

Phosphoinositide kinase signaling controls ER-PM cross-talk

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ABSTRACT Membrane lipid dynamics must be precisely regulated for normal cellular function, and disruptions in lipid homeostasis are linked to the progression of several diseases. However, little is known about the sensory mechanisms for detecting membrane composition and how lipid metabolism is regulated in response to membrane stress. We find that phosphoinositide (PI) kinase signaling controls a conserved PDK-TORC2-Akt signaling cascade as part of a homeostasis network that allows the endoplasmic reticulum (ER) to modulate essential responses, including Ca²⁺-regulated lipid biogenesis, upon plasma membrane (PM) stress. Furthermore, loss of ER-PM junctions impairs this protective response, leading to PM integrity defects upon heat stress. Thus PI kinase-mediated ER-PM cross-talk comprises a regulatory system that ensures cellular integrity under membrane stress conditions.

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INTRODUCTION

The plasma membrane (PM) is highly organized and undergoes extensive remodeling via the delivery and removal of proteins and lipids. During membrane stress, PM quality control and recalibration systems ensure the integrity of the PM through the clearance of damaged PM components and by the delivery of newly synthesized materials (Zhao *et al.*, 2013). The biogenesis of proteins and lipids destined for the PM takes place in the endoplasmic reticulum (ER), which has essential roles in protein quality control, lipid biosynthesis, and calcium (Ca²⁺) signaling (Friedman and Voeltz, 2011). ER metabolism must be modulated to adjust PM composition as needed. However, we do not fully understand how changes in PM composition and status are communicated to the ER.

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The authors declare that they have no conflict of interest.

Abbreviations used: ER, endoplasmic reticulum; PI, phosphoinositide; PM, plasma membrane.

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The ER forms a continuous membrane network throughout the cell, consisting of nuclear ER, cytoplasmic sheets and tubules, and an extensive cortical meshwork (Friedman and Voeltz, 2011; Figure 1A depicts ER architecture in yeast). The ER engages in cross-talk with the PM through cortical ER-PM junctions, where the ER and PM form close connections without undergoing membrane fusion (Stefan *et al.*, 2013). ER-PM junctions are extensively formed in yeast by three conserved tether protein families: the VAP orthologues Scs2/Scs22, the extended synaptotagmin-like (E-Syt) proteins Tcb1/2/3, and the TMEM16 family member Ist2 (Manford *et al.*, 2012; Figure 1B). Loss of these ER-PM tethers results in a dramatic reduction of the cortical ER network and ER stress, indicating that ER-PM junctions control ER structure and function (Manford *et al.*, 2012; Δ tether cells, Figure 1B). However, roles for ER-PM junctions in the regulation of PM organization and integrity have not been extensively characterized.

Here we show that ER-PM junctions are needed to maintain cellular integrity upon membrane stress. We find that phosphoinositide (PI) kinase signaling activates the yeast phosphoinositide-dependent kinase (PDK) orthologues Pkh1/Pkh2 and the target of rapamycin complex 2 (TORC2) upon stress conditions. Pkh1/2 and TORC2 activate the Akt/SGK protein kinase orthologues Ypk1/2 necessary for stress-induced sphingolipid synthesis in the ER (Roelants *et al.*, 2011; Berchtold *et al.*, 2012; Niles *et al.*, 2012; Sun *et al.*, 2012). Our results show that in cells lacking ER-PM tether proteins, Ypk1/2-stimulated sphingolipid synthesis in the ER is compromised, resulting in PM integrity defects. This is due to misregulation of Ca²⁺ dynamics and

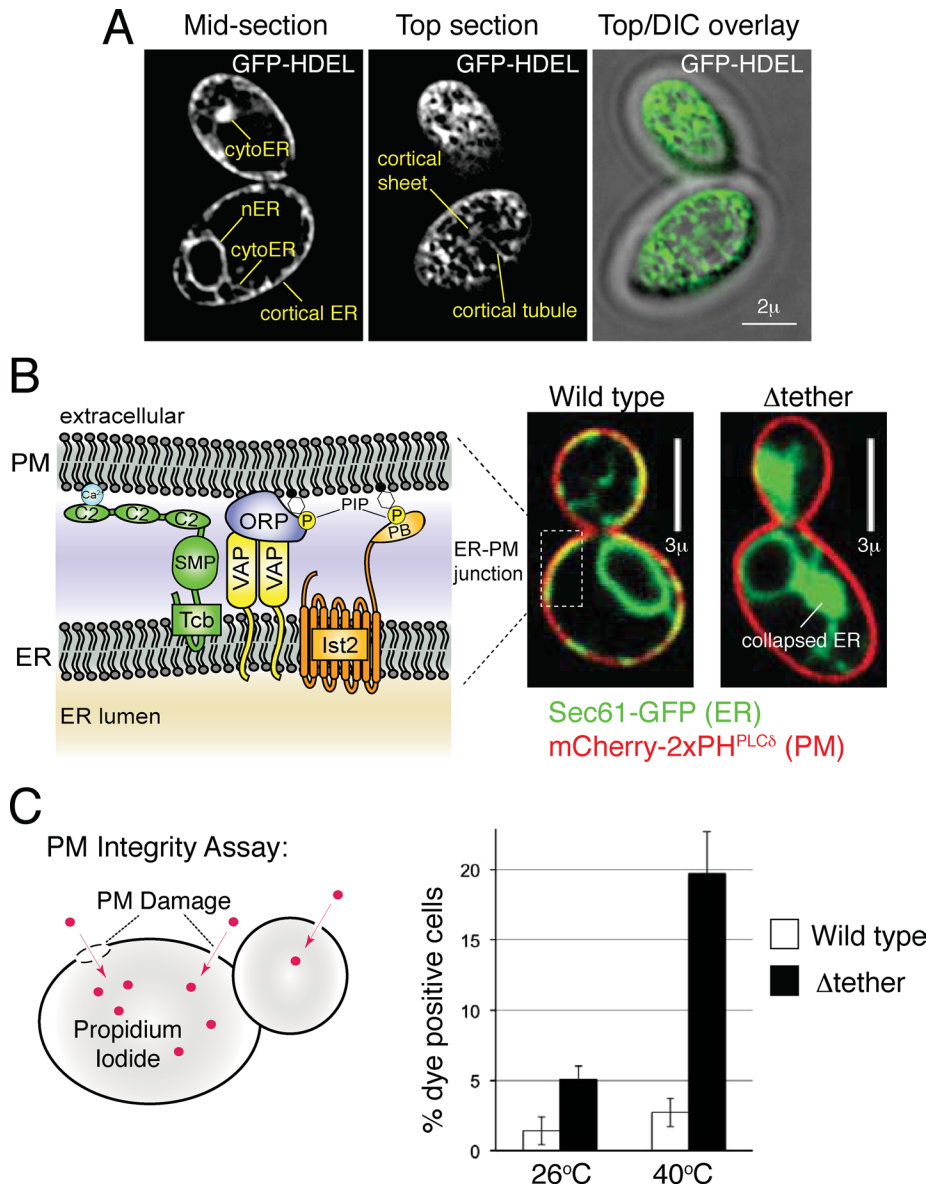


FIGURE 1: ER-PM junctions maintain PM integrity upon heat stress. (A) The ER forms an extensive network of sheets and tubules. Localization of the ER marker GFP-HDEL in wild-type diploid cells. Cortical ER (cER), cytoplasmic ER (cytoER), and nuclear ER (nER) are labeled. Scale bar, 2 μ m. (B) Left, three protein families tether the cortical ER to the PM: the VAP proteins Scs2/22, Ist2, and the tricalbins (Tcb1/2/3). The VAP proteins bind PI4P-binding ORP family members. PB, polybasic domain; SMP, synaptotagmin-like mitochondrial lipid-binding protein. Right, loss of ER-PM tethers alters ER morphology. Localization of the ER marker Sec61-GFP (green) and PM marker mCherry-2xPH^{PLC δ} (red) in wild-type and Δ tether cells (*ist2 Δ* , *scs2/22 Δ* , *tcb1/2/3 Δ*). An ER-PM junction is indicated in wild-type cells and collapsed ER in Δ tether cells. Scale bars, 3 μ m. (C) Cells incubated at 26 or 40 $^{\circ}$ C for 2 h were stained with propidium iodide to monitor PM integrity. Staining of wild-type (white bars) and Δ tether (black bars) cells was analyzed by flow cytometry. See Supplemental Figure S1.

calcineurin signaling, which inhibits sphingolipid synthesis in the ER. Thus ER architecture and PI kinase-mediated ER-PM cross-talk play a central role in membrane homeostasis and cellular integrity.

RESULTS

ER-PM junctions maintain PM integrity during heat stress

The ER is a major site for protein biogenesis, lipid synthesis, and Ca²⁺ homeostasis. Cross-talk between the ER and PM at membrane contact sites may regulate these important ER functions in response

to changes in PM composition. Consistent with this, the cortical ER network is continuously remodeled in yeast cells and forms numerous contacts with the PM (Supplemental Movie S1). When followed over time (30 min), the cortical ER cumulatively covers >80% of the PM (Supplemental Movie S2). Accordingly, ER-PM contacts may allow the ER to sense the state of the PM (damage, composition) and launch responses (Ca²⁺ dynamics, protein and lipid synthesis) to ensure PM integrity on changes in PM organization and stress conditions.

