Phospholipid flippases and Sfk1p, a novel regulator of phospholipid asymmetry, contribute to low permeability of the plasma membrane

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ABSTRACT Phospholipid flippase (type 4 P-type ATPase) plays a major role in the generation of phospholipid asymmetry in eukaryotic cell membranes. Loss of Lem3p-Dnf1/2p flippases leads to the exposure of phosphatidylserine (PS) and phosphatidylethanolamine (PE) on the cell surface in yeast, resulting in sensitivity to PS- or PE-binding peptides. We isolated Sfk1p, a conserved membrane protein in the TMEM150/FRAG1/DRAM family, as a multicopy suppressor of this sensitivity. Overexpression of *SFK1* decreased PS/PE exposure in *lem3Δ* mutant cells. Consistent with this, *lem3Δ sfk1Δ* double mutant cells exposed more PS/PE than the *lem3Δ* mutant. Sfk1p was previously implicated in the regulation of the phosphatidylinositol-4 kinase Stt4p, but the effect of Sfk1p on PS/PE exposure in *lem3Δ* was independent of Stt4p. Surprisingly, Sfk1p did not facilitate phospholipid flipping but instead repressed it, even under ATP-depleted conditions. We propose that Sfk1p negatively regulates transbilayer movement of phospholipids irrespective of directions. In addition, we showed that the permeability of the plasma membrane was dramatically elevated in the *lem3Δ sfk1Δ* double mutant in comparison with the corresponding single mutants. Interestingly, total ergosterol was decreased in the *lem3Δ sfk1Δ* mutant. Our results suggest that phospholipid asymmetry is required for the maintenance of low plasma membrane permeability.

INTRODUCTION

In eukaryotic cells, the composition of phospholipids is quite different between the exoplasmic (outer) and the cytosolic (inner) membrane leaflets of the plasma membrane. The bulk of phosphatidylcholine (PC) and sphingolipids are contained in the exoplasmic leaflet, whereas phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphoinositides are predominantly distributed in the cytosolic leaflet (Devaux, 1991; Zachowski, 1993; Murate *et al.*, 2015). In the model membranes, phospholipid transfer between the bilayer hardly occurs spontaneously, because polar heads of phospholipids are hydrophilic, whereas the center of the bilayer is hydrophobic region. Therefore, transbilayer movement of phospholipids in cell membranes is catalyzed by three types of phospholipid translocases: flippase, floppase, and scramblase. Flippases are type 4 P-type ATPases that translocate phospholipids from the exoplasmic to cytosolic leaflet (flip) (Tanaka *et al.*, 2011; Sebastian *et al.*, 2012; Panatala *et al.*, 2015). Some of the ABC transporters are

reported to act as floppases that translocate phospholipids from the cytosolic to exoplasmic leaflet (flop) (Coleman *et al.*, 2013). Scramblase catalyzes bidirectional transbilayer movement in an energy-independent manner (Montigny *et al.*, 2016).

Flippases are implicated in the fundamental body functions including vision, hearing, and learning and their diseases (Levano *et al.*, 2012; van der Mark *et al.*, 2013; Coleman *et al.*, 2014). At the cellular level, flippases in endomembranes are important for the membrane trafficking processes: endosomal trafficking (Hua *et al.*, 2002; Pomorski *et al.*, 2003; Furuta *et al.*, 2007; Lee *et al.*, 2015; Tanaka *et al.*, 2016), biogenesis of secretory vesicles (Gall *et al.*, 2002; Poulsen *et al.*, 2008; Mioka *et al.*, 2014), and protein sorting in the *trans*-Golgi network (TGN) (Hachiro *et al.*, 2013; Hankins *et al.*, 2015). On the other hand, flippases located in the plasma membrane are not only involved in the membrane trafficking (Hua *et al.*, 2002; Pomorski *et al.*, 2003; Furuta *et al.*, 2007) but also participate in other various functions: apoptosis signaling (Segawa *et al.*, 2014), mating signaling (Sartorel *et al.*, 2015), apical membrane barrier (Paulusma *et al.*, 2006), cell polarity (Iwamoto *et al.*, 2004; Saito *et al.*, 2007; Das *et al.*, 2012), and cell migration (Kato *et al.*, 2013).

The relevance of flippases to cellular functions has been mostly found in studies using a model organism, budding yeast. Budding yeast has five flippases: Drs2p, Dnf1p, Dnf2p, Dnf3p, and Neo1p. Drs2p, Dnf1/2p, and Dnf3p form complexes with Cdc50p, Lem3p, and Crf1p noncatalytic subunits, respectively (Saito *et al.*, 2004; Furuta *et al.*, 2007). These interactions are required for ER exit, proper localization, function, and translocase activity (Saito *et al.*, 2004; Furuta *et al.*, 2007; Lenoir *et al.*, 2009; Takahashi *et al.*, 2011; Puts *et al.*, 2012). Therefore, defects in *drs2Δ*, *dnf1Δ dnf2Δ*, and *dnf3Δ* mutants are phenocopied by *cdc50Δ*, *lem3Δ*, and *crf1Δ* mutants, respectively (Saito *et al.*, 2004; Furuta *et al.*, 2007). Of the five flippases, only Neo1p is essential, and does not bind to any of Cdc50 family proteins (Saito *et al.*, 2004; Furuta *et al.*, 2007). Cdc50p–Drs2p, Crf1p–Dnf3p, and Neo1p are located primarily in the TGN and endosomes (Chen *et al.*, 1999; Saito *et al.*, 2004; Wicky *et al.*, 2004; Furuta *et al.*, 2007), while Lem3p–Dnf1/2p is mainly localized to the plasma membrane (Kato *et al.*, 2002; Pomorski *et al.*, 2003). Loss of the Lem3p–Dnf1/2p causes the decrease in uptake of exogenous phospholipid analogues, and the exposure of PE and PS (Kato *et al.*, 2002; Hanson *et al.*, 2003; Parsons *et al.*, 2006). Lem3p–Dnf1/2p are also required for the uptake of exogenous lysophospholipids (Riekhof and Voelker, 2006; Riekhof *et al.*, 2007).

In addition to Lem3p–Dnf1/2p, some factors affecting the phospholipid asymmetry in the plasma membrane have been identified. Serine/threonine kinases Fpk1/2p phosphorylate Dnf1/2p and up-regulate their flippase activity (Nakano *et al.*, 2008). Two other kinases, Ypk1p and Gin4p, and sphingolipids indirectly modulate the phospholipid asymmetry through the regulation of Fpk1/2p (Roelants *et al.*, 2010, 2015). Pdr5p and Yor1p, two multidrug ABC transporters, contribute to PE flop in the plasma membrane (Decottignies *et al.*, 1998; Pomorski *et al.*, 2003). Opt2p, a member of the oligopeptide transporter family, is implicated in phospholipid flop and plays a role in maintenance of phospholipid asymmetry mediated by the *RIM101* pathway (Yamauchi *et al.*, 2015).

In this study, to further understand the regulatory mechanisms and the physiological role of the phospholipid asymmetry in the plasma membrane, we searched for novel regulators of the phospholipid asymmetry and identified Sfk1p, a conserved transmembrane protein belonging to the TMEM150/FRAG1/DRAM family (Chung *et al.*, 2015).Our results suggest an unprecedented function of Sfk1p; Sfk1p may negatively regulate the rate of spontaneous flip

and flop of phospholipids in the plasma membrane. Moreover, we found that the order of phospholipid asymmetry correlated with passive permeability of the plasma membrane.

