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RESEARCH ARTICLE

Ypk1 and Ypk2 kinases maintain Rho1 at the plasma membrane by flippase-dependent lipid remodeling after membrane stresses

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ABSTRACT

The plasma membrane (PM) is frequently challenged by mechanical stresses. In budding yeast, TORC2-Ypk1/Ypk2 kinase cascade plays a crucial role in PM stress responses by reorganizing the actin cytoskeleton via Rho1 GTPase. However, the molecular mechanism by which TORC2-Ypk1/Ypk2 regulates Rho1 is not well defined. Here, we found that Ypk1/Ypk2 maintain PM localization of Rho1 under PM stress via spatial reorganization of the lipids including phosphatidylserine. Genetic evidence suggests that this process is mediated by the Lem3-containing lipid flippase. We propose that lipid remodeling mediated by the TORC2-Ypk1/Ypk2-Lem3 axis is a backup mechanism for PM anchoring of Rho1 after PM stress-induced acute degradation of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2], which is responsible for Rho1 localization under normal conditions. Since all the signaling molecules studied here are conserved in higher eukaryotes, our findings might represent a general mechanism to cope with PM stress.

KEY WORDS: Akt, Rho, Flippase, Phosphatidylserine, Membrane stress, Actin cytoskeleton

INTRODUCTION

The plasma membrane (PM) frequently suffers from physical stress, including turgor pressure caused by imbalanced osmolality and membrane stretching/shrinking caused by muscle contraction (Lessey et al., 2012; Hohmann, 2002). Moreover, abnormal lipid composition causes PM stress even without environmental perturbations (Berchtold et al., 2012). To quickly manage such PM stress, eukaryotic cells have developed sophisticated signaling circuits that involve the target of rapamycin complex 2 (TORC2) kinase complex. TORC2 is activated by PM stress, such as hypotonic shock or inhibition of sphingolipid biosynthesis (Berchtold et al., 2012), and regulates various cellular functions, including actin organization, cell motility and morphogenesis (Loewith and Hall, 2011; Cybulski and Hall, 2009; Loewith et al., 2002; Zoncu et al., 2011).

In both yeast and mammals, the Rho-family of GTPases is a crucial target of TORC2 (Schmidt et al., 1997; Jacinto et al., 2004; Ho et al., 2008; Helliwell et al., 1998a). In budding yeast, the RhoA homolog Rho1 plays essential roles in actin organization and in stress responses (Levin, 2011). After activation by its guanine nucleotide exchange factors (GEFs), Rho1 binds to and recruits its effector Pkc1 (Andrews and Stark, 2000; Kamada et al., 1996), the only protein kinase C (PKC) in budding yeast, to the PM. Then, Rho1-GTP and the membrane lipid phosphatidylserine cooperatively activate Pkc1 (Kamada et al., 1996), which in turn activates the downstream MAP kinase cascade for transcriptional responses (Kamada et al., 1995) and remodels actin organization partly through downregulation of the formin Bni1 and the exocyst subunit Sec3 (Kono et al., 2012). Several models have been proposed to explain how TORC2 regulates Rho1 and Pkc1, including TORC2-dependent activation of Rho1 GEFs (Schmidt et al., 1997; Ho et al., 2008) and Pkc1 phosphorylation by TORC2 (Nomura and Inoue, 2015). However, the physiological significance of these mechanisms is not well understood.

The best-characterized TORC2 targets are the Akt/SGK families of protein kinases, which are directly phosphorylated and activated by TORC2 (Kamada et al., 2005). The yeast Akt (and/or SGK) homologs Ypk1/Ypk2 regulate sphingolipid synthesis via the homologs Orm1 and Orm2, and Lac1 and Lag1 (Roelants et al., 2011; Muir et al., 2014; Sun et al., 2012; Aronova et al., 2008), phospholipid flipping via the flippase kinases Fpk1/Fpk2 (Roelants et al., 2010), and production and efflux of glycerol via Gpd1 and Fps1, respectively (Lee et al., 2012; Muir et al., 2015). Among these effectors, Fpk1/Fpk2, whose kinase activities are inhibited by phosphorylation through Ypk1/Ypk2 (Roelants et al., 2010), are of particular importance because a recent quantitative phosphoproteomic approach revealed that regulation of actin polarization and endocytosis through the TORC2-Ypk1/Ypk2 pathway is largely mediated by the Fpk1/Fpk2 axis (Rispal et al., 2015). A recent study demonstrated that Fpk1/Fpk2 negatively regulate the Rho1-Pkc1 pathway, at least in part via the Rho1 GEF Rom2 (Niles and Powers, 2014). However, the Fpk1/Fpk2 substrates that regulate Rho1-Pkc1 remain unknown.

In this study, we define a key signaling mechanism that links the TORC2-Ypk1/Ypk2-Fpk1/Fpk2 kinase cascade to Rho1-Pkc1. Ypk1/Ypk2 promote cortical localization of Rho1 through inhibition of the Lem3-containing lipid flippase complex, an established target of Fpk1/Fpk2. The flippase complex determines subcellular distribution of phosphatidylserine that is required for Rho1 localization and cell viability under stress. We propose that Ypk1/Ypk2-dependent rearrangement of phosphatidylserine compensates for the reduction of anionic charge at the PM caused by stress-triggered degradation of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2].

RESULTS

Ypk1/Ypk2 support Rho1 localization to the bud cortex during PM stress

The TORC2-Ypk1/Ypk2 cascade regulates actin polarity (Rispal et al., 2015). To examine the relationship between *YPK1/YPK2* and

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RHO1, we first tested their genetic interaction. Overexpression of *RHO1* rescued the temperature-sensitive (ts) growth defect of the *ypk1-1 ypk2Δ* strain (hereafter referred to as *ypk^ts^*) (Fig. 1A). Moreover, the actin organization defect of *ypk^ts^* cells was largely rescued by overexpression of *RHO1* (Fig. 1B). These results suggest that Ypk1/Ypk2 regulate actin organization via or in parallel with Rho1.

TORC2 activates Rho1 GEFs through an unknown mechanism (Schmidt et al., 1997; Ho et al., 2008), and Fpk1/Fpk2 regulate the localization of the Rho1 GEF Rom2 (Niles and Powers, 2014). If Ypk1/Ypk2 regulate Rho1 solely via GEFs, then active mutants of Rho1 would rescue *ypk^ts^*. To test this possibility, we expressed two Rho1 active forms in *ypk^ts^* cells: the *RHO1-Q68H* mutant, which is defective in GTP hydrolysis, and the *RHO1-F35L* mutant, which has high intrinsic nucleotide exchange activity. These *RHO1* mutants are dominantly active and rescue the lethality of the cells that lack all three Rho1 GEFs, i.e. Rom1, Rom2 and Tus1 (Yoshida et al., 2009). Neither of these Rho1 active mutations suppressed the *ypk^ts^* growth defect (Fig. 1C). A plausible interpretation of these results is that Ypk1/Ypk2 regulates Rho1 through GEF-independent mechanisms.