We examined whether ER-PM junctions are necessary to maintain PM integrity under stress conditions. These experiments used a quantitative flow cytometry assay measuring entry of the dye propidium iodide into yeast cells under various conditions (Figure 1 and Supplemental Figure S1; Zhao et al., 2013). Propidium iodide is membrane impermeant and can be used to score cells in a population that have lost cellular integrity. Wild-type yeast cells grown at 26 $^{\circ}$ C exhibited negligible staining (<3% cells within the population; Figure 1C, 26 $^{\circ}$ C). At 26 $^{\circ}$ C, 5% of mutant cells lacking the ER-PM tether proteins (Δ tether cells) stained with the dye, comparable to wild-type cells (Figure 1C). Wild-type cells were resistant to mild heat stress conditions (2 h at 40 $^{\circ}$ C), with the PM remaining intact in the vast majority of the population (<4% of wild-type cells stain positive at 40 $^{\circ}$ C; Figure 1C). However, Δ tether cells showed increased sensitivity to heat stress. On incubation at 40 $^{\circ}$ C, nearly 20% of the Δ tether cells scored positive (fivefold greater than wild-type cells; Figure 1C). The Δ tether cells also displayed dramatic sensitivity to brief heat shock at 42 $^{\circ}$ C. Nearly 70% of the mutant cells scored as positive, compared with 5% of wild-type cells under these conditions (Supplemental Figure S1A). Among the individual tether family members, the Tcb1/2/3 proteins were particularly important. Approximately 45% of cells lacking Tcb1/2/3 displayed PM integrity defects upon brief heat shock at 42 $^{\circ}$ C (compared with 15 and 5% of cells lacking the Scs2/22 VAP orthologues and Ist2 respectively; Supplemental Figure S1A). Cells lacking Tcb1/2/3 do not display an obvious reduction in cortical ER (Manford et al., 2012), suggesting that the tricalbins may be specifically needed for PM integrity rather than another ER-localized protein. Consistent with this, an artificial ER-PM tether protein (tandem PM-binding PH^{PLC δ} domains linked to the Scs2 ER-anchored transmembrane domain) formed cortical ER in the Δ tether cells but did not restore PM integrity upon heat shock (Supplemental Figure S1, B and C). Thus proteins that form and function at ER-PM junctions (Tcb1/2/3 and Scs2/22) are specifically needed to maintain cellular integrity upon heat-induced PM damage.

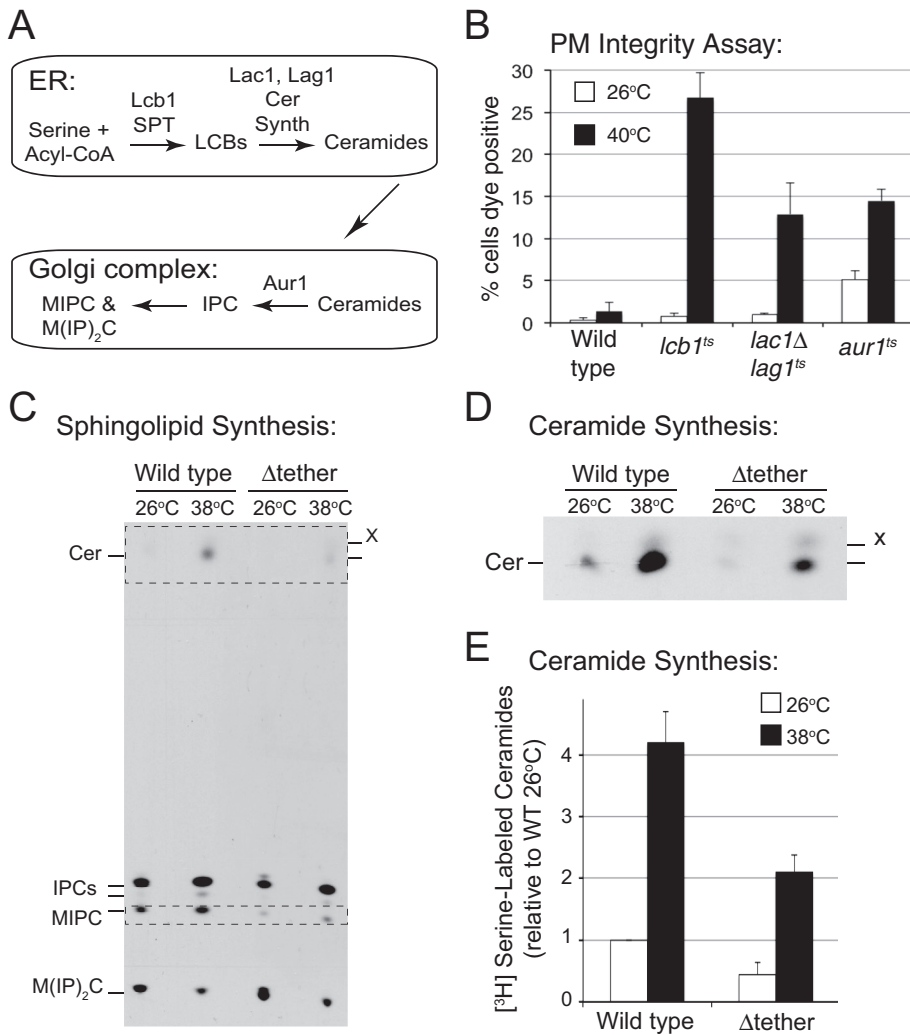


FIGURE 2: ER-PM junctions regulate sphingolipid synthesis. (A) The yeast biosynthetic pathway for sphingolipid synthesis in the ER and Golgi network. (B) Sphingolipid synthesis in the ER and Golgi complex protects PM integrity during heat stress. Wild-type, *lcb1^{ts}*, *lac1 lag1^{ts}*, and *aur1^{ts}* cells incubated at 26 (white bars) or 40°C for 2 h (black bars) were stained with propidium iodide and analyzed by flow cytometry. (C) Analysis of sphingolipid synthesis in wild-type and Δ tether cells. Wild-type and Δ tether cells were preincubated at the appropriate temperature for 10 min and labeled with [³H]serine for 60 min. Sphingolipids were extracted and analyzed by TLC. Ceramides (Cer) and the complex sphingolipids IPCs, MIPC, and M(IP)₂C are indicated. An unknown lipid, X, was also observed. The hatched areas point out reduced ceramides and MIPC in the Δ tether mutant cells. (D) Synthesis of ceramides in wild-type and Δ tether cells. Results shown here are from a long exposure from an independent experiment to better visualize ceramides. As in Figure 2C, cells were preincubated at the appropriate temperature for 10 min and labeled with [³H]serine for 60 min. Sphingolipids were extracted and analyzed by TLC. Ceramides (Cer) and an unknown lipid, X, are indicated. (E) Quantitation of ceramide synthesis in wild-type and Δ tether cells normalized to wild type at 26°C. The data represent the mean \pm SD from three independent experiments.

Elevated temperatures damage integral PM proteins, such as ion channels and nutrient transporters, activating a PM quality control pathway mediated by the ART protein E3 ubiquitin ligase adaptors (Zhao *et al.*, 2013). Misfolded PM proteins detected by the ARTs are marked with ubiquitin and targeted for endocytic internalization. After internalization, ubiquitinated damaged proteins are delivered to the endosomal sorting complexes required for transport (ESCRT)-mediated sorting pathway for degradation in vacuoles. Clearance of misfolded proteins from the cell surface is needed to maintain PM integrity, and loss of the ART proteins confers

reduced viability upon heat stress (Zhao *et al.*, 2013). However the ART- and ESCRT-mediated quality control pathways still function in cells lacking the ER-PM tether proteins. On heat stress (40°C), a green fluorescent protein (GFP)-tagged amino acid transporter, Can1-GFP, was internalized from the PM and delivered to the vacuole in both wild-type and Δ tether cells (Supplemental Figure S1D). Thus the role for ER-PM contacts in PM integrity may be distinct from the quality control pathways carried out by the ART and ESCRT proteins.

ER-PM junctions regulate sphingolipid synthesis necessary for PM integrity

Cells up-regulate de novo sphingolipid synthesis in the ER during heat-induced membrane stress (Tabuchi *et al.*, 2006; Cowart and Hannun, 2007; Cowart and Obeid, 2007; Sun *et al.*, 2012; Muir *et al.*, 2014). We examined whether de novo sphingolipid synthesis is necessary to maintain PM integrity upon heat stress and whether ER-PM cross-talk is involved in this membrane stress response pathway. Sphingolipid synthesis is initiated in the ER, where long-chain sphingoid bases (LCBs) and ceramides are generated by the ER-localized serine palmitoyltransferase (SPT) and ceramide synthases, respectively (Figure 2A). We first examined whether de novo sphingolipid synthesis was necessary to maintain PM integrity upon heat stress. At 40°C, >25% of *lcb1^{ts}* mutant cells (with impaired SPT activity) and 12% of *lac1 lag1^{ts}* mutant cells (with impaired ceramide synthase activity) stained with propidium iodide (20- and 10-fold greater than wild-type cells, respectively; Figure 2B). In addition, 15% of *aur1^{ts}* cells impaired in complex sphingolipid synthesis in the Golgi network displayed PM integrity defects upon shift to 40°C (Figure 2, A and B). Thus sphingolipid synthesis in the ER and Golgi compartments is needed for PM integrity during heat stress.