RESULTS

Sfk1p, in conjunction with Lem3p-Dnf1/2p, regulates PS and PE asymmetry in the plasma membrane

PC, PE, and PS are thought to be potential substrates for Lem3p-Dnf1/2p, but unlike nitrobenzoxadiazole (NBD)-PC and -PE, the internalization of NBD-PS occurs even in *lem3Δ* or *dnf1Δ dnf2Δ* cells (Kato *et al.*, 2002; Saito *et al.*, 2004; Stevens *et al.*, 2008). To examine the contribution of Lem3p-Dnf1/2p to the flip of endogenous PS, we tested growth sensitivity to papuamide B (pap B), a depsipeptide, that binds to PS in biological membranes (Parsons *et al.*, 2006). *lem3Δ* and *dnf1Δ dnf2Δ* exhibited high sensitivity to pap B and duramycin, a small tetracyclic peptide which binds to PE (Iwamoto *et al.*, 2007) (Figure 1A). These results strongly suggest that Lem3p-Dnf1/2p actively translocates endogenous PS, as well as PE, in the plasma membrane. Lem3p-Dnf1/2p is recycled through the endocytic recycling pathway from the plasma membrane to the TGN via early endosomes (Saito *et al.*, 2004; Takagi *et al.*, 2012), and this pathway is impaired in *cdc50Δ* and *drs2Δ* cells (Saito *et al.*, 2004; Liu *et al.*, 2007). Therefore, the milder sensitivity of *cdc50Δ* and *drs2Δ* to pap B and duramycin may be due to reduced levels of Lem3p-Dnf1/2p in the plasma membrane due to the recycling defect (Figure 1A). Consistent with this idea, mutants defective in the endocytic recycling pathway (e.g., *vps51Δ* and *ypt6Δ*) exhibited a sensitivity to a PE-binding peptide and intracellularly accumulated Dnf2p-green fluorescent protein (GFP) (Takagi *et al.*, 2012).

To investigate the regulatory mechanism and physiological role of PS asymmetry, we screened for multicopy suppressor genes that rescue the pap B sensitivity of the *lem3Δ* mutant. The screen identified *SFK1* (YKL051W), which encodes a conserved plasma membrane protein of the TMEM150/FRAG1/DRAM family with six membrane-spanning domains (Audhya and Emr, 2002; Chung *et al.*, 2015). Overexpression of *SFK1* suppressed growth sensitivity to pap B in *lem3Δ* and *cdc50Δ/drs2Δ* mutants (Figure 1B) and also suppressed the duramycin sensitivity in the *lem3Δ* mutant (Figure 1C). Overexpression of *SFK1* did not suppress growth sensitivity of wild-type cells to a high concentration of pap B or duramycin (Supplemental Figure S1A). To confirm the effect of overexpression of *SFK1* to the exposure of PE in the plasma membrane, we visualized exposed PE using biotinylated Ro 09-0198 peptide (Bio-Ro), an analogue of duramycin, and Alexa Fluor 488–conjugated streptavidin (Emoto *et al.*, 1996; Iwamoto *et al.*, 2004; Figure 1D). We observed fluorescence signals in the bud tip of *lem3Δ* cells (40.3%, *n* > 100 cells), but these signals were significantly reduced in *SFK1*-overexpressing *lem3Δ* cells (5.2%, *n* > 100 cells) (Figure 1E).

Consistent with the effects of overexpression, deletion of *SFK1* aggravated the growth sensitivities to pap B and duramycin in *lem3Δ* cells at lower concentrations of these drugs (Figure 1F). The increase in exposed PE in *lem3Δ sfk1Δ* cells was confirmed by Bio-Ro staining: fluorescence signals were detected all over the plasma membrane in 73.6% of *lem3Δ sfk1Δ* cells (*n* > 100 cells) (Figure 1G). Quantitative analysis of fluorescence signals suggested that ~80% of *lem3Δ sfk1Δ* cells exhibited Alexa Fluor 488 signal in more than 70% of cell surface area (Supplemental Figure S1B). *sfk1Δ* single-mutant cells were not sensitive to either pap B or duramycin at concentrations at which *lem3Δ* mutant cells were sensitive (Figure 1F) and did not expose PE (Figure 1G). These results suggest that Sfk1p, in conjunction with Lem3p-Dnf1/2p, regulates the asymmetric distribution of PS and PE.

FIGURE 1: Identification of *SFK1* as a multicopy suppressor of pap B sensitivity in *lem3*Δ mutant cells. (A) High sensitivity to pap B and duramycin in *lem3*Δ and *dnf1*Δ *dnf2*Δ cells. Cells were cultured in YPDA medium at 30°C, serially diluted, and spotted onto YPDA plates containing 0.5 μg/ml pap B or 5 μM duramycin, followed by incubation for 1.5 d at 30°C. For the pap B assay including (B), YKT2112 (*cdc50*Δ) and YKT2000 (*drs2*Δ) strains were used, whereas YKT249 (*cdc50*Δ) and YKT745 (*drs2*Δ) were used for the duramycin assay. (B) Suppression of pap B sensitivity in flippase mutants by overexpression of *SFK1*. Cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C, and cell growth was examined as in A for 2 d at 30°C. (C) Suppression of duramycin sensitivity in *lem3*Δ by overexpression of *SFK1*. *lem3*Δ cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C, and cell growth was examined as in A for 2 d at

30°C. (D) Staining of exposed PE in the *lem3*Δ mutant with biotinylated Ro-peptide (Bio-Ro) and Alexa Fluor 488–labeled streptavidin. Arrowheads indicate Alexa Fluor 488 signal on the bud tip. Dashed lines indicate cell edges. Scale bar: 5 μm. (E) PE exposure in *lem3*Δ is decreased by overexpression of *SFK1*. Wild-type and *lem3*Δ cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C and then treated with Bio-Ro. Cells were categorized into three patterns: 1) cells with no signal, 2) those with signal at the bud, and 3) those with signal all over the cell surface. Percentages for each pattern are shown (n > 100 cells). (F) Deletion of *SFK1* increases sensitivity to pap B and duramycin in *lem3*Δ. Cell growth was examined as in A for 2-d at 30°C. (G) Deletion of *SFK1* increases exposure of PE in *lem3*Δ. Cells were cultured in YPDA medium at 30°C, and exposed PE was visualized as in D. Dashed lines indicate cell edges. Scale bar: 5 μm. Cells were categorized as in E (n > 100 cells).

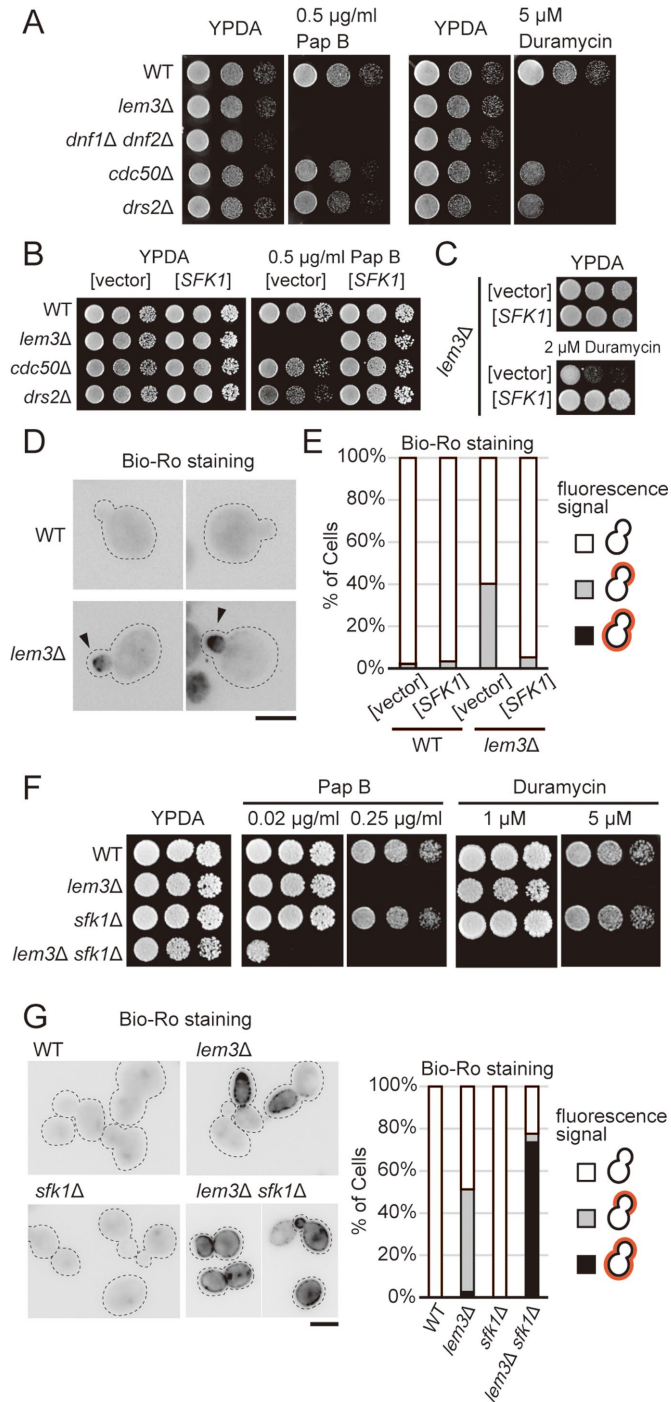## Reduced exposure of PS and PE by *SFK1* overexpression is independent of phosphoinositides, ABC transporters, and phospholipase B

