotted on YPD plates and grown at 30 °C and 37 °C.



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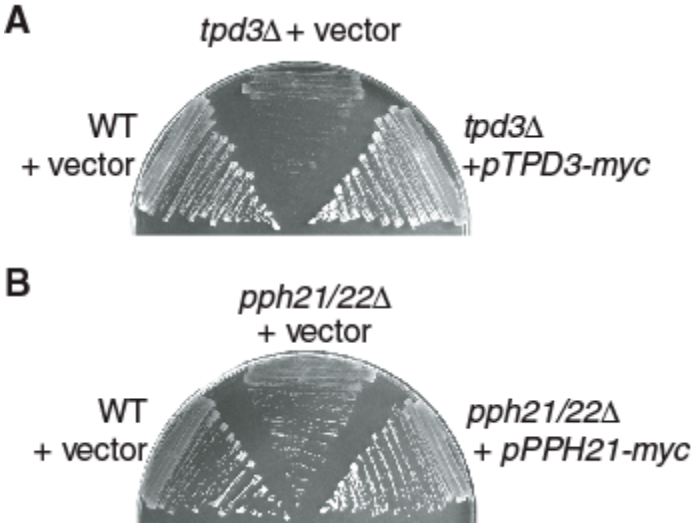

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(B) Pph21-myc is functional. Wild type (BY4741) and isogenic *pph21/22Δ* mutant (BY4741 pph21/22) cells carrying an empty vector (pRS415) or *PPH21-myc* plasmid (pTP244) were analyzed for cell growth as described for panel A.

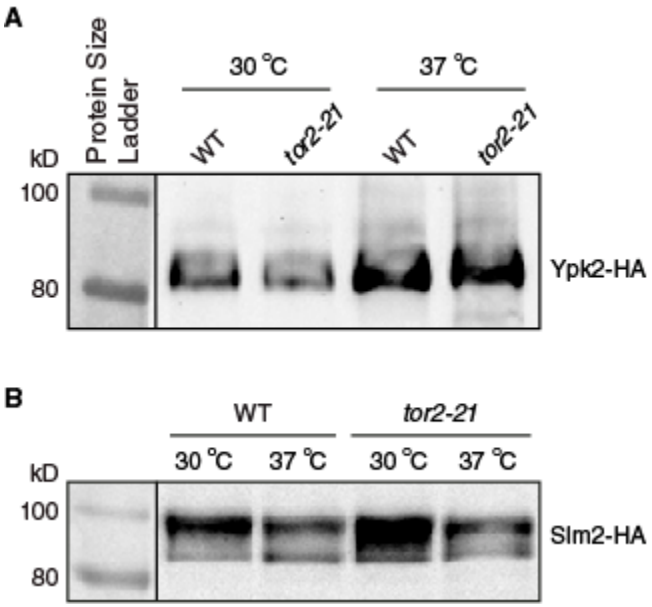

Figure S8 Immunoblot analysis of HA-tagged Ypk2 (panel A) and Slm2 (panel B). Wild-type (WT, SH100) and temperature-sensitive *tor2-21* mutant cells (SH121) expressing C-terminal 3xHA-tagged Ypk2 or Slm2 from a centromeric plasmid (*YPK2-HA*, pTP271; *SLM2-HA*, pTP377) were grown in YNBcasD medium at 30 °C to mid-log phase and switched to 37 °C for 3h before cellular proteins were processed for Western blotting.

Table S1 Quantitative analysis of polarization of the actin cytoskeleton in wild-type and isogenic *tor2-21* (SH121), *tor2-21 sac7Δ* (TPY110), *tor2-21 far11Δ* (TPY311), *tor2-21 sac7Δ far11Δ* (TPY301) mutant cells. Cells were grown in YPD medium at 30 °C to mid-log phase and then switched to 37 °C for 3 h before cells were fixed by formaldehyde and stained with rhodamine phalloidin. The percentage of cells with polarized actin cytoskeleton was tabulated.