We next examined whether ER-PM junctions play a role in the regulation of sphingolipid synthesis. In [³H]serine radiolabelling experiments, we observed that ³H-labeled ceramides increased greater than fourfold in wild-type cells upon heat stress conditions (Figure 2, C–E, 26 vs. 38°C), consistent with previous studies (Tabuchi *et al.*, 2006; Cowart and Hannun, 2007; Cowart and Obeid, 2007; Sun *et al.*, 2012; Muir *et al.*, 2014). However, [³H]serine-labeled ceramides were significantly reduced in the Δ tether cells (50% of wild-type levels at both 26 and 38°C; Figure 2, C–E). Ceramides are converted to more complex sphingolipids in the Golgi network (Figure 2A), including the yeast complex sphingolipids inositolphosphoceramides (IPCs) and mannosylinositolphosphoceramides (MIPC and M(IP)₂C). Incorporation of ³H-labeled serine into IPC and M(IP)₂C was not significantly

affected, but MIPC synthesis was reduced in the Δ tether cells as compared with wild-type cells (48 and 70% of wild-type levels at 26 and 38°C, respectively; Supplemental Figure S2, A and B). The Δ tether mutant cells were also hypersensitive to aureobasidin A, an inhibitor of Aur1 activity and sphingolipid synthesis in the Golgi complex (Supplemental Figure S2C), further suggesting that sphingolipid synthesis is impaired in these cells. Expression of the artificial ER-PM tether protein did not rescue the hypersensitivity of the Δ tether mutant cells to aureobasidin A (Supplemental Figure S2C). Taken together, these results suggest that 1) ER-PM tether proteins are specifically involved in the control of sphingolipid synthesis and 2) sphingolipid synthesis defects in the Δ tether mutant cells may result in PM integrity defects upon stress conditions.

Pkh1 and TORC2 signaling link PI kinase signaling at the PM to sphingolipid synthesis in the ER

We next investigated how ER-PM cross-talk is regulated and the role for ER-PM junctions in membrane stress responses. PI kinases localized at the PM are required for cellular integrity and responses to heat stress (Audhya *et al.*, 2000; Audhya and Emr, 2002). At elevated temperature, levels of phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P₂) generated by the Stt4 PI4K and the Mss4 PI4P 5-kinase are modestly increased (Audhya and Emr, 2002). Stt4 PI4K and Mss4 PI4P5K activities have also been implicated in sphingolipid metabolism (Tabuchi *et al.*, 2006). However, roles for PI kinase signaling in the regulation of ER-PM cross-talk and membrane stress responses have not been fully elucidated.

The yeast protein kinases 1 and 2 (Ypk1/2), orthologues of mammalian Akt, regulate sphingolipid synthesis in the ER. Ypk1/2 phosphorylate and inactivate the Orm1/2 proteins, integral ER membrane proteins that inhibit SPT, the first enzyme in sphingolipid biosynthesis (Roelants *et al.*, 2011; Berchtold *et al.*, 2012; Sun *et al.*, 2012; Gururaj *et al.*, 2013). Ypk1/2 also phosphorylate and activate ceramide synthases in the ER (Muir *et al.*, 2014). Activation of Ypk1/2 occurs through two upstream protein kinases, the PDK orthologues Pkh1/Pkh2 and TORC2; Roelants *et al.*, 2004; Niles *et al.*, 2012). TORC2 signaling in yeast cells is regulated by the PI(4,5)P₂-binding Slm1 and Slm2 proteins (Audhya *et al.*, 2004; see later discussion of Figure 5). However, it is unclear whether PI kinase signaling regulates the yeast PDK orthologues Pkh1 and Pkh2.

We addressed whether PI kinase and TORC2-Pkh1/2-Ypk1/2 signaling function together in ER-PM cross-talk. Loss of Stt4, Mss4, Pkh1/2, or Ypk1/2 function similarly resulted in severe PM integrity defects even upon brief heat shock conditions (42°C, 10 min). At 42°C, 60% of *stt4*^{ts} mutant cells and 50% of *mss4*^{ts}, *pkh1*^{ts} *pkh2*, and *ypk1*^{ts} *ypk2* mutant cells stained with propidium iodide (10-fold greater than wild-type cells; Figure 3A). Moreover, overexpression of Ypk1 partially rescued the growth defects of *stt4*^{ts} mutant cells (Supplemental Figure S3A), suggesting that Ypk1 function may be impaired in cells with reduced Stt4 PI4K activity. Altogether, these results suggested that Stt4, Mss4, Pkh1/2, and Ypk1/2 function in a similar pathway, and we further investigated roles for PI kinases in Pkh1/2 and Ypk1/2 signaling.

The PDK orthologues Pkh1/2 phosphorylate the activation loops of Ypk1 and Ypk2 (residue T504 in Ypk1; Roelants *et al.*, 2004). We tested whether Stt4 and Mss4 PI kinase activities control Pkh1/2 signaling, using an antibody that recognizes Ypk1 phosphorylated at residue T504. On heat stress (at 38°C), phospho-Ypk1(T504) increased more than threefold in wild-type cells (Figure 3B and Supplemental Figure S3B). As a control, phospho-Ypk1(T504) levels were barely detectable in *pkh1*^{ts} *pkh2* mutant cells (Figure 3B and

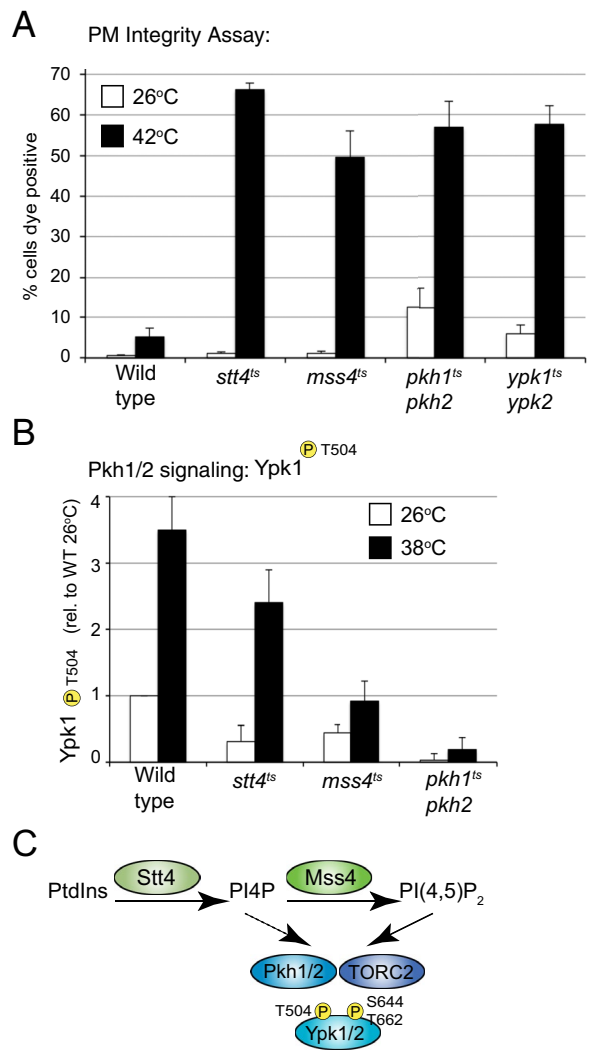


FIGURE 3: PI kinase signaling regulates the protein kinase Pkh1. (A) Wild-type, *stt4*^{ts}, *mss4*^{ts}, *pkh1*^{ts} *pkh2* Δ , and *ypk1*^{ts} *ypk2* Δ cells incubated at 26 (white bars) or 42°C for 10 min (black bars) were stained with propidium iodide and analyzed by flow cytometry to monitor PM integrity. (B) Pkh1/2 signaling is modestly impaired in *stt4*^{ts} and strongly impaired in *mss4*^{ts} cells. Wild-type, *stt4*^{ts}, *mss4*^{ts}, and *pkh1*^{ts} *pkh2* Δ cells were incubated at 26 or 38°C for 2 h. Protein extracts were analyzed by immunoblotting using antisera that recognize phospho-Ypk1(T504). Levels of phospho-Ypk1(T504), normalized to a protein loading control, are shown relative to wild-type cells at 26°C. Results are the mean \pm SD from three independent experiments. See Supplemental Figure S3B. (C) Speculative model for regulation of Ypk1/2 signaling by Pkh1/2, TORC2, and the PI isoforms PI4P and PI(4,5)P₂. PI(4,5)P₂ is essential for Pkh1/2 signaling, but PI4P may be involved in Pkh1/2 regulation as well (dashed arrow).