SFK1 was originally isolated as a multicopy suppressor of a temperature-sensitive mutation of *STT4*, which encodes a type III phosphatidylinositol 4-kinase. Sfk1p was implicated in the proper localization of Stt4p to the plasma membrane and Stt4p-mediated PI4P production. However, Sfk1p was not essential for either localization to the plasma membrane or kinase activity of Stt4p (Audhya and Emr, 2002). On the other hand, Ypp1p and Efr3p play essential roles in the assembly of the functional Stt4p complex at the plasma membrane (Baird *et al.*, 2008). Thus, Sfk1p may have another function not related to Stt4p. Overexpression of neither *STT4* nor *MSS4*, which encodes a phosphatidylinositol-4-phosphate 5-kinase (Desrivieres *et al.*, 1998; Homma *et al.*, 1998), rescued growth sensitivity to pap B or duramycin in the *lem3*Δ mutant (Figure 2A). We further examined the involvement of Stt4p in the effect of overexpression of *SFK1*. TMEM150A, a mammalian homologue of Sfk1p, binds to PI4KIIIα via its C-terminal cytoplasmic region (Chung *et al.*, 2015). We constructed the *SFK1ΔC* mutant that lacks the C-terminal cytoplasmic region (Figure 2B), which was properly expressed and localized to the plasma membrane (Figure 2, C and D). Consistent with the results in mammalian cells, overexpression of *SFK1ΔC* hardly suppressed the temperature sensitivity of *stt4-1* (Figure 2E). In contrast, overexpression of *SFK1ΔC* suppressed the pap B sensitivity of *lem3*Δ (Figure 2F). Furthermore, the *stt4-1* mutation did not aggravate sensitivity to pap B in the *lem3*Δ mutant (Figure 2G). These results indicate that the effects of overexpression or deletion of *SFK1* on the PS and PE exposure in the *lem3*Δ mutant are not mediated by Stt4p.

Some ABC transporters act as floppases in both mammalian cells (Aye *et al.*, 2009) and yeast (Pomorski *et al.*, 2003). To investigate the possibility that Sfk1p inhibits flop of PS and PE by down-regulating an ABC transporter, we created a *lem3*Δ *abc*[8]Δ strain deleted for all eight known plasma membrane-localized ABC transporters, *YOR1*, *PDR5*, *SNQ2*, *PDR10*, *PDR11*, *PDR12*, *PDR15*, and *AUS1* (Jungwirth and Kuchler, 2006) (see *Materials and Methods*). The deletion of the ABC transporters did not suppress the pap B sensitivity in *lem3*Δ but did partially suppress the duramycin sensitivity, suggesting that these ABC transporters are not responsible for PS flop but may make some contribution to PE flop (Pomorski *et al.*, 2003). Overexpression of *SFK1* suppressed both pap B and duramycin sensitivity in the *lem3*Δ *abc*[8]Δ mutant (Figure 2H). These results suggest that ABC transporters are not involved in the reductions in PS and PE exposure on overexpression of *SFK1*.

We next examined the possibility that Sfk1p contributes to the degradation of exposed PS and PE. Budding yeast has three phospholipase Bs (PLBs), *PLB1*, *PLB2*, and *PLB3*. Yeast PLBs are localized in the periplasmic space or the cell wall and have hydrolase activity toward PC, PE, PS, and PI (Lee *et al.*, 1994; Merkel *et al.*, 1999). To test the possibility that Sfk1p accelerates the PLB-mediated hydrolysis of exposed PS and PE, we generated the *lem3Δ plb1Δ plb2Δ plb3Δ* mutant and checked its sensitivity to pap B. Deletion of PLBs slightly increased the pap B sensitivity of *lem3Δ*, suggesting that PLBs contribute to the hydrolysis of exposed PS to a minor extent (Figure 2I). Overexpression of *SFK1* suppressed the pap B sensitivity of *lem3Δ plb1Δ plb2Δ plb3Δ*, suggesting that the suppression was not mediated by up-regulation of PLBs.

Phospholipid flipping is decreased by the overexpression of *SFK1*

In the next series of experiments, we investigated whether overexpression of *SFK1* would promote PS and PE flipping. We first examined the localization of other flippases, as *SFK1* overexpression might recruit another flippase to the plasma membrane in *lem3Δ*. Enhanced GFP (EGFP)-tagged Dnf3p, Drs2p, and Neo1p were distributed normally in punctate structures corresponding to the TGN/endosomes (Hua *et al.*, 2002; Wicky *et al.*, 2004), and they were not localized to the plasma membrane in *SFK1*-overexpressing *lem3Δ* cells, suggesting that the effect of *SFK1* overexpression on *lem3Δ* cells is not mediated by these flippases (Figure 3A). We also confirmed that Dnf1p-EGFP and Dnf2p-EGFP were trapped in the ER in *lem3Δ* (Saito *et al.*, 2004) on overexpression of *SFK1* (Figure 3A).

We next determined the internalization of NBD-labeled phospholipids. As previously reported, the bulk of internalization of NBD-PC and -PE, but not -PS, depended on Lem3p-Dnf1/2p (Kato *et al.*, 2002; Saito *et al.*, 2004; Stevens *et al.*, 2008) (Figure 3B). Considering that *lem3Δ* and *dnf1Δ dnf2Δ* were hypersensitive to pap B