Next, we tested the possibility that Ypk1/Ypk2 regulate Rho1 localization. In wild type cells, Rho1 is concentrated at the bud cortex

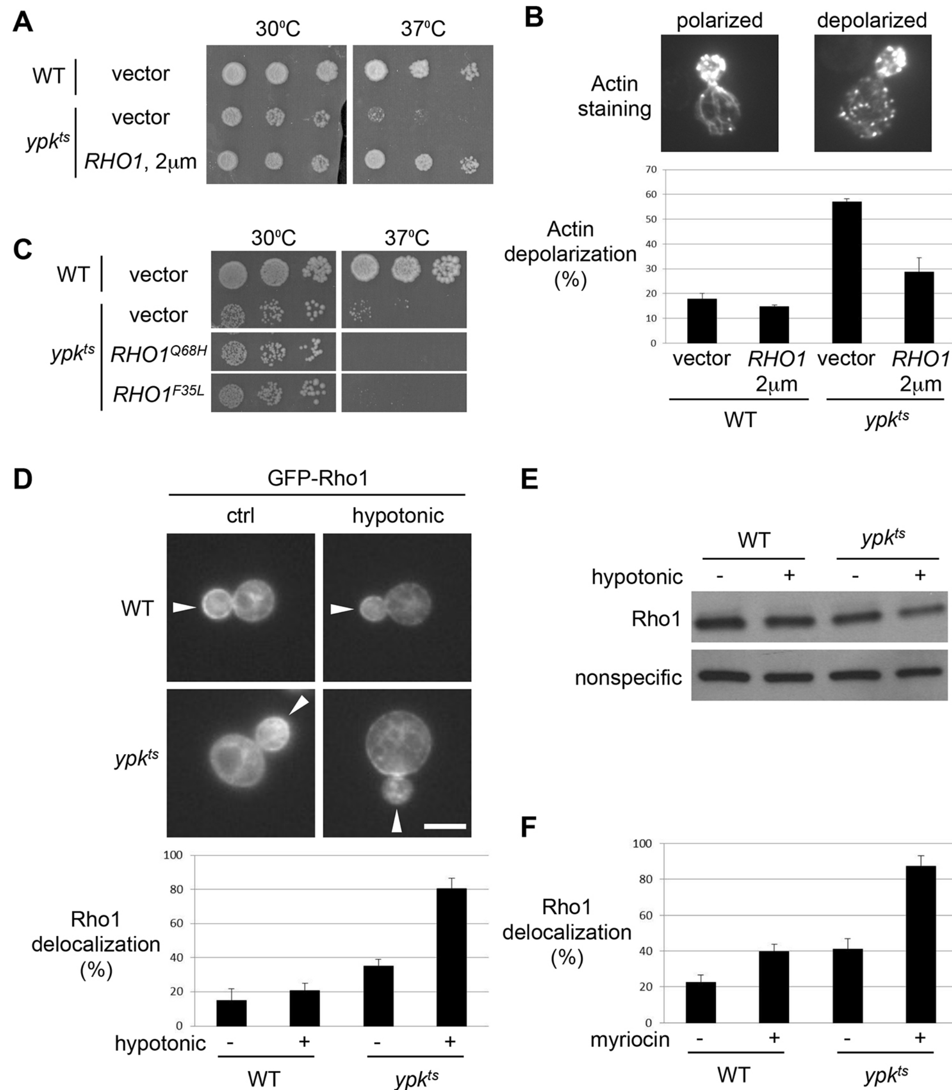

Fig. 1. Ypk1/Ypk2 regulate Rho1 localization. (A) Growth rescue of the *ypk^ts^* mutant by *RHO1* overexpression. Cells of the *ypk^ts^* strain were grown at either permissive (30°C) or non-permissive (37°C) temperature for 3 days. (B) Rescue of the actin polarization defect in the *ypk^ts^* mutant by *RHO1* overexpression. Cells were grown at 30°C and then shifted to 37°C for 2 h. The actin cytoskeleton was stained with Alexa Fluor 488-conjugated phalloidin. Representative images of polarized (WT) and depolarized (*ypk^ts^*) actin are shown. In the chart, the percentage of small- to medium-budded cells with a depolarized actin cytoskeleton is shown. Data are presented as the mean±s.d. of three independent experiments (*n*>50 cells were counted in each experiment). (C) Failure in growth rescue of *ypk^ts^* mutant by *RHO1^Q68H^* or *RHO1^F35L^* expression. Cells were grown at either 30°C or 37°C for 4 days. (D) Effect of hypotonic shock on GFP-Rho1 localization. Cells were grown at 30°C and then shifted to 37°C for 2 h. Images were taken 5 min after hypotonic shock. The percentage of small to medium-budded cells without Rho1 localization at the bud cortex is shown in the chart. Data are presented as the mean±s.d. of three independent experiments (*n*>50 cells were counted in each experiment). Scale bar: 5 µm. (E) Protein expression levels of Rho1. Cells were grown at 30°C and then shifted to 37°C for 2.5 h. Cell lysates were prepared 10 min after hypotonic shock. Rho1 protein level was analysed using western blotting with anti-Rho1 antibody. Nonspecific signals are shown as a loading control. (F) Effect of myriocin on Rho1 localization. Cells were grown at 30°C and then treated with 0.5 µg/ml myriocin at 37°C for 2.5 h. The percentage of small- to medium-budded cells without Rho1 localization at the bud cortex is shown. Data are presented as the mean±s.d. of three independent experiments (*n*>50 cells were counted in each experiment).

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during polarized growth; but we found that localization of GFP-Rho1 at the bud cortex was slightly reduced in *ypk^{ts}* cells at the restrictive temperature, although Rho1 protein was expressed at normal levels (Fig. 1D,E). Ypk1/Ypk2 are activated upon PM stresses, such as hypotonic shock or treatment with myriocin (an inhibitor of sphingolipid synthesis), and are essential for the resistance to these stresses (Berchtold et al., 2012). Based on these notions, we examined GFP-Rho1 localization under these stresses. Under both stress conditions, *ypk^{ts}* cells showed a prominent impairment of cortical localization of Rho1 compared with that seen in unstressed cells (Fig. 1D,F). These results suggest that Ypk1/Ypk2 support PM localization of Rho1, especially when the PM is stressed.

Ypk1/Ypk2 regulate subcellular distribution of phosphatidylserine

Rho GTPases Rho1 and Cdc42 are delivered to the inner cortex of growing daughter cell tips through polarized secretion (Wedlich-Soldner et al., 2003; Abe et al., 2003). Once delivered, Rho1 and Cdc42 are anchored to the inner PM by two mechanisms: by a hydrophobic interaction with the PM via covalently attached geranylgeranyl moiety and by an electrostatic interaction with anionic PM lipids via the poly-basic sequence (Heo et al., 2006). Therefore, elaborate regulation of anionic lipids is essential for stable localization of Rho1. Although Ypk1/Ypk2 are well-established regulators of sphingolipids and neutral glycerophospholipids (Roelants et al., 2010, 2011; Muir et al., 2014; Sun et al., 2012; Aronova et al., 2008), it is unclear whether and how anionic lipids are regulated by Ypk1/Ypk2.

We have previously shown that Rho1 localizes to the PM in part by interaction of the C-terminal Rho1 poly-basic sequence with PI(4,5)P_2 (Yoshida et al., 2009). Therefore, we first tested the role of Ypk1/Ypk2 in the regulation of PI(4,5)P_2. GFP-2PH^{PLC}, a specific biosensor for PI(4,5)P_2 (Stefan et al., 2002), exclusively decorated the PM in both wild type and *ypk^{ts}* cells (Fig. 2A), suggesting that Ypk1/Ypk2 are not required to maintain PI(4,5)P_2 levels.

Next, we examined the role of Ypk1/Ypk2 in the subcellular distribution of phosphatidylserine, another important anionic lipid, by using the specific phosphatidylserine biosensor GFP-2PH$^{evt\text{-}2}$ (Uchida et al., 2011; Lee et al., 2015). Phosphatidylserine has been implicated in polarized growth through PM recruitment of the Rho GTPase Cdc42 in both yeast and humans (Fairn et al., 2011; Das et al., 2012; Bruurs et al., 2015). GFP-2PH$^{evt\text{-}2}$ showed peripheral localization in wild type cells, reflecting enrichment of phosphatidylserine at the inner leaflet of the PM (Fig. 2B). In sharp contrast, GFP-2PH$^{evt\text{-}2}$ was mainly lost from the PM and relocated to the early endosomes in *ypk^{ts}* cells (Fig. 2B,C). A similar relocation of GFP-2PH$^{evt\text{-}2}$ has been observed in *ypk1(L424A) ypk2Δ*, the analog-sensitive *ypk* mutant (Roelants et al., 2011) (Fig. 2D). Displacement of GFP-2PH$^{evt\text{-}2}$ from the PM suggests that either the total amount of phosphatidylserine at the inner PM is reduced or that the ability of phosphatidylserine to recruit GFP-2PH$^{evt\text{-}2}$ is impaired. The latter event can be caused by an altered lateral distribution of phosphatidylserine, because recent evidence suggested that nanoclustering of phosphatidylserine at the inner PM increases its ability to recruit proteins, such as human K-Ras (Zhou et al., 2015). In either case, our data suggests that Ypk1/Ypk2 regulate the spatial organization of phosphatidylserine.