Supplemental Figure S3B). Phospho-Ypk1(T504) was modestly reduced in *stt4*^{ts} cells (30 and 68% of control levels at 26 and 38°C, respectively; Figure 3B and Supplemental Figure S3B). In *mss4*^{ts} mutant cells, phospho-Ypk1(T504) levels were significantly reduced (44 and 26% of control levels at 26 and 38°C, respectively; Figure 3B and Supplemental Figure S3B). As a control, Ypk1 expression levels were slightly elevated in *pkh1*^{ts} *pkh2*, *stt4*^{ts}, and *mss4*^{ts} mutant cells (2-, 1.3-, and 1.6-fold, respectively, at 38°C; Supplemental Figure S3C), indicating that reduced phospho-Ypk1(T504) levels in the

mutant cells were not simply due to lower Ypk1 expression. Taking Ypk1 levels into account, phospho-Ypk1(T504):Ypk1 ratiometric levels in *stt4^{ts}* and *mss4^{ts}* mutant cells were 52 and 16% of wild-type levels, respectively, at the restrictive temperature. These results suggested that both Stt4 and Mss4 were needed for full Pkh1/2 signaling, although the Mss4 PI4P 5-kinase and its product, PI(4,5)P₂, are critical regulators of Pkh1/2 signaling.

Next we examined Pkh1 subcellular localization under normal and heat shock conditions using a functional GFP-Pkh1 fusion expressed from its own promoter (Figure 4 and Supplemental Figure S4, A and B). In wild-type cells at 26°C, GFP-Pkh1 was mainly diffuse throughout the cytoplasm (Figure 4, A and C, and Supplemental Figure S4, C and F), but small, cortical puncta (one to three per cell) could be observed in cells (Figure 4, A and C arrows). On heat shock at 42°C, there was a measurable increase in the number of cortical GFP-Pkh1 foci per cell (Figure 4, A and B, and Supplemental Figure S4, C and F), as well as of puncta size and intensity. The enlarged GFP-Pkh1 foci at 42°C appeared to be mainly cortical, although intracellular puncta were also observed (Figure 4 and Supplemental Figure S4).

A previous study demonstrated that the Pkh1 protein binds liposomes containing various PI lipids and LCBs in vitro (Gallego *et al.*, 2010). We examined roles for the PI isoforms PI4P and PI(4,5)P₂ as well as LCBs in Pkh1 localization in vivo. Pretreatment with the PI4K inhibitor phenylarsine oxide (PAO) impaired heat shock-induced formation of GFP-Pkh1 puncta (Supplemental Table S3), suggesting a role for PI lipids. However, upon brief heat shock conditions (10 min at 42°C; Supplemental Table S3), neither Stt4 nor Mss4 activity appeared to be specifically required, suggesting that both PI4P and PI(4,5)P₂ may target Pkh1 to the PM. Accordingly, GFP-Pkh1 puncta were increased in *sjl1 sjl2^{ts} sjl3* mutant cells impaired in synaptojanin activity required for efficient PI(4,5)P₂ turnover (Supplemental Table S3). PI4P availability is increased at the PM upon heat-induced membrane stress (Supplemental Figure S4C; Jesch *et al.*, 2010), and in support of a role for PI4P in GFP-Pkh1 localization, the size and number of GFP-Pkh1 foci at 26°C increased in *sac1Δ* cells and Δ tether cells, which have elevated PI4P levels but not PI(4,5)P₂ (Figure 4, C and D, and Supplemental Table S3; Manford *et al.*, 2012). As a control, expression of GFP-Pkh1 was not increased in *sac1* cells and Δ tether cells as compared with wild type (Supplemental Figure S4B).

LCBs have also been suggested to regulate Pkh1/2 function in vivo (Liu *et al.*, 2005), and LCBs are elevated in cells lacking the Sac1 PI4P phosphatase activity (see later discussion of Figure 6A; Brice *et al.*, 2009; Breslow *et al.*, 2010). However, GFP-Pkh1 remained localized to punctate structures in *sac1* mutant cells treated with myriocin, which blocks LCB synthesis (Supplemental Figure S4D; 3–10 puncta/cell), indicating that increased PI4P levels may be sufficient for Pkh1 membrane recruitment. Consistent with this, the intensity of GFP-Pkh1 assemblies decreased in *stt4^{ts} sac1Δ* double mutant cells as compared with *sac1Δ* single-mutant cells (Supplemental Figure S4E). Moreover, GFP-Pkh1 puncta were not significantly increased at 26 or 42°C in *orm1 orm2* mutant cells, which have elevated LCB levels (Supplemental Figure S4F and Supplemental Table S3; see later discussion of Figure 6A; Brice *et al.*, 2009; Breslow *et al.*, 2010).

Taken together, these results suggest that both PI4P and PI(4,5)P₂ may be involved in targeting Pkh1 to the PM, although PI(4,5)P₂ is a primary regulator of phosphorylation of Ypk1 by Pkh1 (Figure 3; see later discussion). In addition, the data indicate that Pkh1 signaling is not disrupted in the Δ tether mutant cells. The Scs2/22, Tcb1/2/3, and Ist2 proteins apparently are not required for formation of cortical Pkh1 assemblies, as Pkh1 was constitutively recruited to the PM in

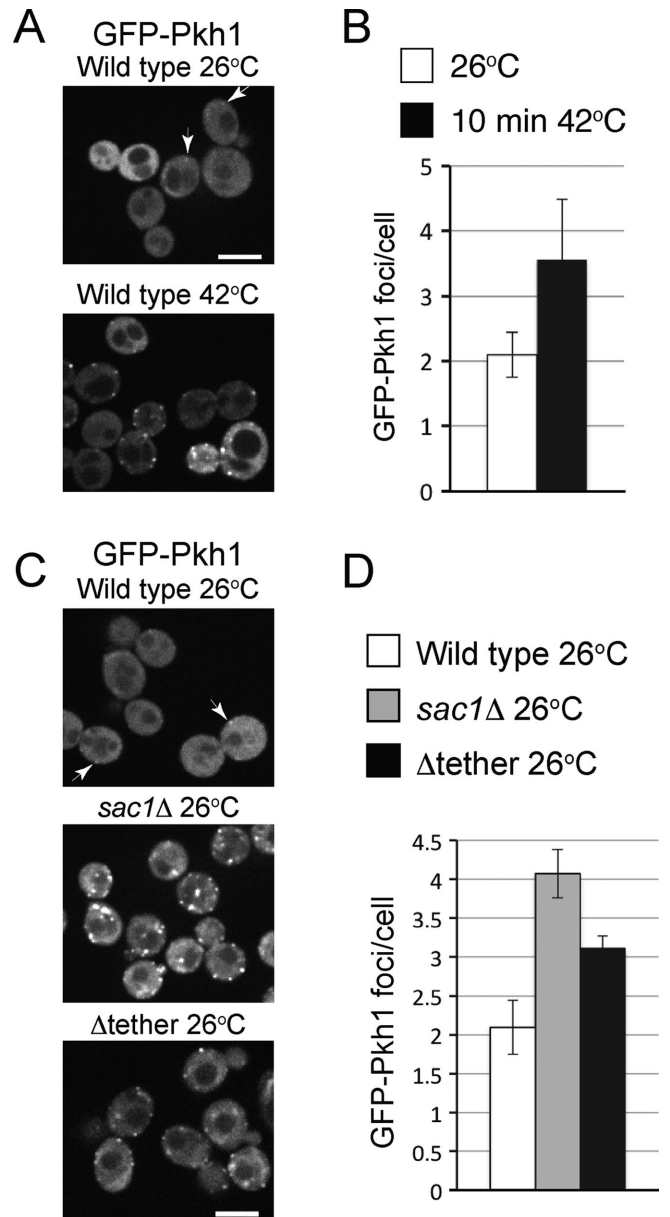


FIGURE 4: Heat-induced membrane stress and PI metabolism regulate Pkh1 localization. (A) Pkh1 assembles into cortical patches upon heat shock. Wild-type cells expressing GFP-Pkh1 were grown at 26°C (top) and shifted to 42°C for 10 min (bottom). Arrows show examples of small GFP-Pkh1 cortical patches in wild-type cells at 26°C. The majority of GFP-Pkh1 foci are cortical, but internal patches are also observed. Scale bar, 5 μ m. See Supplemental Figure S4C. (B) High-content quantitative analysis of GFP-Pkh1 distribution. Maxima for GFP-Pkh1 foci in single focal planes (1482 foci in total from 720 cells at 26 and 3742 foci in total from 1095 cells after heat shock at 42°C) were identified using Fiji. Results show the mean and SD from three independent experiments. (C) PI4P metabolism controls Pkh1 localization. GFP-Pkh1 localization in wild-type, *sac1Δ*, and Δ tether cells. Arrows show examples of small GFP-Pkh1 cortical patches in wild-type cells at 26°C. GFP-Pkh1 puncta are increased in *sac1Δ* and Δ tether cells at 26°C. Scale bar, 5 μ m. (D) GFP-Pkh1 puncta are increased in *sac1Δ* and Δ tether cells. Maxima for GFP-Pkh1 foci in single focal planes (1482 in total from 720 wild-type cells, 7258 in total from 1802 *sac1Δ* cells, and 3921 in total from 1261 Δ tether cells at 26°C) were identified using Fiji. Results show the mean and SD from three independent experiments. See Supplemental Figure S4, D–F.