amino acid of Sfk1ΔCp is shown in red for comparison. (C) Expression of Sfk1ΔCp. Wild-type cells expressing *SFK1*-3HA or *SFK1ΔC*-3HA under the control of the *GAL1* promoter were cultured in YPGA for 6 h. Total lysates were prepared and separated by SDS–PAGE, followed by immunoblotting with antibody against HA. (D) Sfk1ΔCp is properly localized to the plasma membrane. Wild-type cells expressing Sfk1p-EGFP or Sfk1ΔCp-EGFP under the control of the *GAL1* promoter were cultured in YPGA for 12 h, followed by fluorescence microscopic observation. Scale bar: 5 μm. (E) Overexpression of *SFK1ΔC* hardly suppresses the temperature-sensitive growth of *stt4-1*. Wild-type and *stt4-1* cells transformed with YEplac195, YEp352-*STT4*, YEplac195-*SFK1*, or YEplac195-*SFK1ΔC* were cultured in SDA-U medium at 30°C, serially diluted, and spotted onto SDA-U plates, followed by incubation for 2 d at 30°C or 34°C. (F) Overexpression of *SFK1ΔC* suppresses pap B sensitivity of *lem3Δ*. *lem3Δ* cells transformed with YEplac195, YEplac195-*SFK1*, or YEplac195-*SFK1ΔC* were cultured in SDA-U medium at 30°C, and cell growth was examined as in A for 1 day at 30°C. (G) The *stt4-1* mutation does not affect the sensitivity to pap B in *lem3Δ* cells. Cells were cultured in YPDA medium at 30°C, and cell growth was examined as in A. (H) The effect of *SFK1* overexpression is independent of ABC transporters. *lem3Δ* and *lem3Δ abc8Δ* cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C, and cell growth was examined as in A. (I) The effect of *SFK1* overexpression is independent of phospholipase Bs. *lem3Δ* and *lem3Δ plb1/2/3Δ* cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C, and cell growth was examined as in A.
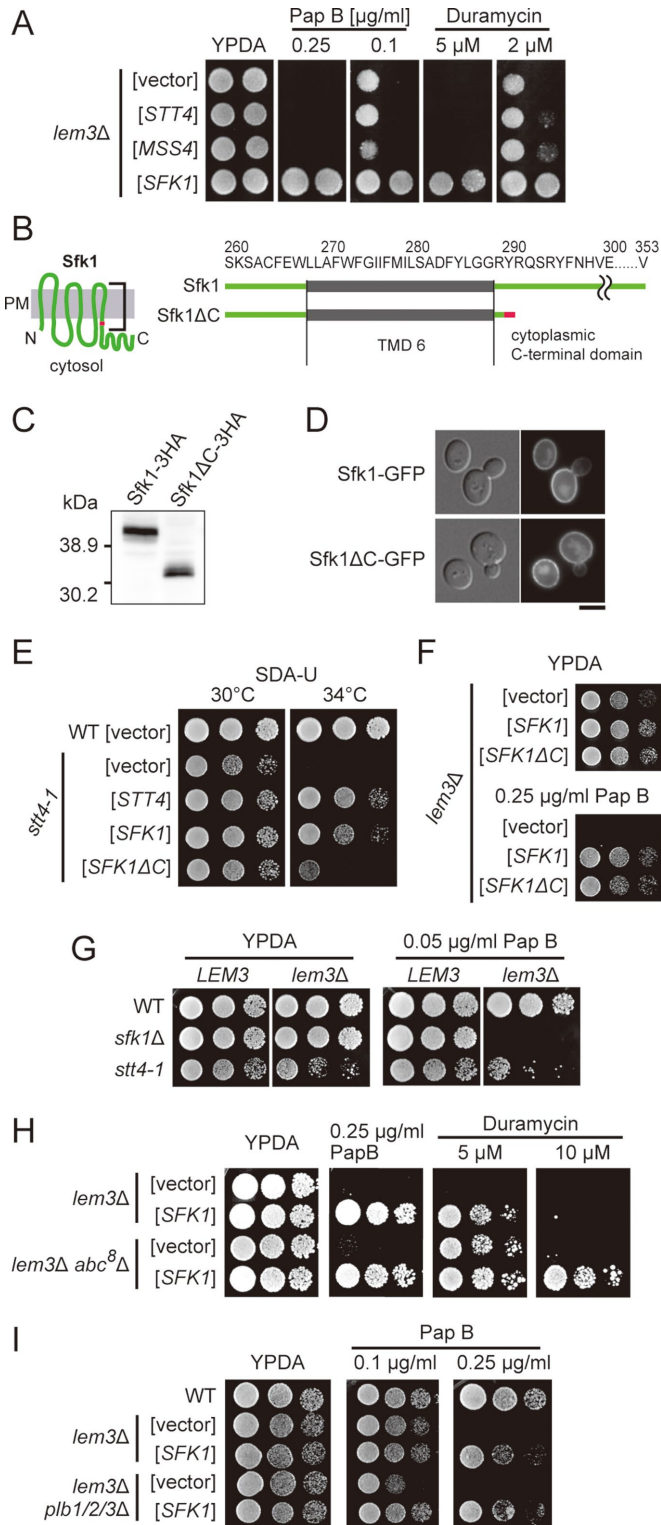
FIGURE 2: The effect of *SFK1* overexpression is independent of phosphoinositides, ABC transporters, and phospholipase B. (A) Pap B and duramycin sensitivity of *lem3Δ* are not suppressed by overexpression of either PI 4-kinase Stt4p or PI(4)P 5-kinase Mss4p. *lem3Δ* cells transformed with YEp24, YEp352-*STT4*, YEp352-*MSS4*, or YEp24-*SFK1* were cultured in SDA-U medium at 30°C, serially diluted, and spotted onto YPDA plates containing 0.25 or 0.1 μg/ml pap B, or 5 or 2 μM duramycin, followed by incubation for 2 d at 30°C. (B) Structures of Sfk1p and Sfk1ΔCp. Sfk1ΔCp was generated by deleting the C-terminal 64 amino acids of Sfk1p. The C-terminal
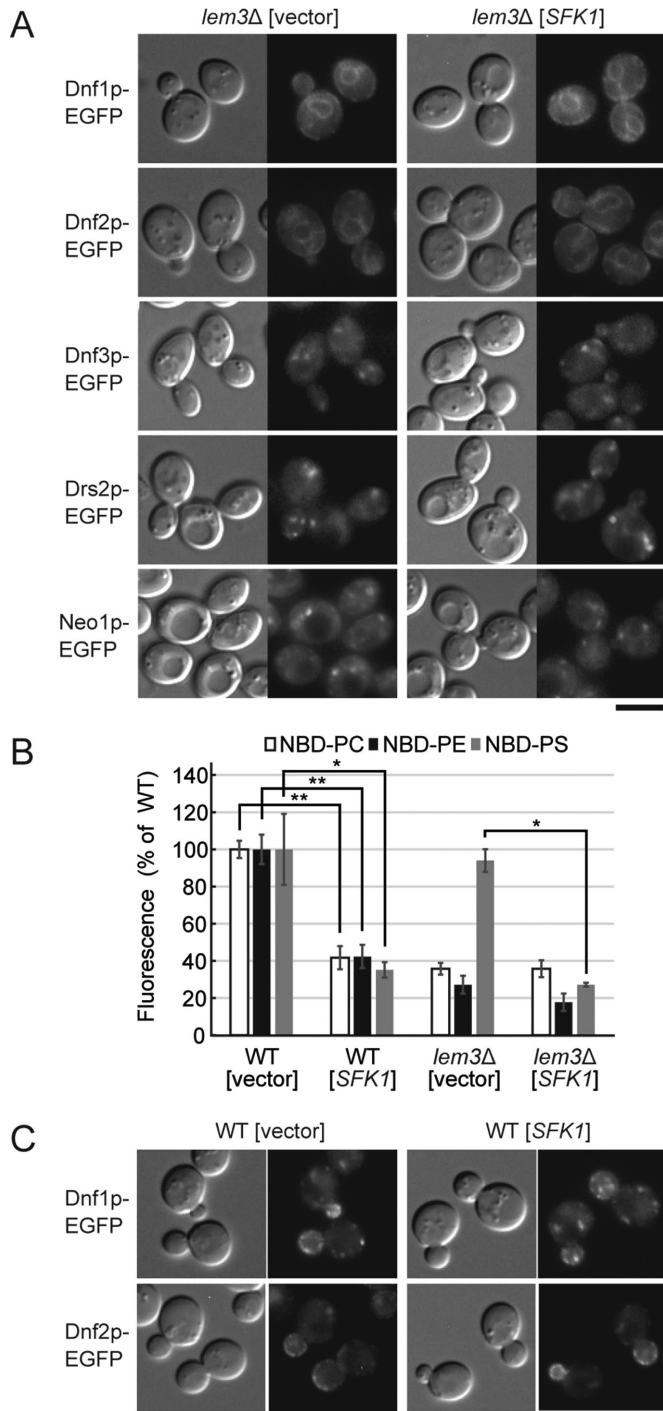
FIGURE 3: Phospholipid flipping is repressed by overexpression of *SFK1*. (A) Overexpression of *SFK1* does not alter the localization of flippases Dnf1p, Dnf2p, Dnf3p, Drs2p, and Neo1p in *lem3Δ*. EGFP-tagged flippase-expressing *lem3Δ* cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C and subjected to fluorescence microscopy. Scale bar: 5 μm. (B) Overexpression of *SFK1* decreases uptake of NBD-PC, -PE, and -PS. Wild-type and *lem3Δ* cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C and labeled with either NBD-PC, -PE, or -PS for 30 min at 30°C. The percentage of average accumulation of NBD-lipids relative to wild-type carrying YEp24 vector is presented with SD of three independent experiments (*$p < 0.05$, **$p < 0.01$; Tukey's test). (C) Overexpression of *SFK1* does not alter the