Ypk1/Ypk2-dependent flippase inhibition is required for phosphatidylserine organization and Rho1 localization

Next, we sought the Ypk1/Ypk2 effector(s) of phosphatidylserine regulation. Ypk1/Ypk2 regulate phospholipid asymmetry by

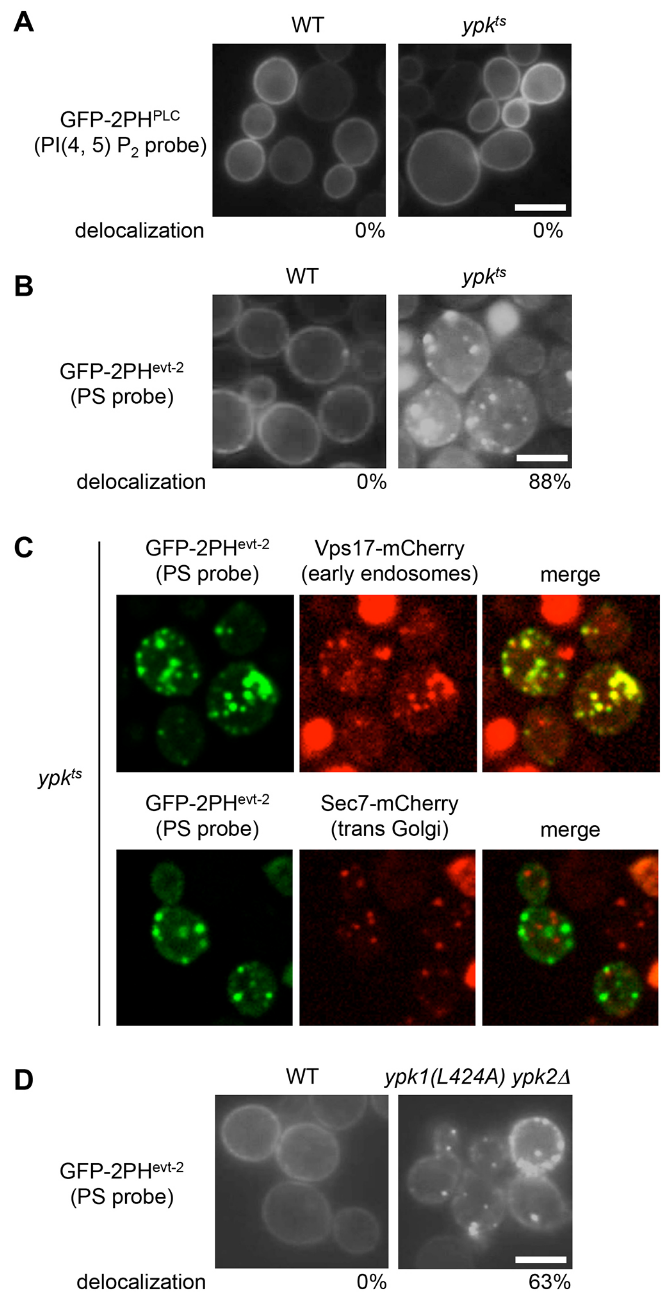

Fig. 2. Ypk1/Ypk2 regulate distribution of phosphatidylserine. (A) Localization of the GFP-2PH^{PLC} PI(4,5)P_2 probe after a 2 h incubation at 37°C. The percentage of cells lacking a PM signal is shown (*n*>150 cells). (B) Localization of the phosphatidylserine (PS) probe GFP-2PH$^{evt\text{-}2}$ after incubation for 2 h at 37°C. The percentage of cells lacking a PM signal is shown (*n*>150 cells). (C) Colocalization analysis of the PS probe with the indicated organelle markers after 2 h of incubation at 37°C. Adenine (0.07%) was added to the medium to prevent accumulation of the red pigment caused by *ade2* mutation. (D) Localization of the phosphatidylserine (PS) probe GFP-2PH$^{evt\text{-}2}$ after 1NM-PP1 treatment for 5 min. The percentage of cells lacking PM signal is shown (*n*>50 cells). All scale bars: 5 μm.

inhibiting Fpk1/Fpk2 kinases (Roelants et al., 2010). Fpk1/Fpk2 phosphorylate and activate the Dnf1/Dnf2-Lem3 lipid flippase complex, which flips the neutral lipids phosphatidylcholine and phosphatidylethanolamine from the outer leaflet to the inner leaflet of the PM (Nakano et al., 2008; Saito et al., 2004; Kato et al., 2002; Baldridge and Graham, 2012, 2013; Baldridge et al., 2013; Panatala et al., 2015; Iwamoto et al., 2004). In human cells,

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phosphatidylcholine flipping is proposed to dilute the local concentration of phosphatidylserine at the inner PM (Miyano et al., 2016). We therefore examined the involvement of the flippase complex in phosphatidylserine regulation. We found that deletion of *LEM3* largely restores PM localization of the phosphatidylserine probe GFP-2PH$^{evt\text{-}2}$ in *ypk^{ts}* (Fig. 3A). This result suggests that Ypk1/Ypk2 regulate phosphatidylserine distribution via inhibition of the Lem3-containing flippase complex.

We also found that *LEM3* deletion efficiently rescues delocalization of GFP-Rho1 upon hypotonic shock or myriocin treatment, as well as temperature-sensitive growth defects and myriocin hypersensitivity of *ypk^{ts}* (Fig. 3B,C,D), raising the