Strain	# cells imaged	# polarized cells	% polarized cells
Wild-type (SH100)	474	357	75.3%
tor2-21 (SH121)	424	89	21.0%
tor2-21 sac7Δ (TPY110)	504	267	53.0%
tor2-21 far11Δ (TPY311)	461	240	52.1%
tor2-21 sac7Δ far11Δ (TPY301)	451	318	70.5%

Table S2 Strains used.

Strain	Genotype	Source/Reference
TPY114 (*far11*)	*MATa ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 far11::kanMX4 [pRS412-LST8]*	This study
SY4078	SY2227 *FAR7-myc13-KAN <pSL2771>*	(KEMP and SPRAGUE 2003)
SH100 (WT)	*MATa leu2-3,112 ura3-52 rme1 trp1 his4 HMLa ade2 tor2::ADE2 [YCplac111::TOR2]*	(HELLIWELL *et al.* 1998)
SH121 (*tor2-21*)	*MATa leu2-3,112 ura3-52 rme1 trp1 his4 HMLa ade2 tor2::ADE2 [YCplac111::tor2-21]*	
SH221 (*tor1 tor2-21*)	*MATa leu2-3,112 ura3-52 rme1 trp1 his3 HMLa ade2 tor1::HIS3 tor2::ADE2 [YCplac111::tor2-21]*	
TPY110 (*tor2-21 sac7*)	SH121 *sac7::kanMX4*	This study
TPY311 (*tor2-21 far11*)	SH121 *far11::TRP1*	This study
TPY301 (*tor2-21 sac7 far11*)	SH121 *sac7::kanMX4 far11::TRP1*	This study
TPY112	SH221 *sac7::kanMX4*	This study
TPY118	SH221 *far11::kanMX4*	This study
TPY207 (*tor2-21 far9*)	SH121 *far9::kanMX4*	This study
TPY264 (*tor2-21 far10*)	SH121 *far10::URA3*	This study
TPY220 (*tor2-21 far9 far10*)	SH121 far9::kanMX4 *far10::URA3*	This study
ZLY423 (WT)	*MATa ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 [pRS412-LST8]*	This study.
TPY122 (*fpr1*)	*MATa ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 fpr1::kanMX4 [pRS412-LST8]*	This study
ZLY2404 (*sac7*)	*MATa ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 sac7::kanMX4 [pRS412-LST8]*	This study
TPY114 (*far11*)	*MATa ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 far11::kanMX4 [pRS412-LST8]*	This study
BY4741 (*pph21/22*)	*MATa ura3 leu2 his3 met15 pph21::kanMX4 pph22::kanMX4*	This study
BY4741	*MATa ura3 leu2 his3 met15*	Yeast genome
BY4741 *tpd3*	BY4741 *tpd3::kanMX4*	deletion project
BY4743	*MATa/MATalpha ura3/ura3 leu2/leu2 his3/his3 lys2/LYS2 met15/MET15*	
BY4743 *tor2/TOR2*	BY4743 *tor2::kanMX4/TOR2*	
BY4743 *avo1/AVO1*	BY4743 *avo1::kanMX4/AVO1*	
BY4743 *avo3/AVO3*	BY4743 *avo3::kanMX4/AVO3*	

TPY654	BY4743 *tor2::kanMX4/TOR2 far11::HIS3/FAR11*	This study
TPY652	BY4743 *avo1::kanMX4/AVO1 far11::HIS3/FAR11*	This study
TPY653	BY4743 *avo3::kanMX4/AVO3 far11::HIS3/FAR11*	This study

Table S3 Plasmids used.