(Figure 1A), the internalization of NBD-PS may be achieved by an NBD-PS-specific ATP-dependent translocase other than Dnf1/2p (Stevens *et al.*, 2008). Surprisingly, overexpression of *SFK1* in wild-type cells decreased uptake of all three NBD-phospholipids by ~60% (Figure 3B). This was not due to the internalization of Dnf1p or Dnf2p: EGFP-tagged versions of these proteins were localized normally to the plasma membrane at polarized sites in *SFK1*-overexpressing cells (Figure 3C). In *lem3Δ* cells, overexpression of *SFK1* significantly decreased the NBD-PS uptake. These results rule out the possibility that Sfk1p enhances flip of PS and PE and instead suggest that Sfk1p represses phospholipid flipping.

Sfk1p may negatively regulate transbilayer movement of phospholipids in both directions

If Sfk1p represses phospholipid flip, then this repression would be released by deletion of *SFK1*. Consistent with this, deletion of *SFK1* increased uptake of NBD-PC and -PE in wild-type and *lem3Δ* cells (Figure 4A, left panel). In an assay for flip of a fluorescently labeled PS, we employed TopFluor-PS, with properties more similar to PS than NBD-PS (Kay *et al.*, 2012). TopFluor-PS, like NBD-PS, was internalized into cells in a *LEM3*-independent manner (Figure 4A, right panel). Deletion of *SFK1* increased uptake of NBD-PS, but not of TopFluor-PS, in wild-type cells. However, deletion of *SFK1* increased uptake of both NBD-PS and TopFluor-PS in the *lem3Δ* mutant (Figure 4A, right panel). These effects by deletion of *SFK1* were also observed when cells were washed at the last step of the assay with bovine serum albumin (BSA) that extracts NBD-lipids from the outer leaflet of the plasma membrane, suggesting that Sfk1p affects flipping but not the insertion of NBD-lipids into the outer leaflet of the plasma membrane (Figure 4B). Interestingly, the increase in internalization of NBD-PC, -PE, and -PS by *sfk1Δ* occurred even under ATP-depleted conditions (Figure 4C). These results suggest that Sfk1p represses NBD-phospholipid uptake in a manner independent of flippases and other ATP-dependent phospholipid translocases.

To summarize the effect of *SFK1*, PS/PE exposure in *lem3Δ* cells was decreased by overexpression of *SFK1*, but increased by deletion of *SFK1*. These results seemed to be consistent with the hypothesis that Sfk1p, like flippases, enhances phospholipid flipping. However, we obtained the opposite results: internalization of NBD-phospholipids was decreased by overexpression of *SFK1* but increased by deletion of *SFK1*. On the basis of these seemingly contradictory results, we propose an unprecedented function for *SFK1*, namely that Sfk1p restricts transbilayer movement of phospholipids independently of direction. That is, Sfk1p may negatively regulate both flip and flop in the plasma membrane (see *Discussion*). We attempted to determine the effect of Sfk1p on the flop of preinternalized NBD-phospholipids, as described previously (Hanson and Nichols, 2001). However, because overexpression or deletion of *SFK1* interfered with equivalent loading of NBD-phospholipids between the tested strains, we were unable to accurately measure the flop rate.

LEM3 and *SFK1* are required for maintenance of membrane impermeability

Deletion of *SFK1* exacerbated the disruption of phospholipid asymmetry in *lem3Δ* cells. To understand the physiological significance

localization of flippases Dnf1/2p. Dnf1p-EGFP- or Dnf2p-EGFP-expressing wild-type cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C and subjected to fluorescence microscopy. Scale bar: 5 μm.
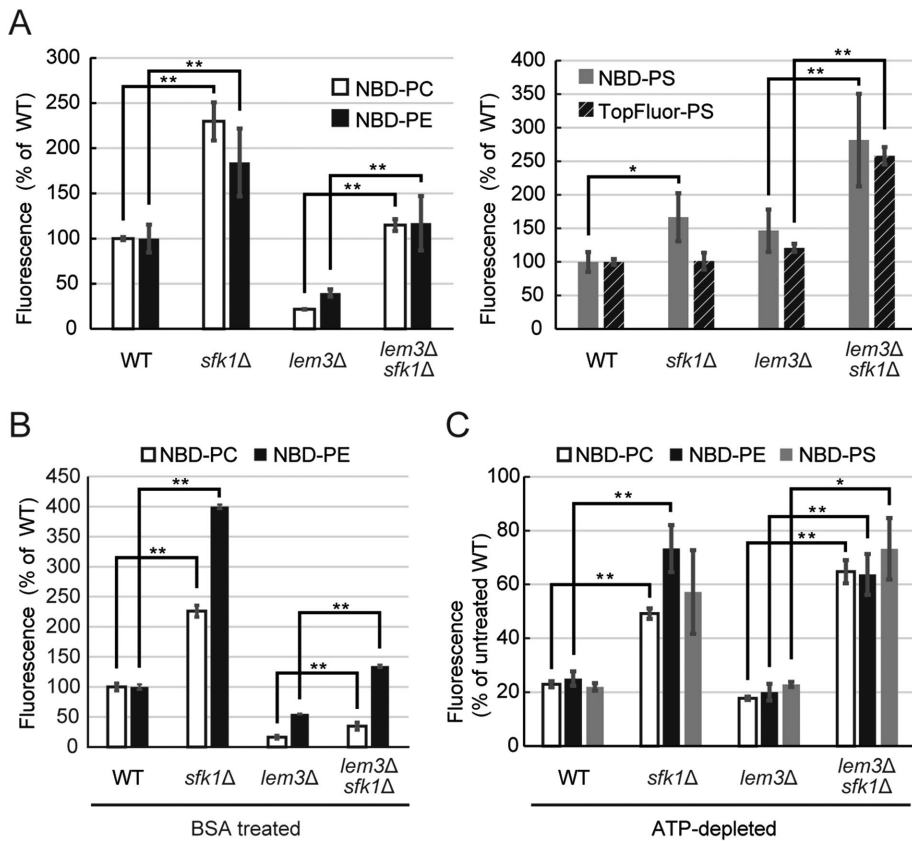
FIGURE 4: Phospholipid flipping is increased by deletion of *SFK1*. Increased uptake of NBD- or TopFluor-labeled phospholipids by deletion of *SFK1*. (A–C) Fluorescent phospholipid uptake assay was performed as described under *Materials and Methods*. Cells were cultured in SD medium at 30°C and labeled with each fluorescent lipid analogue for 30 min at 30°C. For assays with BSA treatment (B), cells were washed with PBS containing 2.5% BSA before flow cytometry. For assays under ATP-depleted conditions (C), cells were preincubated with 1 mM sodium azide for 30 min at 30°C. Percentage average accumulation of fluorescent lipid analogues relative to wild type in the presence of ATP is presented, with SD of three independent experiments (*$p < 0.05$, **$p < 0.01$; Tukey's test).