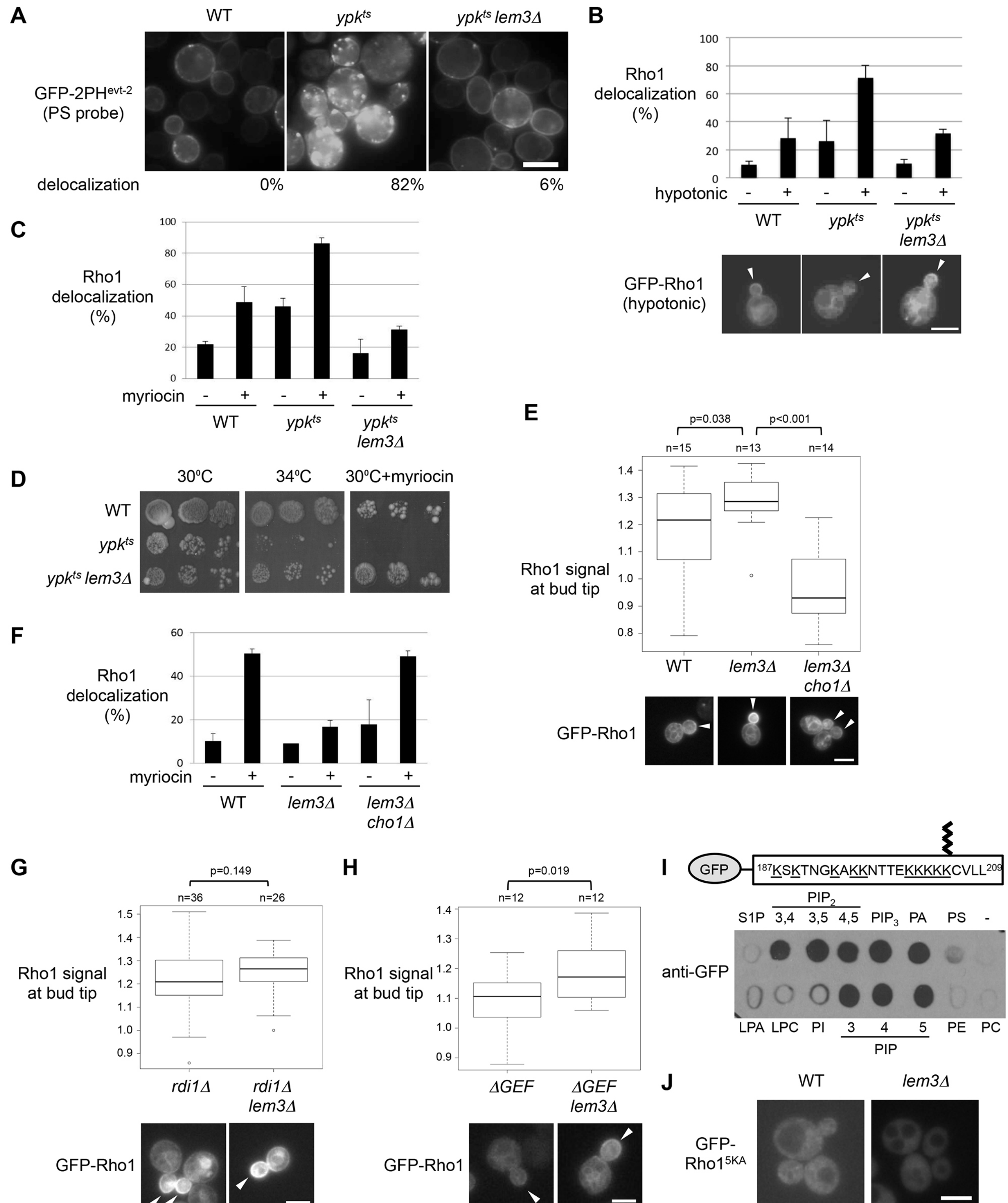

Fig. 3. See next page for legend.

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Fig. 3. Phosphatidylserine contributes to Rho1 regulation through the Ypk1/Ypk2-flippase pathway. (A) Rescue of phosphatidylserine (PS) distribution in the *ypk^ts* mutant by deletion of *LEM3*. Cells were grown at 30°C and then shifted to 37°C for 2 h. The percentage of cells that lack a PM signal is shown (*n*>150 cells). (B,C) Rescue of GFP-Rho1 localization in the *ypk^ts* mutant by *LEM3* deletion. In B, cells were grown at 30°C and then shifted to 37°C for 2 h. Images were taken 5 min after hypotonic shock. Arrowheads indicate the cellular growth site (bud tip). In C, cells were grown at 30°C and then treated with 0.5 µg/ml myriocin at 37°C for 2.5 h. The percentage of small to medium-budded cells without Rho1 localization at the bud cortex is shown in the chart. Data are presented as the mean±s.d. of three independent experiments (*n*>50 cells were counted in each experiment). (D) Growth rescue of the *ypk^ts* mutant by *LEM3* deletion. Cells of the *ypk^ts* strain were grown at either permissive (30°C) or non-permissive (34°C) temperature, or in the presence of 250 ng/ml myriocin for 5 days. (E) Enhanced cortical accumulation of GFP-Rho1 in the *lem3Δ* mutant and its suppression by *CHO1* deletion. The intensity of the GFP-Rho1 signal at the bud tip normalized by the mean intensity of the intracellular signal in buds is shown in the box plot. Representative cell images are shown. Arrowheads indicate the cellular growth site (bud tip). (F) Effect of myriocin on Rho1 localization. Cells were treated with 10 µg/ml myriocin for 2 h. The percentage of small to medium-budded cells lacking Rho1 at the bud cortex is shown. Data are presented as the mean±s.d. of three independent experiments (*n*>30 cells were counted in each experiment). (G,H) The effect of *LEM3* deletion on cortical localization of GFP-Rho1 in the absence of *RDI1* or three Rho1GEFs. The box plot shows the intensity of the GFP-Rho1 signal at the bud tip normalized by the mean intensity of the intracellular signal in buds. Representative cell images are shown. Because yeast cells that lack all three RhoGEFs (Rom1, Rom2 and Tus1) are lethal, Lrg1 – an important Rho1 GAP – was also deleted in this experiment. (I) *In vitro* binding of the Rho1 poly-basic sequence to membrane lipids. The poly-basic sequence of GFP-Rho1 from yeast lysates was incubated with a PIP strips membrane (Echelon) and its affinity to specific lipids was analysed. PI, phosphatidylinositol; PA, phosphatidyl acid. (J) Diffused localization of GFP-Rho1-5KA. In E, G and H, the box represents the 25–75th percentiles, and the median is indicated. The whiskers represent all samples lying within 1.5× of the interquartile range. Outliers are indicated by dots. All scale bars: 5 µm.

possibility that redistribution of phosphatidylserine contributes to Rho1 regulation by Ypk1/Ypk2. To test the requirement of phosphatidylserine in Rho1 regulation, we depleted cellular phosphatidylserine by deleting *CHO1*, the only gene encoding phosphatidylserine synthase in budding yeast. GFP-Rho1 was excessively enriched in the bud cortex in unstressed *lem3Δ* cells, whereas *CHO1* deletion cancelled this effect (Fig. 3E). Furthermore, in *lem3Δ* cells, GFP-Rho1 was maintained in the bud cortex even after myriocin treatment; this phenotype was also suppressed by *CHO1* deletion (Fig. 3F). Thus, phosphatidylserine is required for the flippase inhibition-dependent cortical localization of Rho1.

We further analysed the mechanism of Rho1 regulation by Lem3. Phosphatidylserine supports cortical localization of yeast Cdc42 by preventing its extraction by Rho guanine nucleotide dissociation inhibitor (GDI) (Das et al., 2012). Rho GDI is potentially involved in flippase-mediated Rho1 regulation as well, because enhancement of PM localization of Rho1 by *LEM3* deletion was not significant in *rdi1Δ* cells, which lack the only Rho GDI in budding yeast (Fig. 3G). A recent study revealed that Ypk1/Ypk2 maintain the Rho1 GEF Rom2 at the PM through inhibition of Fpk1/Fpk2 (Niles and Powers, 2014), suggesting that Rom2 contributes to Rho1 regulation by Ypk1/Ypk2. However, enhanced PM localization of Rho1 in by *LEM3* deletion was still evident in the yeast strains that lack all three Rho1 GEFs Rom1, Rom2 and Tus1 (*ΔGEF*) (Fig. 3H), suggesting that Rho1 regulation by flippase is not solely mediated by GEFs.

Next, we examined whether Rho1 physically associates with phosphatidylserine. An *in vitro* binding assay using PIP strips indicated that the Rho1 poly-basic sequence has an affinity for phosphatidylserine, supporting the idea that phosphatidylserine contributes to the cortical localization of Rho1 through electrostatic interaction – although the interaction was weaker than that of other anionic lipids (Fig. 3I). *In vivo*, a high concentration of phosphatidylserine at the PM may compensate for the low affinity of phosphatidylserine for the Rho1 poly-basic sequence, as phosphatidylserine accounts for more than 10% of PM phospholipids, whereas $PI(4,5)P_2$ composes only 1% (Fairn and Grinstein, 2012; Leventis and Grinstein, 2010; van Meer et al., 2008; Martin, 2015). Nanoclustering of phosphatidylserine at the PM might also contribute to Rho1 binding, as is the case for human K-Ras (Zhou et al., 2015). We confirmed that the effect of *lem3Δ* on Rho1 PM localization *in vivo* is mediated by the poly-basic sequence, as the $Rho1^{5KA}$ mutant, whose five lysine residues within its poly-basic sequence are mutated to alanine (Yoshida et al., 2009), did not accumulate in the PM in *lem3Δ* cells (Fig. 3J). Taken together, our data supports the model that flippase inhibition by Ypk1/Ypk2 promotes PM localization of Rho1 by enhancing the electrostatic interaction between the Rho1 poly-basic sequence and anionic lipids, including phosphatidylserine.