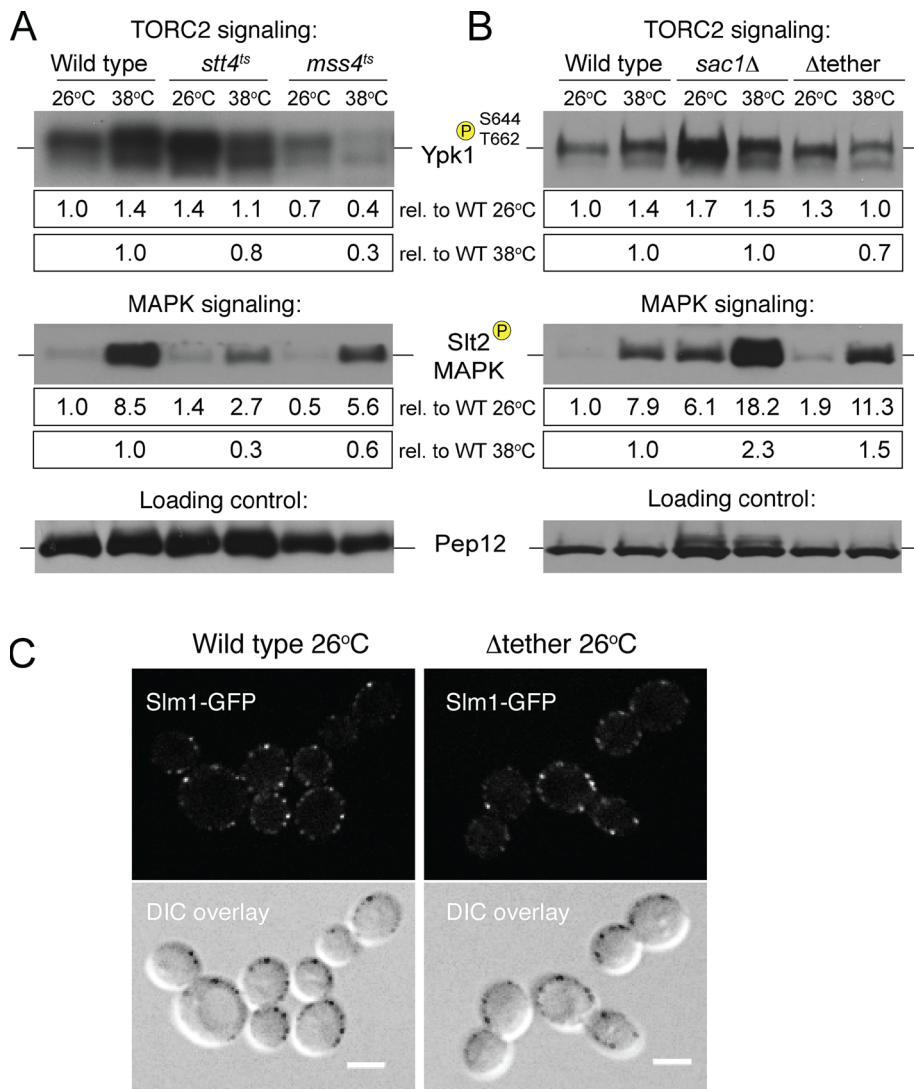


FIGURE 5: PI4P and PI(4,5)P₂ metabolism control TORC2 signaling. (A) TORC2 signaling and Slt2 MAPK phosphorylation in wild-type, *stt4^{ts}*, and *mss4^{ts}* cells. Wild-type (WT), *stt4^{ts}*, and *mss4^{ts}* cells were incubated at 26 or 38°C for 2 h. Protein extracts were analyzed by immunoblotting using antisera that recognize phospho-Ypk1(T662) or phospho-Slt2. Quantifications below the blot report the difference relative to WT after normalizing to a protein loading control; results are the mean of three independent experiments. (B) TORC2 signaling and Slt2 MAPK phosphorylation in cells lacking the PI4P phosphatase *Sac1* or the ER-PM tether proteins. WT, *sac1Δ*, and Δ tether cells were incubated at 26 or 38°C for 2 h. Protein extracts were analyzed by immunoblotting using antisera that recognize phospho-Ypk1(T662) or phospho-Slt2. Quantifications below the blot report the difference relative to WT after normalizing to a protein loading control; results are the mean of three independent experiments. See Supplemental Figure S5. (C) Slm1-GFP localization in WT cells (left) and Δ tether cells (right). Bottom, differential interference contrast overlays. Scale bar, 5 μ m.

cells lacking these proteins. In line with this, phospho-Ypk1(T504) levels in the Δ tether mutant cells resembled wild-type control levels (Supplemental Figure S3B). Thus the sphingolipid synthesis defects in the Δ tether mutant cells cannot simply be explained by reduced Pkh1 signaling or defects in targeting Pkh1 to the PM.

Ypk1 activation also requires phosphorylation at residues S644 and T664 by TORC2. Phosphorylation of Ypk1 by TORC2 facilitates phosphorylation of Ypk1 by Pkh1, resulting in full Ypk1 activity (Roelants *et al.*, 2011; Niles *et al.*, 2012). TORC2 signaling is regulated by the PH domain-containing proteins Slm1 and Slm2, which bind PI(4,5)P₂ and recruit Ypk1 to the PM for phosphorylation by

TORC2 (Audhya *et al.*, 2004; Niles *et al.*, 2012). We further investigated roles for PI lipids and ER-PM junctions in TORC2 signaling during heat-induced membrane stress. Phosphorylation of Ypk1 by TORC2 increased upon heat stress, as measured by antisera that detect phospho-Ypk1(S644, T662; Figure 5). As expected, *mss4^{ts}* mutant cells displayed significantly reduced TORC2 signaling (30% of wild-type levels at 38°C; Figure 5A). This could also result in significantly reduced phospho-Ypk1(T504) levels observed in *mss4^{ts}* mutant cells (Figure 3), because the PI(4,5)P₂-binding Slm1/2 proteins and TORC2 promote phosphorylation of Ypk1 by Pkh1/2 (Roelants *et al.*, 2004; Niles *et al.*, 2012). Heat-induced Ypk1 phosphorylation by TORC2 was also impaired in *stt4^{ts}* mutant cells (38°C, Figure 5A), possibly because *Stt4*-generated PI4P is used for PI(4,5)P₂ synthesis by *Mss4* (Audhya *et al.*, 2000; Audhya and Emr, 2002). As a control, the MAPK Slt2 was hypophosphorylated in *stt4* and *mss4* mutant cells at the restrictive temperature (Figure 5A), consistent with a previous study (Audhya and Emr, 2002).

Of interest, levels of phospho-Ypk1(S644, T662) were increased in *stt4^{ts}*, *sac1Δ*, and the Δ tether mutant cells at 26°C (Figure 5, A and B). Membrane stress caused by disruptions in sphingolipid metabolism results in activation of TORC2 signaling (Berchtold *et al.*, 2012). Accordingly, the constitutive TORC2 signaling at 26°C observed in *stt4^{ts}*, *sac1*, and Δ tether mutant cells alike could be due to alterations in sphingolipid metabolism in these cells (Tabuchi *et al.*, 2006; Brice *et al.*, 2009; Breslow *et al.*, 2010; Figures 3 and 6A). Consistent with this idea, *pkh1^{ts} pkh2* double-mutant cells also displayed increased TORC2 signaling, as phospho-Ypk1(S644, T662) levels were elevated 6.7- and 6.5-fold above wild-type levels at 26 and 38°C, respectively (Supplemental Figure S5). Alternatively, elevated phospho-Ypk1(S644, T662) levels in *pkh1^{ts} pkh2Δ*, *stt4^{ts}*, *sac1Δ*, and Δ tether mutant cells at 26°C could simply be due to increased Ypk1 protein levels in these cells (Supplemental Figure S3; unpublished data).

On heat stress, phospho-Ypk1(S644, T662) levels were slightly reduced in the Δ tether mutant cells (70% of wild-type levels at 38°C; Figure 5B). However, impaired TORC2 signaling cannot readily explain the sphingolipid synthesis defects observed in the Δ tether cells. Sphingolipid synthesis was compromised in the Δ tether cells even at 26°C (Figure 2), whereas TORC2 signaling was not impaired (and even increased) at this temperature (Figure 5B). Consistent with this idea, Slm1-GFP localized to cortical patches in both wild-type and Δ tether mutant cells (Figure 5C). Thus ER-PM junctions are not necessarily required for Pkh1/2 and TORC2 localization and function. In the Δ tether mutant cells, Pkh1 and TORC2 still localize to the PM and phosphorylate Ypk1, but