Emter *et al.* (2002) suggested that ergosterol, a major sterol in budding yeast, restricts the rate of passive diffusion across the plasma membrane. In support of this hypothesis, deletion of *ERG6*, an enzyme in the ergosterol biosynthetic pathway, resulted in high sensitivity to CHX and significant accumulation of rhodamine dye. This effect of *ERG6* on the membrane permeability was independent of *PDR5*, a multidrug ABC transporter acting as an efflux pump. Consistent with the results of Emter *et al.* (2002), *pdr5Δ* and *erg6Δ* cells were highly sensitive to CHX (Figure 5C) but only *erg6Δ* cells exhibited rhodamine accumulation in the absence of ATP, confirming that energy-dependent efflux by Pdr5p was unrelated to membrane permeability (Figure 5D). We observed that *lem3Δ sfk1Δ* cells exhibited high sensitivity to CHX and an ATP-independent accumulation of rhodamine (Figure 5, E and F). Further, the effect of *lem3Δ sfk1Δ* on rhodamine accumulation was additive to the effects of *pdr5Δ* and *erg6Δ* (Figure 5G). These results suggest that membrane permeability is increased by simultaneous deletion of *LEM3* and *SFK1*.

Rhodamine accumulation and sensitivity to cytotoxic compounds, including CHX, did not differ significantly between wild-type and *sfk1Δ* cells but were slightly higher in *lem3Δ* cells and highest in *lem3Δ sfk1Δ* cells (Figure 5, B, E, and F). The pattern of these phenotypes is similar to that of pap B/duramycin sensitivity (Figure 1F), which led us to hypothesize that disruption of phospholipid asymmetry causes an increase in membrane permeability. Interestingly, *lem3Δ sfk1Δ* exhibited a synthetic growth defect with *erg6Δ*, but not *pdr5Δ* (Figure 5H), reflecting the functional relationship between phospholipid asymmetry and ergosterol in the plasma membrane.

Overexpression of *SFK1* suppresses the increase in membrane permeability in *lem3Δ* cells

We found that the *lem3Δ* cells grown in synthetic medium accumulated higher levels of rhodamine than those grown in rich medium (Figure 5F), even under ATP-depleted conditions (Figure 6A). Next, we tested the effect of overexpression of *SFK1* on membrane permeability in the *lem3Δ* mutant. Overexpression of *SFK1* did not change rhodamine uptake in wild-type cells but suppressed the increase in rhodamine accumulation in *lem3Δ* cells, even under ATP-depleted conditions (Figure 6A). Furthermore, the sensitivities of *lem3Δ* cells to CHX and FLC were also suppressed by overexpression of *SFK1* (Figure 6B). This suppression occurred in *lem3Δ cwp1Δ cwp2Δ* triple mutant cells (Supplemental Figure S2B), whereas overexpression of *SFK1* did not suppress sensitivity to calcofluor white (CFW), which binds to cell wall chitin, in the *lem3Δ* cells (Supplemental Figure S2C). These results are consistent with our notion that the increase in membrane permeability is caused by perturbed phospholipid asymmetry in the *lem3Δ* mutant.

of phospholipid asymmetry in the plasma membrane, we investigated the phenotypes of *lem3Δ sfk1Δ* cells. We found that *lem3Δ sfk1Δ* cells were highly sensitive to detergents (Figure 5A). This is consistent with a previous report that the dysfunction of ATP8B1, a P4-ATPase in mammalian cells, leads to high sensitivity to hydrophobic bile salts in liver cells (Paulusuma *et al.*, 2006). Thus, the asymmetric distribution of phospholipids in the plasma membrane may act as a barrier to protect cells against membrane-destabilizing agents. In addition, *lem3Δ sfk1Δ* cells were also highly sensitive to various metal cations and cytotoxic compounds, including hygromycin B, sodium azide, and fluconazole (FLC), which have different intracellular targets (Figure 5B). To exclude the possibility that this drug sensitivity was due to weakened cell wall, we examined effects of mutations in *CWP1* and *CWP2*, which encode cell wall mannoproteins. The *cwp1Δ cwp2Δ* mutant shows a decrease in cell wall thickness and an increase in the permeability to genotoxic agents (Zhang *et al.*, 2008). The *cwp1Δ cwp2Δ* mutations did not confer sensitivity to 5 µM FLC and 10 ng/ml cycloheximide (CHX) on wild-type, *lem3Δ*, and *sfk1Δ* cells, whereas *lem3Δ sfk1Δ cwp1Δ cwp2Δ* cells were sensitive to these drugs (Supplemental Figure S2A). These results raise the possibility that the permeability of the plasma membrane is elevated in *lem3Δ sfk1Δ* cells.
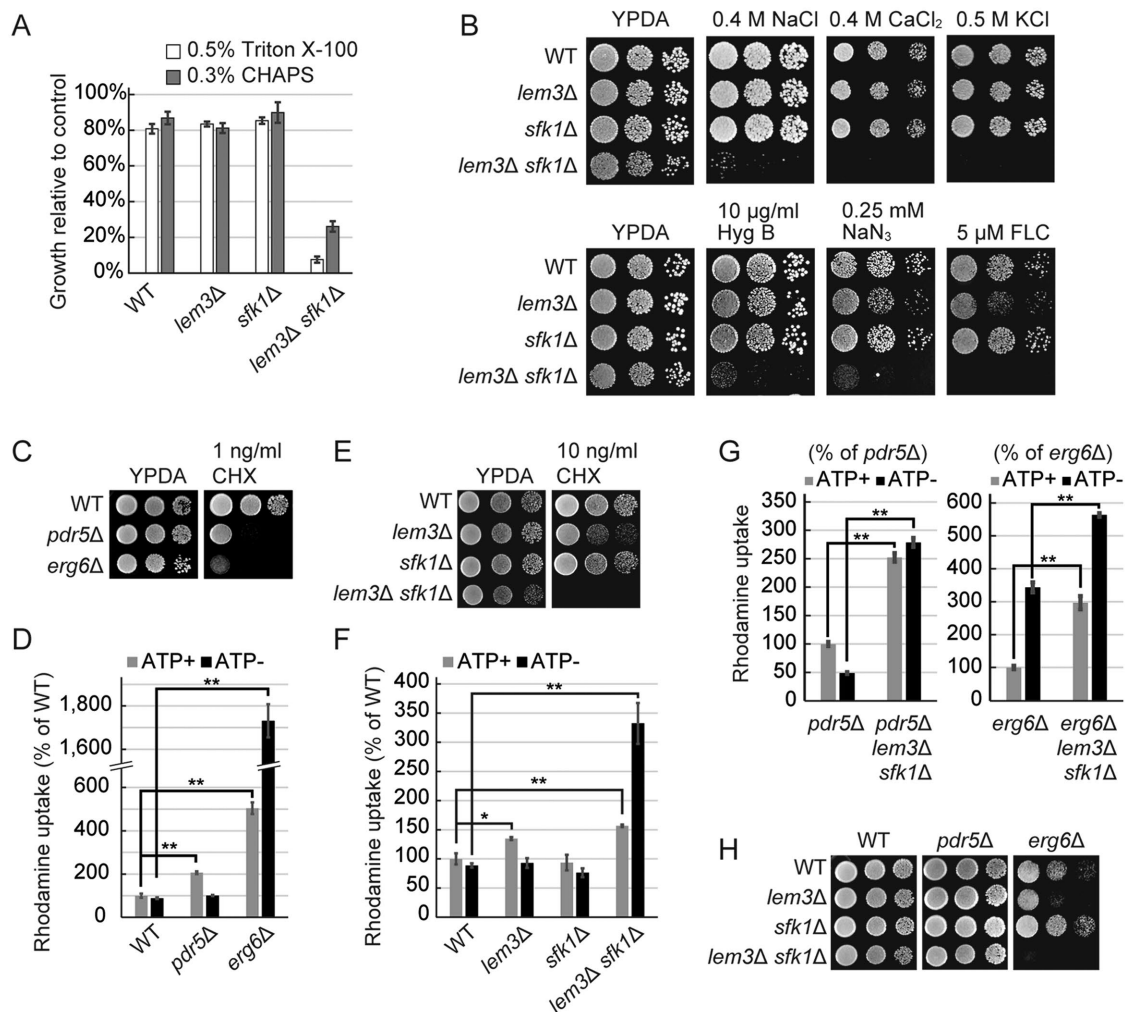
FIGURE 5: Plasma membrane permeability is increased in the *lem3Δ sfk1Δ* double mutant. (A) Sensitivity of *lem3Δ sfk1Δ* to detergents. Cells in exponential phase were grown in YPDA medium with or without detergents for 10 h at 30°C, and optical density at 600 nm was measured. Growth relative to detergent-free control is presented with mean ± SD from three independent experiments. (B) Sensitivity of *lem3Δ sfk1Δ* to metal cations and cytotoxic compounds. Cells were cultured in YPDA medium at 30°C, serially diluted, and spotted onto YPDA plates containing the indicated concentrations of divalent cations and compounds, followed by incubation for 2 d at 30°C. FLC; fluconazole. (C–F) Membrane permeability is increased in *erg6Δ* and *lem3Δ sfk1Δ*. (C, E) Sensitivity to cycloheximide. Cell growth was examined as in B. (D, F) Intracellular accumulation of rhodamine. Cells were cultured in YPDA medium at 30°C, preincubated in SD medium with or without 1 mM sodium azide for 30 min at 30°C, and incubated with rhodamine 6G for 60 min at 30°C. Rhodamine accumulation was measured as described under *Materials and Methods*. Percentage average accumulation of rhodamine relative to wild type in the presence of ATP is presented, with SD from three independent experiments (*$p < 0.05$, **$p < 0.01$; Tukey's test). (G) Elevated membrane permeability in *lem3Δ sfk1Δ* is independent of Pdr5p and Erg6p. Intracellular accumulation of rhodamine was measured as in D and F. Percentage average accumulation of rhodamine relative to *pdr5Δ* or *erg6Δ* in the presence of ATP is presented, with SD from three independent experiments (**$p < 0.01$; Student's *t* test). (H) *lem3Δ sfk1Δ* exhibits a synthetic growth defect with *erg6Δ*. Cell growth was examined as in B.