Plasmid	Description	Source/Reference
pZL2762	pRS416-FAR11-HA, expressing Far11 from its own promoter with a 3xHA tag at the C-terminus.	This study
pTP377	pRS416-SLM2-HA, expressing Slm2 from its own promoter with a 3xHA tag at the C-terminus.	This study
pTP271	pRS416-YPK2-HA, expressing Ypk2 from its own promoter with a 3xHA tag at the C-terminus.	This study
pZL1255	pRS412-LST8	This study
pTP242	pRS415-ADH1-TPD3-myc, expressing Tpd3 from the *ADH1* promoter with a 3xmyc tag at the C-terminus.	This study
pTP244	pRS415-PPH21-myc, expressing Pph21 from its own promoter with a 3xmyc tag at the C-terminus.	This study

Supplemental References

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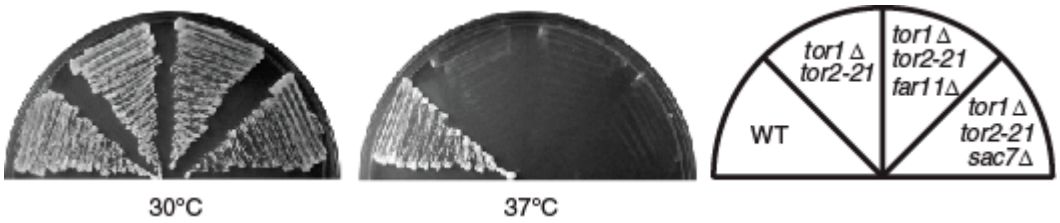

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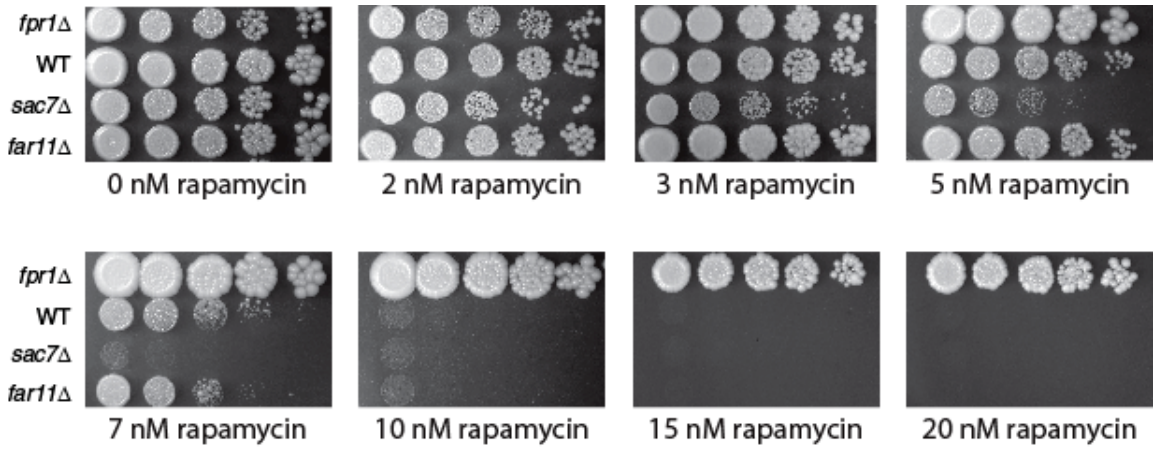

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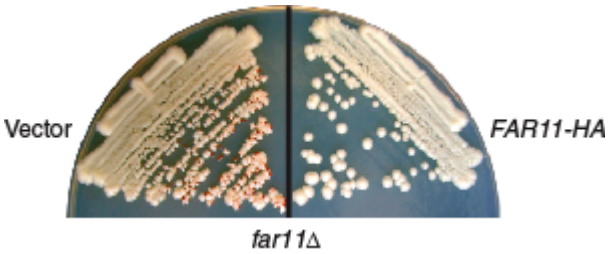

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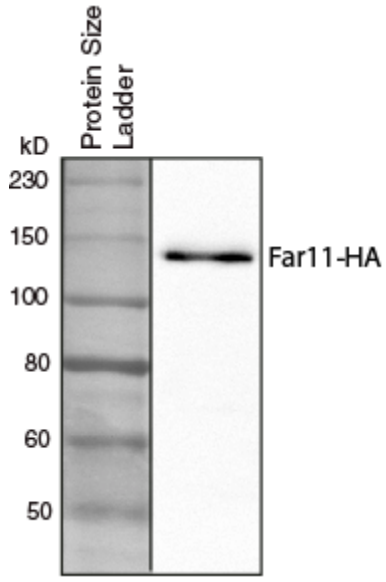