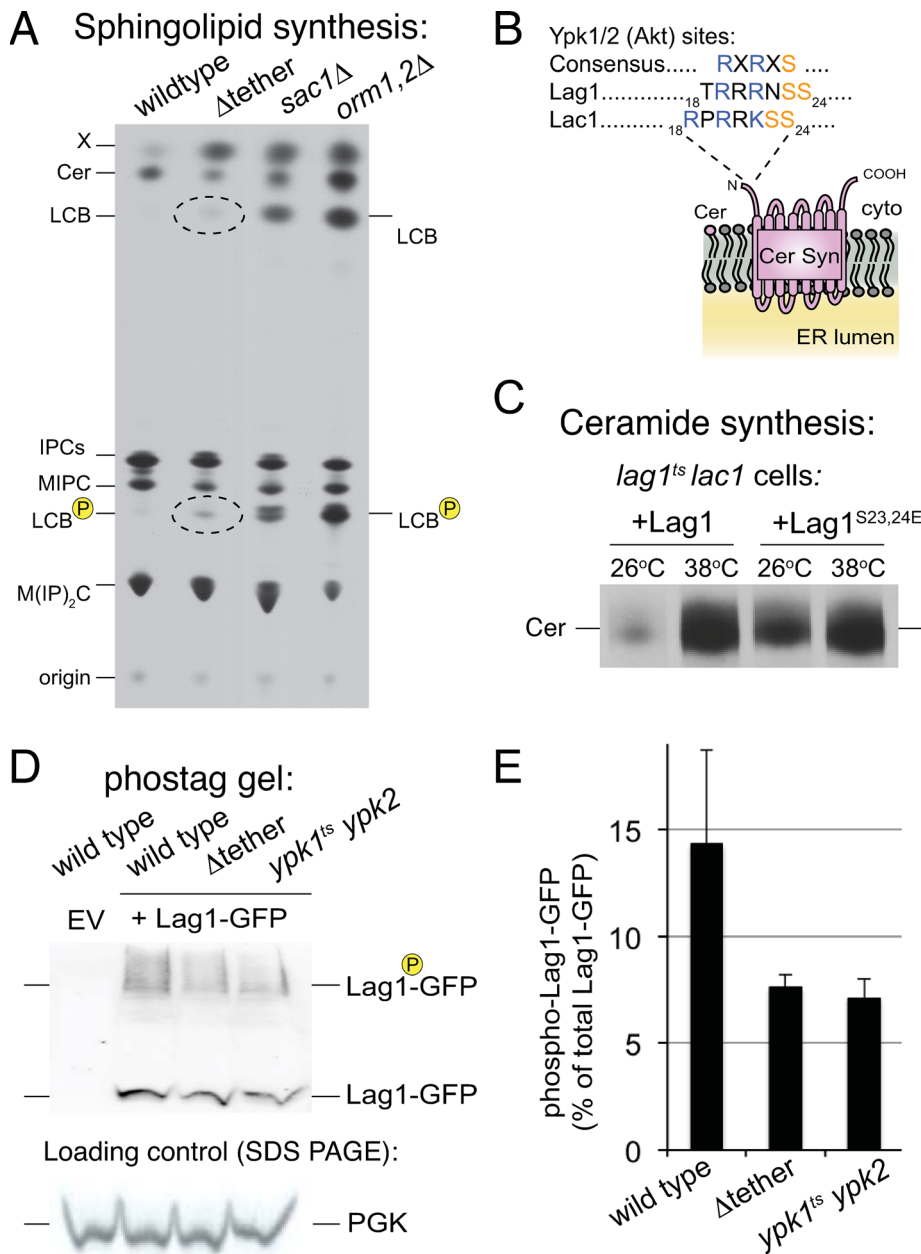


FIGURE 6: ER-PM cross-talk controls ceramide synthase activity in the ER. (A) Synthesis of ceramides, but not LCBs, is compromised in the Δ tether cells. Wild-type, Δ tether, *sac1* Δ , and *orm1* Δ *orm2* Δ cells were labeled with [³H]serine for 60 min at 26°C. Spingolipids were extracted and analyzed by TLC. LCBs, phosphorylated-LCBs (LCB-P), ceramides (Cer), and other spingolipid species are indicated. The hatched ovals point out elevated LCBs and phosphorylated LCBs in the Δ tether mutant cells. In addition, note that ceramides are reduced in the Δ tether mutant cells as compared with wild-type cells (Figure 2). The *sac1* Δ and *orm1* Δ *orm2* Δ cells serve as controls for LCBs and phosphorylated LCBs. See Supplemental Figure S6, A and B. (B) Schematic cartoon depicting topology of Lac1 and Lag1 in the ER. Both proteins possess Ypk1/2 consensus sites in their N-terminal cytoplasmic tails. (C) Double-mutant *lag1*^{ts} *lac1* cells expressing either wild-type Lag1 or the phosphomimetic form Lag1^{S23,24E} from plasmids were preincubated at the appropriate temperature for 10 min and labeled with [³H]serine for 60 min. Spingolipids were extracted and analyzed by TLC. (D) Analysis of Lag1-GFP phosphorylation upon heat stress conditions. Wild-type, Δ tether, and *ypk1*^{ts} *ypk2* Δ cells expressing Lag1-GFP were incubated at 38°C for 2 h. Protein extracts were prepared, separated by Phos-tag gel electrophoresis, and analyzed by immunoblotting using an antibody that specifically recognizes GFP. Wild-type cells carrying empty vector were included as a control for the GFP antisera. SDS-PAGE and immunoblotting were used to determine expressions levels of PGK as a protein loading control. (E) Quantitation of phosphorylated Lag1-GFP level in wild-type and Δ tether cells as a percentage of total Lag1-GFP protein levels. The data represent means \pm SDs from two independent experiments analyzed in duplicate.

Ypk1 signaling may not be effectively transduced to targets involved in spingolipid synthesis in the ER.

ER-PM junctions modulate cytoplasmic Ca²⁺ and calcineurin to control Ypk1-regulated lipid synthesis in the ER

We next investigated why spingolipid synthesis in the ER was impaired in the Δ tether cells (Figure 2). We found that synthesis of LCBs and phosphorylated LCBs was not compromised in the Δ tether cells. Instead, levels of LCBs and phospho-LCBs were increased relative to wild-type cells at 26°C (Figure 6A), although not nearly to the extent as *sac1* and *orm1* *orm2* mutant cells known to have increased LCB species (Figure 6A; Brice et al., 2009; Breslow et al., 2010). The Δ tether cells were also resistant to myriocin, which inhibits LCB synthesis in the ER, and the myriocin-resistant phenotype was not rescued by expression of the artificial tether protein (Supplemental Figure S6, A and B). The accumulation of LCBs (Figure 6A) and decrease in ceramides (Figure 2) suggested that ceramide synthesis might be a rate-limited step in the Δ tether cells.

Ypk1/2 phosphorylate and activate the ceramide synthases Lag1 and Lac1 at consensus sites within their N-terminal cytoplasmic tails (Figure 6B; Muir et al., 2014). Consistent with this, basal ceramide synthesis was increased (nearly 80% of heat-induced levels) in cells expressing a phosphomimetic form of Lag1 from a plasmid (S23E, S24E; Figure 6C). We then examined the expression and phosphorylation status of the Lag1 protein in the Δ tether and *ypk1*^{ts} *ypk2* mutant cells at 38°C by monitoring levels and mobility shifts of Lag1-GFP on Phos-tag acrylamide gels. First, steady-state expression levels of Lag1-GFP were reduced in the Δ tether and *ypk1*^{ts} *ypk2* mutant cells (63 and 62% of wild-type levels respectively, normalized to a protein loading control; Figure 6D). Moreover, the Lag1-GFP protein was hypophosphorylated in the Δ tether and *ypk1*^{ts} *ypk2* mutant cells (53 and 49% of wild-type levels, respectively; Figure 6, D and E). In these analyses, 14% of the total Lag1-GFP protein appeared as slower-migrating phosphorylated forms in wild-type cells (Figure 6E). However, in both Δ tether and *ypk1*^{ts} *ypk2* mutant cells, approximately only 7% of the total Lag1-GFP protein was phosphorylated (Figure 6E). Lag1 is also phosphorylated by additional kinases, and this may explain the residual Lag1 phosphorylation observed in Δ tether and *ypk1*^{ts} *ypk2* mutant cells (Fresques et al., 2015).