We next examined the effect of *SFK1* in *erg6Δ* cells. Overexpression of *SFK1* suppressed the pap B sensitivity of *lem3Δ erg6Δ*, suggesting that Sfk1p was functional in the plasma membrane with accumulated intermediates in ergosterol biosynthesis (Figure 6C). However, the sensitivity of *erg6Δ* to CHX was not suppressed by overexpression of *SFK1*, and, consistent with this, *erg6Δ* did not exhibit high sensitivity to pap B (Figure 6D). These results suggest that the increase in membrane permeability in *erg6Δ* is not associated with perturbation of phospholipid asymmetry.

Fluidity of the plasma membrane is increased in the *lem3Δ sfk1Δ* mutant

Membrane permeability to most nonionic substances is correlated to membrane fluidity (Lande *et al.*, 1995). Membrane fluidity can be assayed by fluorescence anisotropy using a fluorescent probe, trimethylammonium diphenylhexatriene (TMA-DPH). TMA-DPH is anchored at the plasma membrane and does not enter into the cell due to its trimethylammonium group (Abe and Hiraki, 2009; Figure 7A). To assess the fluidity of the plasma membrane, we assayed
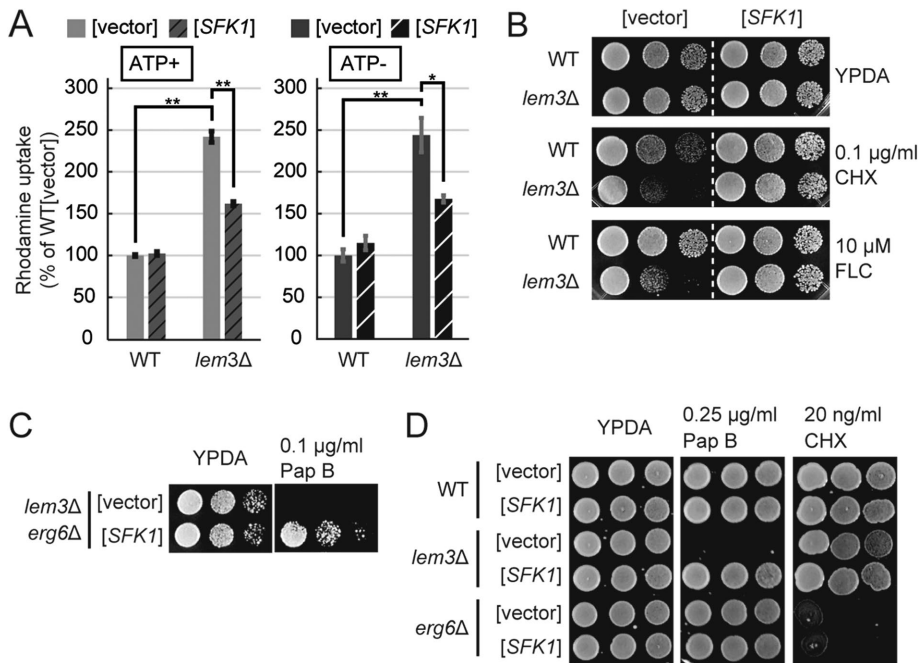
FIGURE 6: Suppression of elevated membrane permeability in *lem3Δ* by overexpression of *SFK1*. (A) Overexpression of *SFK1* reduces the influx of rhodamine into *lem3Δ* cells in an ATP-independent manner. Wild-type and *lem3Δ* cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C, preincubated in SDA-U medium with or without 1 mM sodium azide for 30 min at 30°C, and incubated with rhodamine 6G for 60 min at 30°C. Percentage average accumulation of rhodamine relative to wild-type carrying YEp24 vector is presented, with SD of three independent experiments (*$p < 0.05$, **$p < 0.01$; Tukey's test). (B) Suppression of sensitivity to CHX and FLC in *lem3Δ* by overexpression of *SFK1*. Wild-type and *lem3Δ* cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C, serially diluted, and spotted onto YPDA plates containing 0.1 µg/ml CHX or 10 µM FLC, followed by incubation for 2 d at 30°C. (C) Sfk1p is functional in the *erg6Δ* background. *lem3Δ erg6Δ* cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C, and cell growth was examined as in B. (D) Overexpression of *SFK1* does not suppress the CHX sensitivity in *erg6Δ*. Wild-type, *lem3Δ*, and *erg6Δ* cells transformed with YEp24 or YEp24-*SFK1* were cultured in SDA-U medium at 30°C, and cell growth was examined as in B.
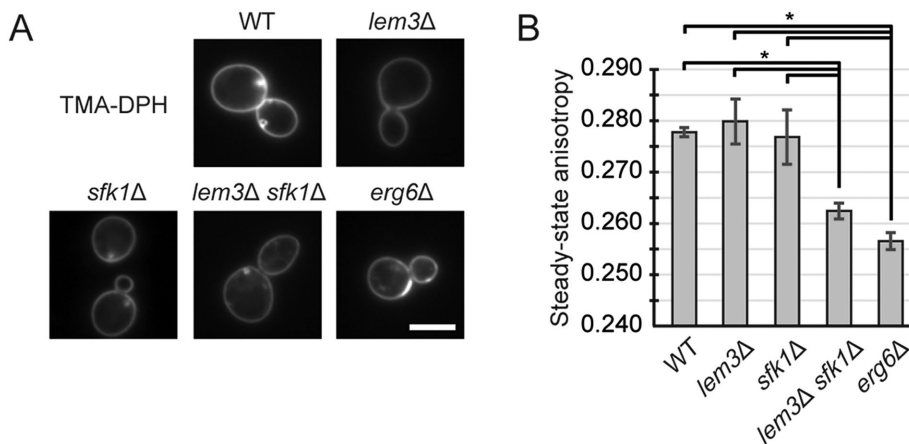