Phosphatidylserine is essential for Rho1 localization and recovery from PM stress

As phosphatidylserine is a highly abundant anionic lipid composing up to 10% of PM phospholipids (Fairn and Grinstein, 2012; Leventis and Grinstein, 2010; van Meer et al., 2008), its contribution to the net negative charge of the PM is significant. Interestingly, however, the *cho1Δ* mutant is viable, indicating that phosphatidylserine is dispensable for normal cell proliferation. Based on our finding that phosphatidylserine is under the control of Ypk1/Ypk2 (Fig. 2), we speculated that phosphatidylserine plays an important role under PM stress. To test this hypothesis, we examined GFP-Rho1 localization in the *cho1Δ* mutant under PM stress. In *cho1Δ* cells, GFP-Rho1 normally localized to the PM in the unstressed condition. In contrast, upon hypotonic shock, cortical localization of GFP-Rho1 was severely impaired (Fig. 4A). Because phosphatidylserine is a precursor of phosphatidylethanolamine, *CHO1* deletion also significantly decreases the level of phosphatidylethanolamine (Fairn et al., 2011). To rule out the possibility that defective GFP-Rho1 localization in *cho1Δ* cells is an indirect consequence of phosphatidylethanolamine reduction, we examined Rho1 localization in the *psd1Δ* mutant, which lacks the main phosphatidylethanolamine synthase (Trotter et al., 1993). Regardless of stress, *psd1Δ* cells were only slightly defective in the cortical localization of GFP-Rho1, suggesting that phosphatidylethanolamine has a minor role in Rho1 localization (Fig. 4B). Importantly, hypotonic shock did not cause severe delocalization of GFP-Rho1 in *psd1Δ* cells. Thus, the loss of cortical localization of Rho1 in *cho1Δ* cells under PM stress is not due to the loss of phosphatidylethanolamine. Analogous to hypotonic shock, Rho1 delocalization induced by treatment with myriocin was exacerbated in *cho1Δ* cells (Fig. 4C). These results suggest that phosphatidylserine is crucial for the maintenance of cortical localization of Rho1, specifically under PM stress.

Rho1 recruits its effector protein Pkc1 to the bud cortex (Andrews and Stark, 2000). Analogous to GFP-Rho1, delocalization of Pkc1-GFP after treatment with myriocin was more prominent in *cho1Δ* cells and less obvious in *lem3Δ* cells (Fig. S1), suggesting that phosphatidylserine ensures not only localization but also function of Rho1 under PM stress.

Delocalization of Pkc1-GFP in *cho1Δ* cells raised the possibility that phosphorylation of Mpk1, the major downstream factor of Pkc1 signaling pathway, could be decreased in these cells. However,

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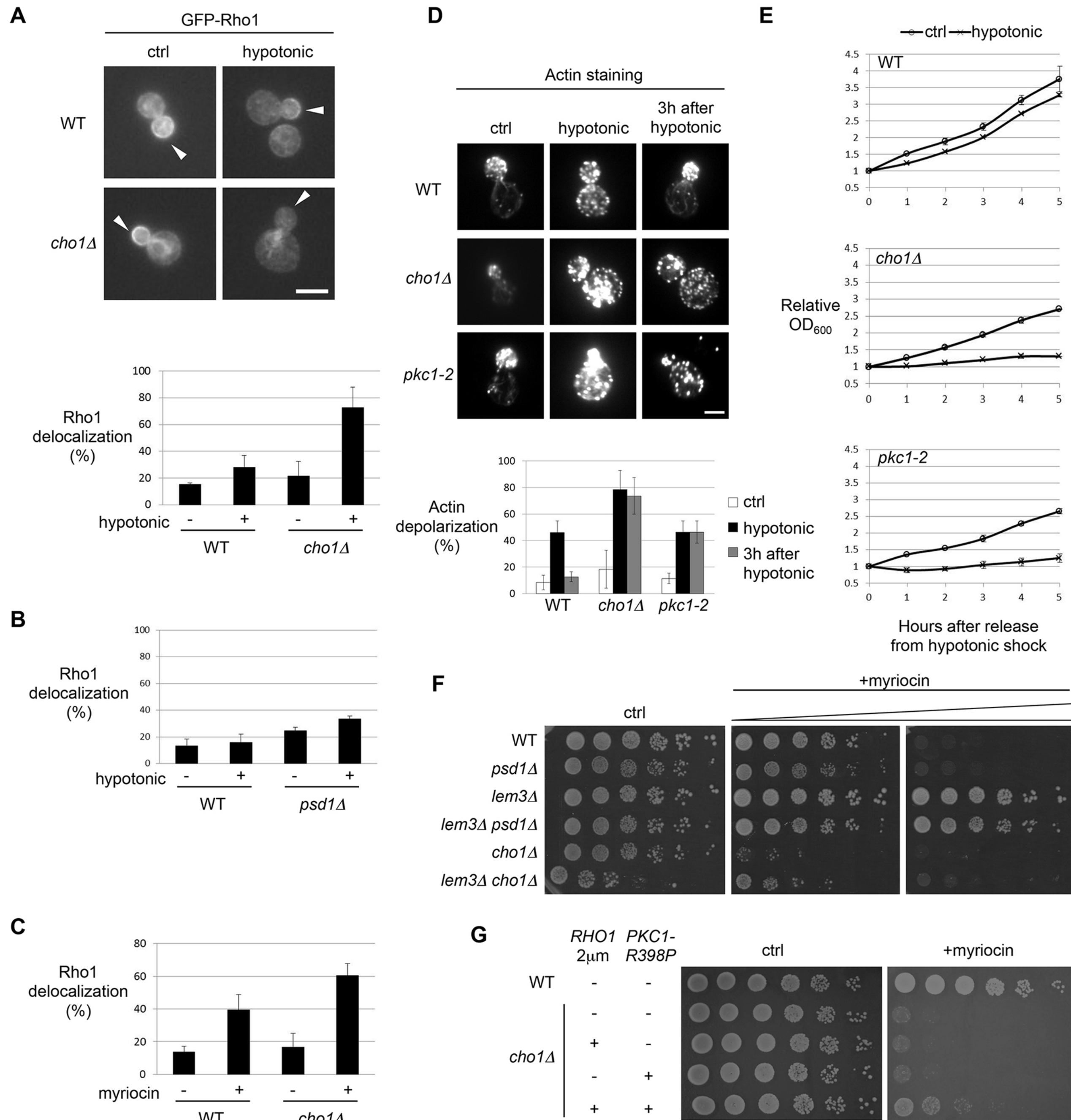

Fig. 4. Phosphatidylserine is essential for recovery from PM stress. (A,B) Effect of hypotonic shock on GFP-Rho1 localization. Images were taken 5 min after hypotonic shock. The percentage of small to medium-budded cells without Rho1 localization at the bud cortex is shown in the chart. Data are presented as the mean±s.d. of three independent experiments (*n*>42 cells were counted in each experiment). (C) Effect of myriocin on Rho1 localization. Cells were treated with 1 µg/ml myriocin for 2.5 h. The percentage of small to medium-budded cells without Rho1 localization at the bud cortex is shown. Data are presented as the mean±s.d. of three independent experiments (*n*>41 cells were counted in each experiment). (D) Actin cytoskeleton after hypotonic shock and its recovery after 3 h. The indicated strains were grown at 40°C, exposed to transient (20 min) hypotonic shock and released to the medium containing 1 M sorbitol. Images were taken after staining actin with Alexa Fluor 488-conjugated phalloidin. The percentage of small- to medium-budded cells showing a depolarized actin cytoskeleton is shown in the chart. Data are presented as the mean±s.d. of three independent experiments (*n*>40 cells were counted in each experiment). (E) Cell proliferation with (cross) or without (circle) transient hypotonic shock performed as in D. Error bars indicate ±s.d. Data are presented as the mean±s.d. of two independent experiments. (F) Growth of the indicated mutants in the presence of 500 or 1000 ng/ml myriocin. Cells were grown at 30°C for 2 days. (G) Growth of the indicated strains in the presence of 500 ng/ml myriocin. Cells were grown at 30°C for 3 days. All scale bars: 5 µm.

phosphorylation of Mpk1 in response to hypotonic shock was observed even in *cho1Δ* cells (data not shown). This suggests that localization of Pkc1 to the bud cortex is not necessarily essential for stress-triggered activation of the downstream MAP kinase cascade, consistent with the recent observation that ectopic activation of the MAP kinase cascade by Pkc1 occurs at endosomes when $PI(4,5)P_2$ is artificially eliminated (Fernández-Acero et al., 2015). It is possible that phosphatidylserine-dependent regulation of Rho1-Pkc1 axis is crucial for Pkc1 targets other than the MAP kinase cascade. As an alternative readout of Rho1-Pkc1 function, we examined organization