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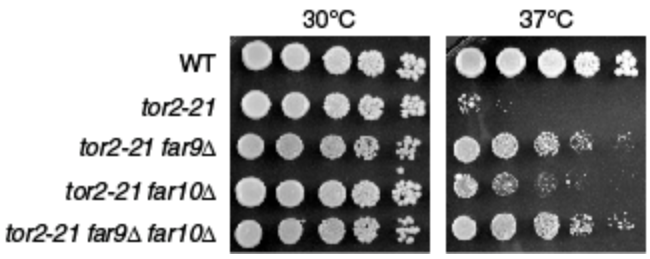

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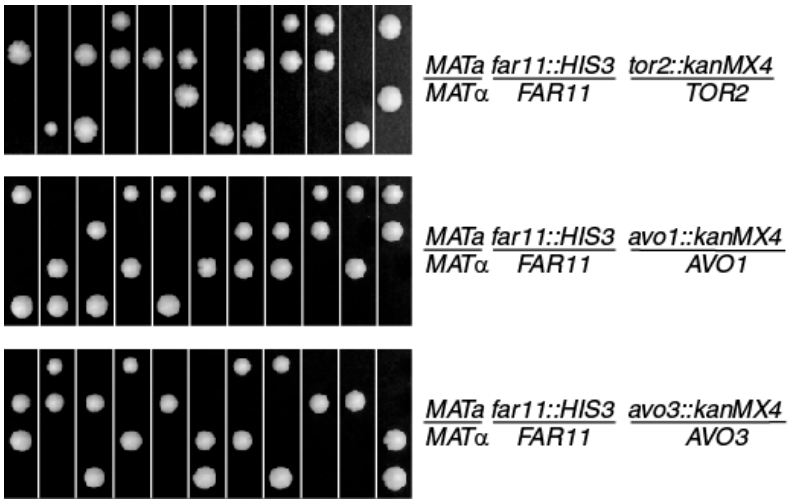

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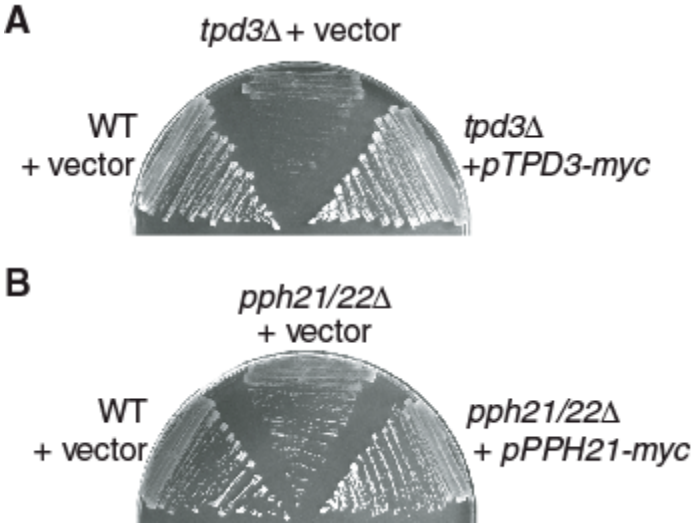