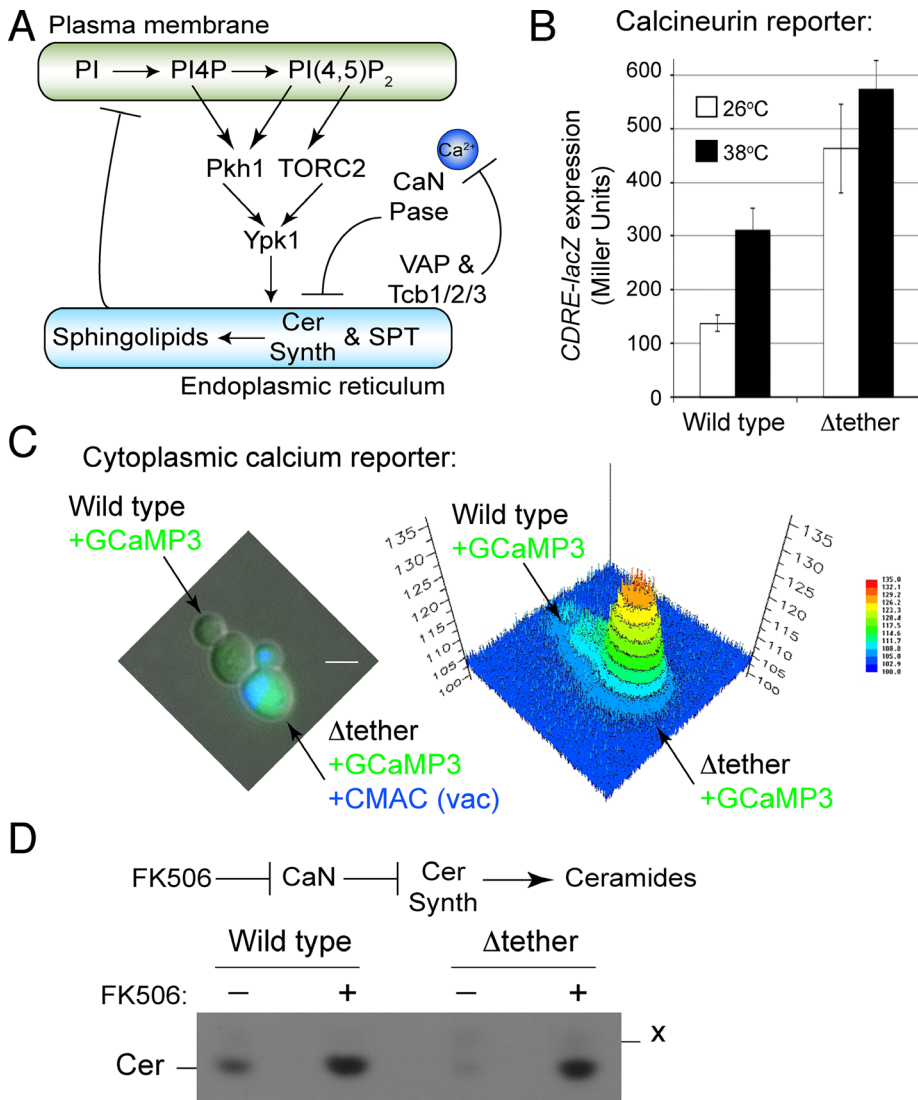


FIGURE 7: ER-PM junctions modulate cytoplasmic Ca²⁺ and calcineurin phosphatase activity during heat-induced ceramide synthesis. (A) Speculative model for a homeostatic regulatory loop in ER-PM cross-talk. Changes in membrane order and composition, including heat-induced membrane stress (Audhya and Emr, 2002) and other conditions, such as sphingolipid depletion (Jesch *et al.*, 2010), increased PI kinase signaling up, regulating the Pkh1-TORC2-Ypk1 cascade. Ypk1 stimulates synthesis of LCBs and ceramides in the ER (via activation of serine palmitoyltransferase, SPT, and ceramide synthases) to adjust membrane lipid composition and protect PM integrity. The ER-localized Scs2/22 and Tcb1/2/3 proteins that form ER-PM junctions modulate cytoplasmic Ca²⁺ and calcineurin activity, which antagonizes sphingolipid synthesis in the ER. On restoration of membrane order, the PI kinase-Pkh1/2-TORC2-Ypk1/2 pathway is maintained at basal signaling levels. (B) Wild-type and Δtether cells carrying a CDRE-lacZ reporter were grown at 26°C and shifted to the indicated temperatures for 2 h, and β-galactosidase activity was determined. Data represent means ± SDs from three independent experiments. See Supplemental Figure S7A. (C) Wild-type and Δtether cells expressing cytoplasmic GCaMP3 were grown at 26°C. Vacuoles in Δtether cells were labeled with the dye CMAC to distinguish mutant cells from wild-type cells. Left, the cultures were mixed and simultaneously observed by fluorescence microscopy. Right, relative GCaMP3 intensities were quantified using soft-WoRx 3.5.0. Scale bar, 3 μm. See Supplemental Figure S7B. (D) Wild-type cells and Δtether cells were labeled with [³H]serine for 60 min at 26°C in the absence or presence of the calcineurin inhibitor FK506. Sphingolipids were extracted and analyzed by TLC. Ceramides (Cer) are indicated; an unknown lipid, X, was also observed.

The ER-localized ceramide synthases Lac1 and Lag1 could potentially be activated by the Ypk1/2 kinases at ER-PM junctions upon membrane stress. Indeed, localization of Lag1 in the cortical ER

responsible for the impaired Lac1/Lag1 function and sphingolipid synthesis defects observed in these cells. Overall our results suggest that PI kinase-Pkh1/2-TORC2 signaling controls ER-PM cross-talk

increased upon heat stress (Supplemental Figure S6C). However Stt4, Mss4, Pkh1/2, and Ypk1/2 activities were not required for heat-induced localization of Lag1 to the cortical ER (unpublished data). Moreover, Lac1 localized to the nuclear ER even under membrane stress conditions (unpublished data), and both Lag1 and Lac1 were involved in maintaining PM integrity upon heat shock (Supplemental Figure S6D). The latter results indicated that Ypk1/2 signaling must be transduced to ceramide synthases localized throughout the ER. For these reasons, we searched for additional roles for the ER-PM tether proteins in regulation of ceramide synthesis.

ER-PM contacts modulate Ca²⁺ dynamics (Stefan *et al.*, 2013), and the Ca²⁺/calmodulin-activated phosphatase calcineurin inhibits ceramide synthesis in the ER (Aronova *et al.*, 2008; Muir *et al.*, 2014; Figure 7A). We addressed whether misregulation of Ca²⁺ and calcineurin might impair ceramide synthesis in the Δtether cells. Basal expression of CDRE::lacZ, a reporter of calcineurin activity via its substrate the Crz1 transcription factor, was increased in the Δtether mutant cells fourfold above control levels (Figure 7B). Basal calcineurin activity in the Δtether cells was even greater than heat stress-induced levels in wild-type cells (Figure 7B). As further evidence for increased calcineurin activity, the Δtether mutant cells displayed resistance to the calcineurin inhibitor FK506 (Supplemental Figure S7A). Cells lacking either the VAPs or the tricalbin proteins were also resistant to FK506, specifically implicating these tether proteins in the control of calcineurin and cellular Ca²⁺ levels (Supplemental Figure S7A). Consistent with this, fluorescence of the cytoplasmic Ca²⁺ reporter GCaMP3 was increased in Δtether cells as compared with wild-type cells (approximately threefold by fluorescence microscopy; Figure 7C). In high-content quantitative flow cytometry assays, the GCaMP3 signal doubled in Δtether cells compared with wild type (Supplemental Figure S7B). Cytoplasmic Ca²⁺ was not significantly elevated in *sac1Δ* cells (Supplemental Figure S7B), suggesting that elevated PI4P or LCBs may not activate calcineurin in the Δtether cells. Thus the ER-PM tether proteins function to modulate cellular Ca²⁺ levels and calcineurin activity. Of interest, the calcineurin inhibitor FK506 increased ceramide production in the Δtether cells (Figure 7D), suggesting that increased calcineurin activity was at least partially responsible for the impaired Lac1/Lag1 function and sphingolipid synthesis defects observed in these cells. Overall our results suggest that PI kinase-Pkh1/2-TORC2 signaling controls ER-PM cross-talk

during membrane stress conditions and that ER-PM junctions modulate cytoplasmic Ca²⁺ levels and calcineurin activity, which antagonizes sphingolipid synthesis in the ER (Figure 7A).

DISCUSSION

PI kinase signaling controls PM homeostasis

We propose that PI kinase signaling controls ER-PM cross-talk necessary for cellular integrity and membrane homeostasis. We identify the PDK orthologue Pkh1 as an effector of Stt4 and Mss4 PI kinase activities at the PM (Figure 3). PI4P and PI(4,5)P₂ may recruit Pkh1 to the PM upon heat-induced membrane stress, as cortical Pkh1 assemblies constitutively formed in mutant cells with elevated PI4P and PI(4,5)P₂ levels (Figure 4 and Supplemental Table S3). Thus PI kinases may regulate both Pkh1/2 and TORC2 at the PM, where they phosphorylate the Akt orthologues Ypk1/2. Our study and previous work (Muir *et al.*, 2014) indicate that Ypk1/2 subsequently activate ceramide synthases localized in the ER for de novo sphingolipid synthesis essential for cellular integrity upon stress conditions. Of interest, control of ceramide synthesis by PDK-Akt-mTor signaling may be conserved. A large-scale proteomic study identified several mammalian ceramide synthase isoforms as targets of the PI3K-PDK-Akt-mTor signaling pathway (Humphrey *et al.*, 2013).

The Mss4 PI4P 5-kinase and PI(4,5)P₂ are critical regulators of Pkh1 and TORC2 signaling (Figures 3 and 5). However, our data suggest that PI4P may also recruit Pkh1 to the PM. Why, then, might yeast cells have redundant mechanisms in place for Pkh1 regulation? On certain membrane stress conditions, including sphingolipid depletion, PI4P signaling may provide important contributions to Ypk1-mediated lipid synthesis in the ER. Sphingolipids are suggested to modulate Mss4 localization and thus PI(4,5)P₂ synthesis (Kobayashi *et al.*, 2005; Gallego *et al.*, 2010). PI4P availability is increased at the PM upon inhibition of sphingolipid synthesis (Jesch *et al.*, 2010), and PI4P may provide a protective mechanism that sustains or even triggers Pkh1-TORC2-Ypk1 signaling upon compromised sphingolipid and PI(4,5)P₂ production. As such, both PI4P and PI(4,5)P₂ may provide important regulatory inputs for the modulation of Ypk1 signaling and ER-PM cross-talk.