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of the actin cytoskeleton, a defect associated with Rho1-Pkc1 malfunction (Delley and Hall, 1999), in *cho1Δ* cells. Under normal growth conditions, the yeast actin cytoskeleton polarizes at the small- to medium-sized buds to sustain polarized bud growth (Pruyne and Bretscher, 2000; Moseley and Goode, 2006). Under conditions such as heat shock or damage of the cell wall, the actin cytoskeleton is rapidly depolarized to suspend polarized cell growth and to repair damage (Levin, 2011). Consistent with a recent report (Gualtieri et al., 2004), the cortical actin cytoskeleton was rapidly depolarized upon transient (20 min) hypotonic shock, and eventually repolarized within 3 h in wild type cells (Fig. 4D). The actin repolarization process required Pkc1 activity, as *pkc1-2* temperature-sensitive mutant cells failed to repolarize actin 3 h after hypotonic shock (Fig. 4D). We found that *cho1Δ* cells show an even more severe actin depolarization phenotype than *pkc1-2* cells. Furthermore, *cho1Δ* cells failed to repolarize even after 3 h (Fig. 4D), suggesting requirement of phosphatidylserine for rapid repolarization and/or the completion of repair processes.

Then, we monitored cell proliferation after hypotonic shock. After the shock, wild type cells exhibited only a transient growth arrest (less than 1 h) (Fig. 4E). In contrast, both *pkc1-2* and *cho1Δ* cells failed to proliferate for at least 5 h after release from the shock (Fig. 4E). This was not simply due to cell death or lysis because these cells remained viable and eventually restarted growth in 24 h (Fig. S2). The *psd1Δ* strain restarted growth within 1 h , which was comparable to the recovery time for wild type cells (data not shown), excluding the possibility that the growth recovery defects in *cho1Δ* are due to the loss of phosphatidylethanolamine. These results suggest that phosphatidylserine and Pkc1 are required for recovery after hypotonic shock.

We found *cho1Δ* cells highly sensitive to PM stress induced by myriocin treatment but not *psd1Δ* cells (Fig. 4F). The myriocin sensitivity of *cho1Δ* cells is likely to be due to the loss of phosphatidylserine but not phosphatidylethanolamine, as the *psd1Δ* mutant is not hypersensitive to myriocin (Fig. 4F). In contrast to *cho1Δ* cells, *lem3Δ* cells are resistant to myriocin – as is the flippase mutant *dnf1Δ dnf2Δ dnf3Δ* (Roelants et al., 2010). Myriocin resistance in the *lem3Δ* strain was largely cancelled by *CHO1* deletion but not by *PSD1* deletion (Fig. 4F), confirming the contribution of phosphatidylserine, but not of phosphatidylethanolamine, to PM stress resistance.

Genetic evidence suggests that one of the crucial targets of phosphatidylserine in the PM stress response is the Rho1-Pkc1 pathway: the myriocin sensitivity of *cho1Δ* cells was partially rescued by overexpressing Rho1 in the presence of Pkc1-R398P, the constitutively active allele of Pkc1 (Nonaka et al., 1995) (Fig. 4G). Neither overexpression of Rho1 alone nor expression of Pkc1-R398P alone detectably rescued myriocin sensitivity, indicating that phosphatidylserine promotes both recruitment of Rho1 to the PM and activation of Pkc1 during PM stress.

Flippase inhibition ensures Rho1 localization in the absence of PI(4,5)P_2

Why is phosphatidylserine only required for Rho1 localization when the PM is stressed (Fig. 4A)? Under normal conditions, PI(4,5)P_2 plays a major role in cortical localization of Rho1 (Yoshida et al., 2009). However, upon various stimuli, such as mechanical stress to the PM, a large fraction (∼50%) of PI(4,5)P_2 is immediately degraded by phospholipase C (PLC) to produce the second messengers diacylglycerol (DAG) and inositol triphosphates (IP_3) (Storch et al., 2012; Perera et al., 2004). Based on these facts, we hypothesized that phosphatidylserine becomes essential only when PI(4,5)P_2 levels are reduced. Consistent with this idea, the *cho1Δ* mutant shows a synthetic growth defect in combination with the temperature-sensitive mutation of *MSS4* (Sun and Drubin, 2012), which encodes the only PI4P 5-kinase in yeast (Homma et al., 1998; Desrivieres et al., 1998).

We confirmed the rapid loss of PI(4,5)P_2 upon PM stress (Perera et al., 2004) with a visual assay using GFP-2PH^{PLC}. We observed a rapid redistribution of GFP-2PH^{PLC} from the PM into the cytoplasm within 2–4 min after hypotonic shock (Fig. 5A).

To test our hypothesis that phosphatidylserine compensates for PI(4,5)P_2, we examined the effect of flippase inhibition on Rho1 localization when PI(4,5)P_2 was absent. In *mss4-1* cells, Rho1 failed to localize to the PM at the restrictive temperature (Fig. 5B), confirming the requirement of PI(4,5)P_2 in Rho1 localization. However, deletion of *LEM3* significantly restored Rho1 localization without detectably restoring PI(4,5)P_2 production in the *mss4-1* mutant (Fig. 5B). Both the temperature-sensitive growth defect and myriocin hypersensitivity of *mss4-1* cells were rescued by deletion of *LEM3* (Fig. 5C). Collectively, our data suggest that inhibition of flippase, which is triggered by activation of TORC2-Ypk1/Ypk2, supports localization of Rho1 to the PM and cell survival when levels of PI(4,5)P_2 are limited. Because enhanced Rho1 localization and myriocin resistance of *lem3Δ* cells are dependent on *CHO1* (Figs 3E,F and 4F), phosphatidylserine should have a significant role in phenotypic rescue of *mss4-1* by *LEM3* deletion.

DISCUSSION

Although previous genetic studies placed TORC2 upstream of the Rho1-Pkc1 pathway (Helliwell et al., 1998a,b), the molecular link between them is poorly understood. Here, we show that Ypk1/Ypk2, essential substrates of TORC2, regulate peripheral localization of Rho1 through flippase-mediated rearrangement of phospholipids. Our results pinpoint the fact that phosphatidylserine is the key lipid responsible for Rho1 localization and function during PM stress, and that the spatial organization of phosphatidylserine is controlled largely by the Ypk1/Ypk2-flippase pathway. Based on our observations that phosphatidylserine is required for Rho1 localization under stress and that flippase inhibition bypasses the requirement of PI(4,5)P_2, we propose that Ypk1/Ypk2 redistribute phosphatidylserine as a backup for PI(4,5)P_2, thereby allowing production of PI(4,5)P_2-derived second messengers without abandoning Rho1-Pkc1 signaling (Fig. 5D).

The precise mechanism by which the Dnf1/Dnf2-Lem3 flippase complex regulates phosphatidylserine distribution needs to be elucidated. Phosphatidylserine is not a good substrate for Dnf1, which rather prefers phosphatidylcholine and phosphatidylethanolamine (Baldridge and Graham, 2012); phosphatidylserine regulation by this flippase might, therefore, be a consequence of phosphatidylcholine/phosphatidylethanolamine flipping.

We notice that *LEM3* deletion only partially rescues the actin polarization defect in *ypk^{ts}* cells (data not shown) that is similar to the defect in response to pharmacological inhibition of TORC2 (Rispal et al., 2015). Therefore, Rho1 targeting does not appear to be the only mechanism by which the TORC2-Ypk1/Ypk2 pathway regulates actin organization. Further studies are needed to fully understand the role for Ypk1/Ypk2 in this process.