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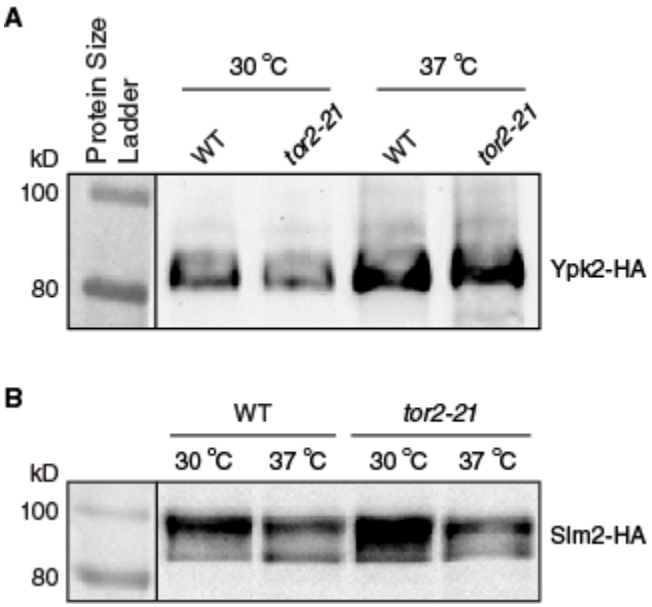

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SH221 (*tor1 tor2-21*)	*MATa leu2-3,112 ura3-52 rme1 trp1 his3 HMLa ade2 tor1::HIS3 tor2::ADE2 [YCplac111::tor2-21]*	
TPY110 (*tor2-21 sac7*)	SH121 *sac7::kanMX4*	This study
TPY311 (*tor2-21 far11*)	SH121 *far11::TRP1*	This study
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TPY112	SH221 *sac7::kanMX4*	This study
TPY118	SH221 *far11::kanMX4*	This study
TPY207 (*tor2-21 far9*)	SH121 *far9::kanMX4*	This study
TPY264 (*tor2-21 far10*)	SH121 *far10::URA3*	This study
TPY220 (*tor2-21 far9 far10*)	SH121 far9::kanMX4 *far10::URA3*	This study
ZLY423 (WT)	*MATa ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 [pRS412-LST8]*	This study.
TPY122 (*fpr1*)	*MATa ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 fpr1::kanMX4 [pRS412-LST8]*	This study
ZLY2404 (*sac7*)	*MATa ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 sac7::kanMX4 [pRS412-LST8]*	This study
TPY114 (*far11*)	*MATa ade2-1 ura3 his3-11,15 leu2 lst8::LEU2 far11::kanMX4 [pRS412-LST8]*	This study
BY4741 (*pph21/22*)	*MATa ura3 leu2 his3 met15 pph21::kanMX4 pph22::kanMX4*	This study
BY4741	*MATa ura3 leu2 his3 met15*	Yeast genome
BY4741 *tpd3*	BY4741 *tpd3::kanMX4*	deletion project
BY4743	*MATa/MATalpha ura3/ura3 leu2/leu2 his3/his3 lys2/LYS2 met15/MET15*	
BY4743 *tor2/TOR2*	BY4743 *tor2::kanMX4/TOR2*	
BY4743 *avo1/AVO1*	BY4743 *avo1::kanMX4/AVO1*	
BY4743 *avo3/AVO3*	BY4743 *avo3::kanMX4/AVO3*	

TPY654	BY4743 *tor2::kanMX4/TOR2 far11::HIS3/FAR11*	This study
TPY652	BY4743 *avo1::kanMX4/AVO1 far11::HIS3/FAR11*	This study
TPY653	BY4743 *avo3::kanMX4/AVO3 far11::HIS3/FAR11*	This study

Table S3 Plasmids used.

Plasmid	Description	Source/Reference
pZL2762	pRS416-FAR11-HA, expressing Far11 from its own promoter with a 3xHA tag at the C-terminus.	This study
pTP377	pRS416-SLM2-HA, expressing Slm2 from its own promoter with a 3xHA tag at the C-terminus.	This study
pTP271	pRS416-YPK2-HA, expressing Ypk2 from its own promoter with a 3xHA tag at the C-terminus.	This study
pZL1255	pRS412-LST8	This study
pTP242	pRS415-ADH1-TPD3-myc, expressing Tpd3 from the *ADH1* promoter with a 3xmyc tag at the C-terminus.	This study
pTP244	pRS415-PPH21-myc, expressing Pph21 from its own promoter with a 3xmyc tag at the C-terminus.	This study

Supplemental References

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