The calcium paradox in cellular stress responses

Cytoplasmic Ca²⁺ and calcineurin activity increase upon heat-induced membrane stress (Figure 7B; Cunningham, 2011), possibly to regulate endocytosis (Wu *et al.*, 2014) for the clearance of damaged PM proteins (Zhao *et al.*, 2013). However, calcineurin inhibits sphingolipid synthesis, which is also essential for cellular integrity upon heat stress (Figures 2 and 7 and Supplemental Figure S6E; Aronova *et al.*, 2008; Muir *et al.*, 2014). Cells may overcome this paradox in Ca²⁺ stress responses through the formation of ER-PM junctions. The PM is organized into distinct functional domains, including ER-free zones and ER-associated PM zones. Ca²⁺- and calcineurin-mediated endocytosis takes place in the ER-free PM zones (Stradalova *et al.*, 2012). ER-PM junctions may serve as sites to modulate cytoplasmic Ca²⁺ signals and calcineurin. In line with this, the Δ tether cells display elevated cytoplasmic Ca²⁺ and calcineurin activity (Figure 7). How the ER-PM tether proteins regulate Ca²⁺ dynamics is unclear. Similarly, the source of increased Ca²⁺ in the Δ tether cells is unknown (i.e., extracellular or intracellular stores). However, the tricalbin proteins (E-Syt orthologues) possess Ca²⁺-binding C2 domains and may be activated by Ca²⁺ for feedback regulation of Ca²⁺ flux into the cytoplasm. Accordingly, loss of the tricalbins resulted in increased calcineurin activity (Supplemental Figure S7) and cell integrity defects upon heat shock (Supplemental Figure S1). Consistent

with this, mammalian E-Syt proteins are regulated by the store-operated Ca²⁺ entry (SOCE) pathway and subsequently inactivate SOCE (Giordano *et al.*, 2013; Maleth *et al.*, 2014; Idevall-Hagren *et al.*, 2015). Moreover, ER-PM junctions are sites for inhibition of voltage-gated Ca²⁺ channels (VGCCs; Park *et al.*, 2010; Wang *et al.*, 2010), but the E-Syt proteins have yet to be implicated in VGCC regulation.

Sphingolipid signaling in membrane stress responses

Cells up-regulate sphingolipid synthesis in the ER in response to heat-induced membrane stress (Figure 2; Tabuchi *et al.*, 2006; Sun *et al.*, 2012; Muir *et al.*, 2014). Increased production of LCBs and ceramides induce several heat stress responses, including transcription of heat stress genes, translation of heat shock factors and chaperones, and membrane trafficking (Coward and Obeid, 2007). Our results indicate that PI kinase-stimulated sphingolipid synthesis in the ER is critical for maintaining PM integrity—but are specialized pools of sphingolipids generated, how are they delivered to the PM, and what are their protective roles during PM stress?

Of interest, the Orm2 protein that inhibits LCB synthesis is depleted from the cortical ER upon membrane stress, whereas the Lag1 ceramide synthesis moves into the cortical ER (Breslow *et al.*, 2010; Supplemental Figure S6). Possibly, localized pools of LCBs and ceramides are generated in the cortical ER at PM contact zones, where they may regulate stress response mechanisms such as a LCB- or ceramide-activated protein kinase or phosphatase. Intriguingly, the PH domain from the Slm1 protein possesses distinct sites that bind PI4P and LCBs (Anand *et al.*, 2012). Our results indicate that basal TORC2 signaling is increased in *sac1* and Δ tether mutant cells, which have elevated levels of PI4P and LCBs (Figures 5B and 6A; Manford *et al.*, 2012), suggesting that PI4P-regulated sphingolipid synthesis might function in a feedforward regulatory loop to control TORC2 signaling and sphingolipid synthesis.

ER-PM junctions are sites for nonvesicular transport where lipid transfer proteins use PI metabolism for membrane lipid delivery (Moser von Filseck *et al.*, 2014). Possibly, LCBs or ceramides generated in the cortical ER are shuttled to the PM by an unidentified lipid transfer protein to provide structural support or maintain lipid-packing order in the PM. Long-acyl-chain lipids, including sphingolipids, are also proposed to serve as a “chemical trap” for sterol lipids that could effectively reduce PM permeability (Holthuis and Menon, 2014). Increased sphingolipid content may also create a membrane environment that favors the proper folding and thermostability of integral PM proteins at elevated temperatures. We do not have a full understanding of how PI kinase signaling controls PM organization and integrity. However, PI kinase-regulated ER-PM cross-talk provides an elegant mechanism to coordinate both the synthesis and traffic of membrane lipids.

Ceramides are transported from the ER by both nonvesicular and vesicular membrane trafficking pathways (Funato and Riezman, 2001). PI kinase-regulated sphingolipid synthesis may be vital for essential functions of the ER as well, including folding and secretion of newly synthesized proteins such as thermoresistant PM proteins. Accordingly, sphingolipids are involved in the ER export and targeting of integral PM proteins, including the yeast PM ATPase Pma1 and the nutrient permeases Can1 and Gap1 (Lee *et al.*, 2002; Wang and Chang, 2002; Daquinag *et al.*, 2007; Lauwers *et al.*, 2007). Consequently, impaired ceramide synthesis in cells lacking ER-PM tether proteins could lead to cargo-specific ER export defects that may contribute to ER stress phenotypes previously observed in Δ tether cells (Manford *et al.*, 2012).

We propose that PI kinase-Pkh1-TORC2 signaling is part of an essential pathway for membrane homeostasis. Of interest, upon loss of the ER tether proteins, additional cellular stress response pathways are activated, including the unfolded protein response in the ER (Manford *et al.*, 2012), a mitogen-activated protein kinase (MAPK)-regulated cell integrity pathway (Figure 5), and calcineurin-mediated transcriptional responses (Figure 7), potentially to compensate for loss of ER-PM crosstalk. Thus PI kinase signaling may coordinately regulate ER-PM cross-talk and other stress response pathways to maintain membrane homeostasis and cellular integrity. It will be important to investigate further how ER-PM cross-talk affects PDK-Akt-mTor signaling and other stress responses in the control of membrane organization.

MATERIALS AND METHODS

Additional details are provided in the Supplemental Materials and Methods.

Strains and plasmids

Strains and plasmids used in this study are listed in Supplemental Tables S1 and S2, respectively.

Fluorescence microscopy

Images were obtained with a DeltaVision RT microscopy system (GE Applied Precision, Issaquah, WA) or spinning-disk confocal microscopy systems (Perkin Elmer, Waltham, MA, and 3i, Denver, CO). See Supplemental Materials and Methods for additional microscopy details.

Plasma membrane integrity assays

PM integrity assays were performed as described (Zhao *et al.*, 2013). Cells were grown at 26°C and shifted to 40°C for 2 h (heat stress conditions) or to 42°C for 10–15 min (heat shock) as indicated. Cells were stained with propidium iodide, washed, and analyzed by flow cytometry. See the Supplemental Materials and Methods for additional details.

Sphingolipid analysis

Metabolic labeling, lipid extraction, and TLC analysis of sphingolipids were performed as described (Tabuchi *et al.*, 2006). See the Supplemental Materials and Methods for additional sphingolipid analysis details.

Analysis of protein phosphorylation and expression

Cells were mechanically lysed in the presence of phosphatase inhibitors. Extracts were analyzed by SDS-PAGE and immunoblotting with the following antibodies: anti-Pep12 (Invitrogen, Thermo Fisher Scientific, Waltham, MA), anti-glucose-6-phosphate dehydrogenase (G6PDH; Sigma-Aldrich, St. Louis, MO), anti-phosphoglycerate kinase (PGK; Novex, Thermo Fisher Scientific, Waltham, MA), anti-GFP (Santa Cruz Biotechnology, Dallas, TX, or Roche, Burgess Hill, UK), anti-PKC (pan) zeta T410 (Cell Signaling, Danvers, MA) for phospho-Ypk1(T504), anti-phospho-Ypk1(T662) (Niles *et al.*, 2012), anti-Ypk1 (Cell Signaling), and anti-phospho-p44/42 MAPK (Cell Signaling) for phospho-Slt2. Levels of GFP-Pkh1, Ypk1, phospho-Ypk1, and phospho-Slt2 were normalized to protein loading controls (Pep12, G6PDH, or PGK). See the Supplemental Materials and Methods for additional details.

β -Galactosidase assays

Strains harboring a *CDRE-lacZ* reporter were grown at 26°C, and indicated samples were shifted to 38°C for 2 h. β -Galactosidase

activity was measured as described in the Supplemental Materials and Methods.

Quantitative GCaMP3 fluorescence assays

Strains harboring a cytoplasmic GCaMP3 reporter were grown at 26°C and transferred to phosphate-buffered saline. Mean fluorescence of 50,000 events was recorded on a BD Accuri C6 flow cytometer. Background was determined using strains harboring vector alone. Results are the mean of three independent experiments. See the Supplemental Materials and Methods for additional details.

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