In mammals, the PM of certain cell types, such as cardiomyocytes and smooth muscles, are regularly exposed to physical stress. Because crucial roles of TORC2 and Rho-PKC pathways in cardiac function and stress response have been demonstrated (Kajimoto et al., 2011; Volkers et al., 2013; Yano et al., 2014; Sciarretta et al., 2015; Zhao et al., 2014; Moschella

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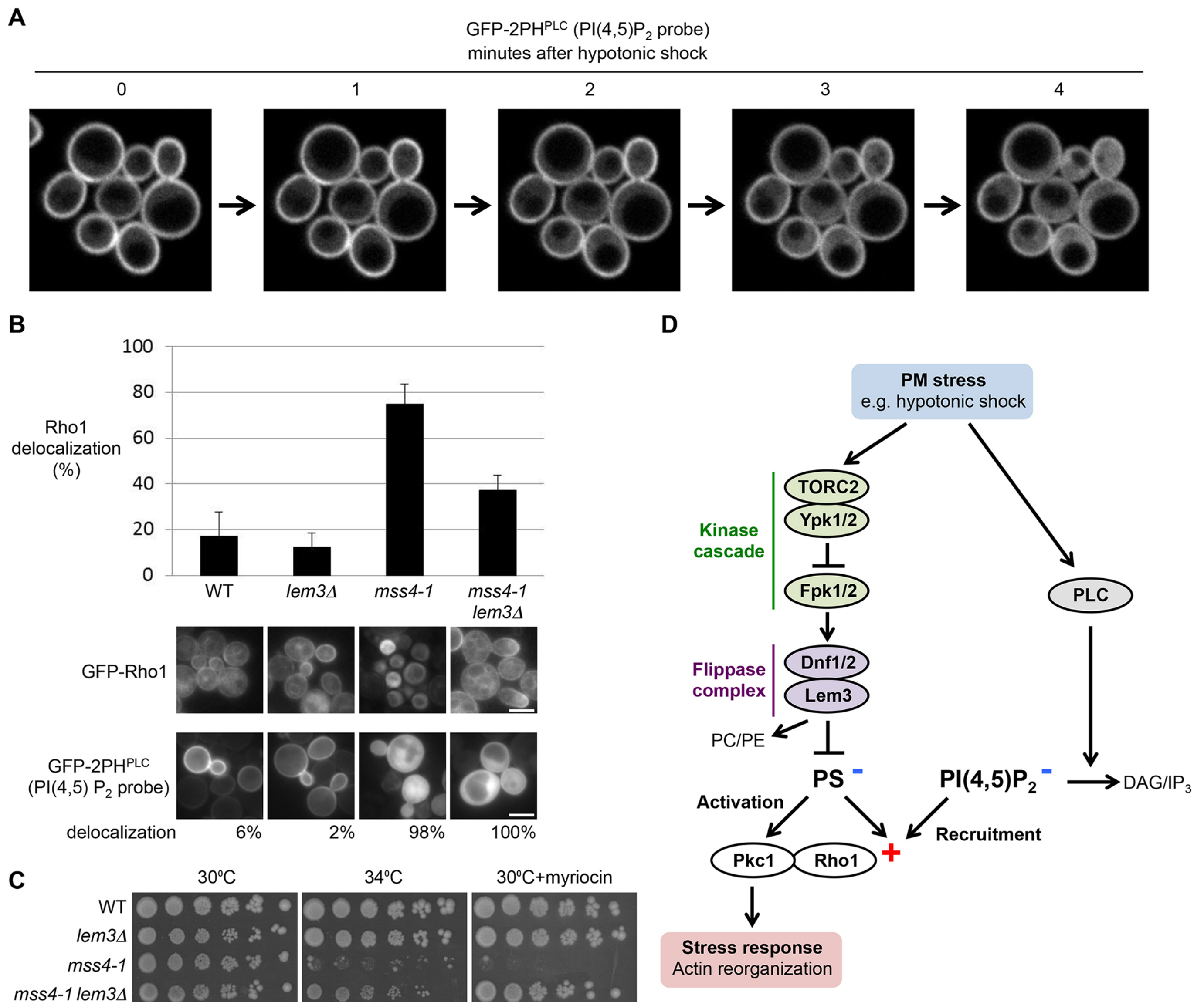

Fig. 5. Inhibition of the Lem3-containing flippase compensates for the loss of $PI(4,5)P_2$. (A) Time-lapse imaging of $PI(4,5)P_2$ degradation upon hypotonic shock using the GFP-2PH^{PLC} $P(4,5)IP_2$ probe. (B) GFP-Rho1 localization after incubating for 3 h at 37°C (top). Percentage of small- to medium-budded cells lacking Rho1 at the bud cortex. Data are presented as the mean±s.d. of three independent experiments (*n*>26 cells were counted in each experiment). Representative cell images are shown. Localization of the GFP-2PH^{PLC} $PI(4,5)P_2$ probe after 2 h of incubation at 37°C (bottom). Percentage of cells that lack localization of GFP-2PH^{PLC} at the PM (*n*>46 cells). Scale bars: 5 µm. (C) Growth rescue of the *mss4-1* mutant by *LEM3* deletion. The *mss4-1* strain was grown at either a permissive (30°C) or non-permissive (34°C) temperature, or in the presence of 250 ng/ml myriocin for 5 days. (D) The schematic summary of Rho1 regulation in PM stress response. The TORC2-Ypk1/Ypk2 pathway regulates the distribution of phosphatidylserine through inhibition of the flippase complex. In stressed conditions, phosphatidylserine compensates for the loss of $PI(4,5)P_2$ by supplying negative charges required for Rho1 localization. PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

et al., 2013), flippases and phosphatidylserine might also be key signaling mediators in these cell types.

MATERIALS AND METHODS

Yeast strains, plasmids and media

The *Saccharomyces cerevisiae* strains used in this study are listed in Table S1. Strains were constructed using standard yeast genetics procedures (Longtine et al., 1998). Plasmids used in this study are listed in Table S2.

For experiments using plasmids, cells were grown in synthetic complete (SC) medium lacking appropriate nutrients for plasmid preservation. In experiments including the *cho1Δ* strain, 1 mM ethanolamine was added to SC medium for growth support (Hikiji et al., 1988). Yeast extract, peptone, dextrose (YPD) medium was used for experiments without plasmids unless specified. Unless specified, cells were grown to mid-log phase at 30°C.

In hypotonic shock experiments, cells were acclimatized to hypertonic conditions by growing them in the presence of 1 M sorbitol. The acclimatized cells in mid-log phase were collected by centrifugation and re-suspended 1:20 volume of the same medium and then diluted 1:20 with distilled water. For actin staining and growth curve assays, which were performed at 40°C in order to inactivate Pkc1 in the *pkc1-2* mutant, cells were collected after hypotonic shock for 20 min and re-cultured in the original medium (with 1 M sorbitol).

Fluorescence microscopy

Fluorescence images were acquired by using an Eclipse E600 fluorescence microscope (Nikon) equipped with a DC350F charge-coupled device

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camera (Andor) and an oil 60× objective (NA 1.4). The images were captured and analysed with NIS-Elements software (Nikon).

Actin staining

Cells were fixed for 40 min with 4% formaldehyde (final concentration). After collecting by centrifugation, cells were stained for 30 min with 0.66 µM Alexa Fluor 488 phalloidin (A12379, Molecular Probes) and washed with phosphate-buffered saline.

PIP strips assay

Yeast cells overexpressing GFP-tagged Rho1 C-terminal tail were collected in mid-log phase. Cell lysate was prepared by disrupting the cell pellet with glass beads in lysis buffer [50 mM Tris-HCl pH 8.0), 10 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and complete Mini Protease Inhibitor Cocktail (Roche)]. The PIP Strips membrane (Echelon) was blocked with 3% bovine serum albumin and incubated with cell lysate (0.5 mg/ml total protein) overnight at 4°C. The signal was detected with anti-GFP antibody (7.1 and 13.1, Roche) and anti-mouse horseradish-peroxidase-conjugated IgG (NA931, GE Healthcare) and developed with ECL Prime (GE Healthcare).

Lysate preparation, SDS-PAGE and western blotting

Lysates were prepared as previously described (Hatakeyama et al., 2010). Briefly, cells were treated with 7.2% w/v trichloroacetic acid (final concentration), pelleted, washed with 70% ethanol and then dissolved in 6 M urea buffer. After boiling in Laemmli SDS sample buffer, samples were subjected to regular SDS-PAGE and immunoblotting experiments.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

R.H. conceived and performed all the experiments. R.H., K.K. and S.Y. designed the experiments and wrote the paper.

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Supplementary information

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Supplemental information

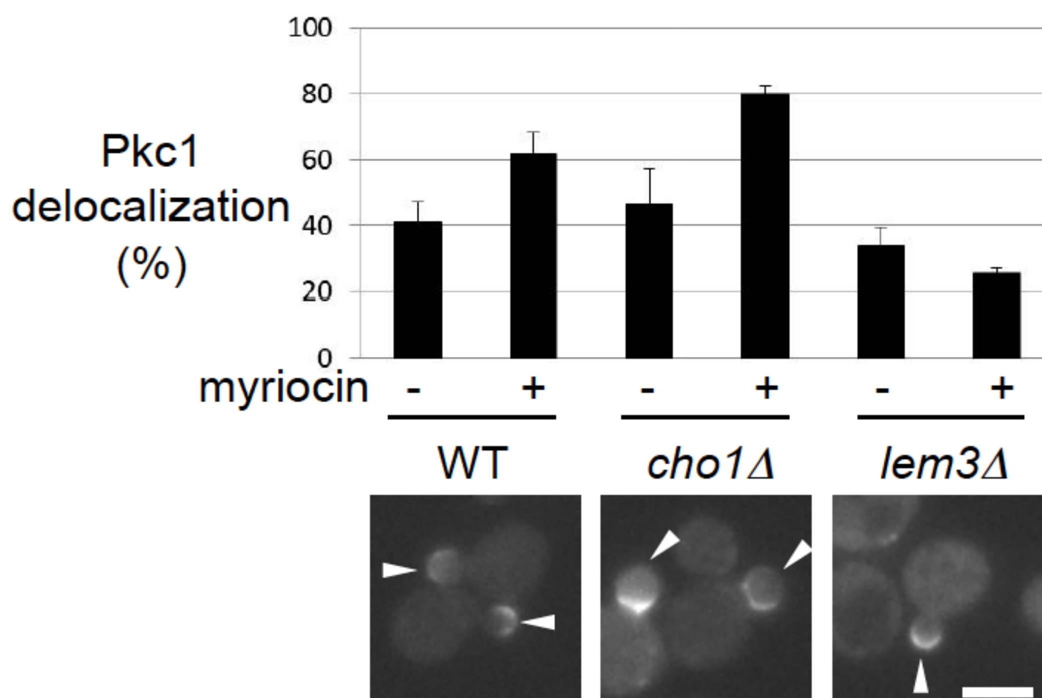

Figure S1, related to Figure 4 Effect of myriocin on Pkc1-GFP localization.

Cells were treated with 1 mg/ml myriocin for 2 hours. Percentage of small to medium-budded cells without Pkc1 localization at the bud cortex is shown. Data are presented as mean +/- SD from three independent experiments (n>58 cells were counted in each experiment). Representative cell images after myriocin treatment are shown. In all the images, arrowheads point to the cellular growth site (bud tip). Scale bars: 5 μm.

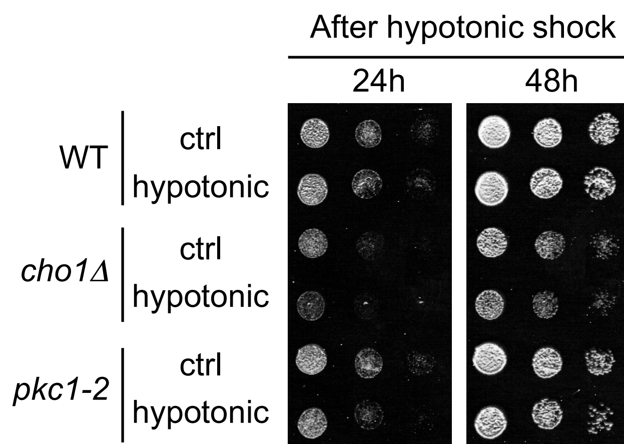

Figure S2, related to Figure 4 Long-term growth after hypotonic shock.

Recovery of cell growth after release from 20 minutes hypotonic shock as in the Fig. 3. Equal number of the cells were serially diluted and spotted on YPD containing 1M Sorbitol at 40°C. After 24 h, *cho1Δ* and *pkc1-2* strains exposed to hypotonic shock barely formed colonies, but they eventually formed equal number of the colonies after 48 h.

Table S1 Strains

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Lab stock
RH310	BY4741 *cho1Δ::kanMX6*	This study
RH314	BY4741 *lem3Δ::kanMX6*	This study
RH376	BY4741 *lem3Δ::hphMX4 cho1Δ::kanMX6*	This study
RH701	BY4741 *psd1Δ::HIS3*	This study
RH702	BY4741 *lem3Δ::kanMX6 psd1Δ::HIS3*	This study
RH35	BY4741 *pkc1-2*	This study
SY1520	BY4741 *rdi1Δ::HIS3MX6*	This study
RH549	BY4741 *rdi1Δ::HIS3MX6 lem3Δ::kanMX6*	This study
SY1416	BY4741 *rom1Δ::kanMX6 rom2Δ::kanMX6 tus1Δ::kanMX6 lrg1Δ::HIS3MX6*	Yoshida et al., 2009
RH384	BY4741 *rom1Δ::kanMX6 rom2Δ::kanMX6 tus1Δ::kanMX6 lrg1Δ::HIS3MX6 lem3Δ::hphMX4*	This study
YOC807	*MATa ade2 leu2 lys2 trp1 ura3 mss4::HIS3 ade3::MSS4::LEU2*	Homma et al., 1998
YOC808	*MATa ade2 leu2 lys2 trp1 ura3 mss4::HIS3 ade3::mss4-1::LEU2*	Homma et al., 1998
RH354	YOC807 *lem3Δ::kanMX6*	This study
RH357	YOC808 *lem3Δ::kanMX6*	This study
YPH499	*MATa ade2-101^{Ochre} his3Δ200 leu2Δ1 lys2-801^{amber} trp1Δ63 ura3-52*	Roelants et al., 2010
YPT40	YPH499 *ypk1-1^{ts}::HIS3 ypk2Δ1::TRP1*	Roelants et al., 2010
RH483	YPT40 *lem3Δ::kanMX6*	This study
yAM123A	BY4741 *ypk1(L424A)::URA3 ypk2Δ::kanMX4*	Gifted from Jeremy Thorner
RKH1	YPT40 *SEC7-mCherry::natMX4*	This study
RKH2	YPT40 *VPS17-mCherry::natMX4*	This study

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Table S2 Plasmids

Plasmid	Gene	Source
SP301	*CEN URA3 GFP-RHO1*	Yoshida et al., 2009
pRH210	*CEN URA3 PKC1-GFP*	This study
pRH176	*2um URA3* P_{PRC1}*-GFP-2PH*^{PLC}	Stefan et al., 2002
SP353	*2um LEU2* P_{GAL1}*-GFP-RHO1*$^{187-209}$	This study
pRH241	*CEN URA3 GFP-RHO1*^{5KA}	This study
pRS316	*CEN URA3* empty vector	Sikorski et al., 1989
YEP352-RHO1	*2um URA3 RHO1*	Gifted from Daniel Lew
pRS315	*CEN LEU2* empty vector	Sikorski et al., 1989
SP336	*CEN LEU2 HA-RHO1*^{Q68H}	Yoshida et al., 2009
SP335	*CEN LEU2 HA-RHO1*^{F35L}	Yoshida et al., 2009
pYO1775	*CEN LEU2 PKC1-R398P*	Nonaka et al., 1995
pAGX-h-evt2-2*PH	*CEN URA3 GFP-2PH*$^{evt-2}$	Uchida et al., 2011
p415-GFP-h-evt2-2*PH	*CEN LEU2 GFP-2PH*$^{evt-2}$	This study

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Supplemental Reference

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