FIGURE 7: Membrane fluidity is increased in the *lem3Δ sfk1Δ* double mutant. (A) TMA-DPH is retained in the plasma membrane. Cells were cultured in SD medium at 25°C and treated with 0.5 µM TMA-DPH. Stained cell suspensions were kept on ice for 45 min and subjected to fluorescence microscopy. Scale bar: 5 µm. (B) Steady-state anisotropy is decreased in the *lem3Δ sfk1Δ* mutant. Cells were cultured in SD medium at 25°C, treated with 0.5 µM TMA-DPH, and subjected to an analysis by spectrophotometry. Measurement of anisotropy was performed as described under *Materials and Methods*. Average of steady-state anisotropy is presented, with SD from three independent experiments (*$p < 0.05$; Tukey's test).

steady-state anisotropy of intact cells with TMA-DPH. Deletion of *ERG6* causes an increase in membrane permeability and membrane fluidity (Emter *et al.*, 2002; Abe and Hiraki, 2009), which is reflected in a decrease in steady-state anisotropy (Abe and Hiraki, 2009; Figure 7B). Steady-state anisotropy was not changed in *lem3Δ* and *sfk1Δ* cells but decreased in *lem3Δ sfk1Δ* as well as in *erg6Δ* cells compared with wild-type cells (Figure 7B). These results suggest that membrane fluidity is also increased in the *lem3Δ sfk1Δ* mutant.

Ergosterol is decreased in the *lem3Δ sfk1Δ* mutant

To examine whether Sfk1p affects membrane lipid composition, we measured phospholipid content in total cellular lipids of mutant cells. Neither deletion nor overexpression of *SFK1* altered the phospholipid composition in wild-type and *lem3Δ* cells (Figure 8A). We next examined ergosterol content in mutant cells. Surprisingly, free ergosterol was decreased in *lem3Δ sfk1Δ* cells compared with wild-type cells (Figure 8B). Because most of the free ergosterol is found in the plasma membrane (Zinser *et al.*, 1993), our results suggest that ergosterol is decreased in the plasma membrane of the *lem3Δ sfk1Δ* mutant, which is consistent with the increased permeability (Figure 5) and increased fluidity (Figure 7) of the plasma membrane in *lem3Δ sfk1Δ* cells. Overexpression of *SFK1* did not change ergosterol levels in wild-type and *lem3Δ* cells (Figure 8B). These results suggest that the decreased level of ergosterol was due to severe defects in phospholipid asymmetry caused by *lem3Δ sfk1Δ* double mutations rather than to a specific effect of Sfk1p on ergosterol levels.

Expression of *SFK1* is up-regulated by some stresses

Growth of cells lacking Lem3p and Sfk1p was sensitive to NaCl (Figure 5B). We checked expressions of Dnf1p, Dnf2p, and Sfk1p under conditions of NaCl stress by fluorescent microscopy and found that expression of Sfk1p-EGFP, but not that of Dnf1/2p-EGFP, was up-regulated by 1 M NaCl (Figure 9A), which was previously reported in a genomewide transcriptome analysis (Yale and Bohnert, 2001). Consistent with this, growth sensitivity to pap B and duramycin in *lem3Δ* cells was suppressed by 0.4 M NaCl, as well as overexpression of *SFK1* (Figure 9B). We confirmed that C-terminally tagged versions of Sfk1p were functional (Supplemental Figure S3).

Up-regulation of Sfk1p-EGFP by NaCl prompted us to investigate whether Sfk1p
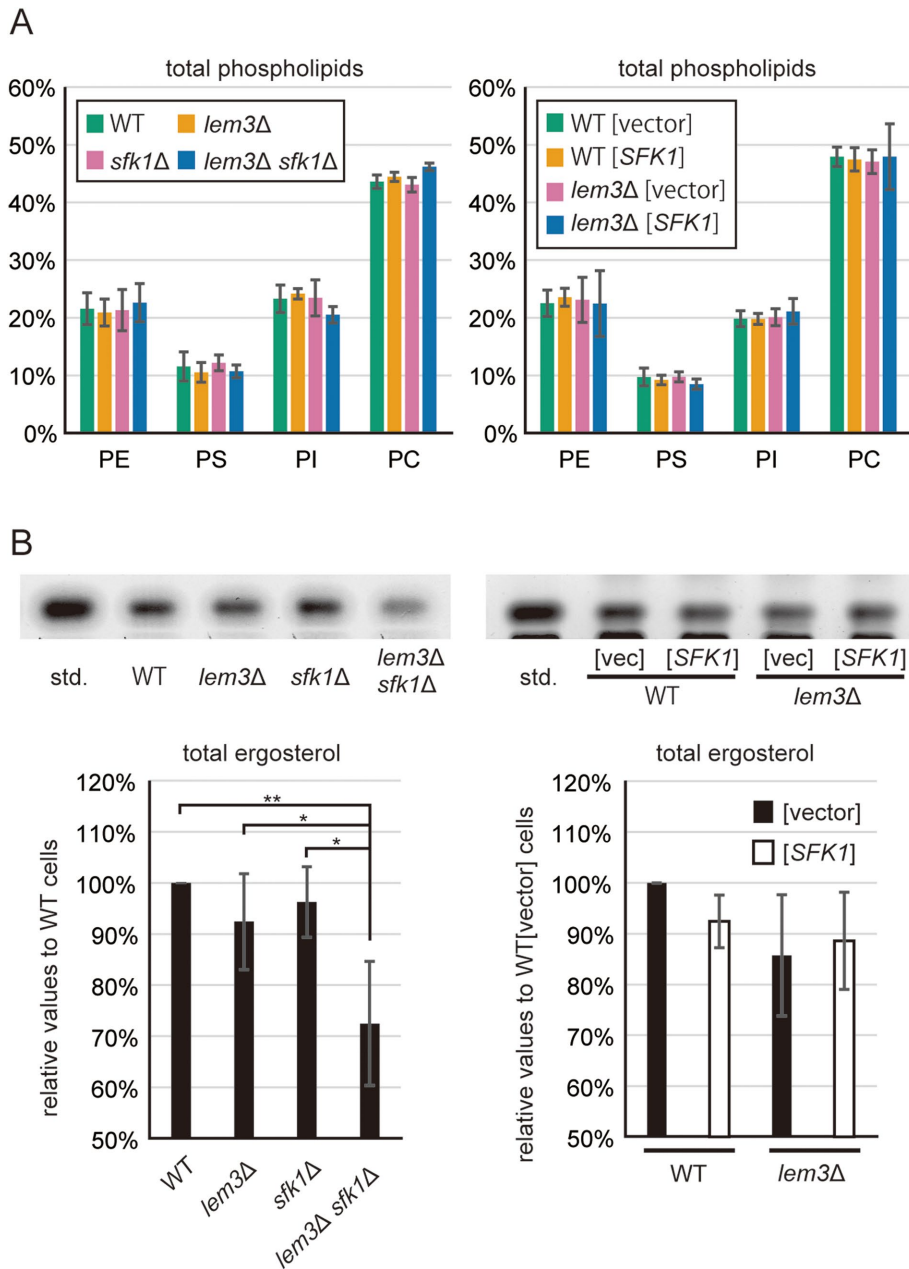
FIGURE 8: Ergosterol is decreased in the *lem3Δ sfk1Δ* mutant. (A) Total phospholipid composition was not altered by either deletion or overexpression of *SFK1*. Cells were grown in YPDA or SDA-U medium, followed by extraction of total cellular lipids. Phospholipid contents were analyzed by TLC as described under *Materials and Methods*. The data present percentages of total phospholipids with SD of three independent experiments. (B) Ergosterol is decreased in *lem3Δ sfk1Δ* cells but not altered in *SFK1*-overexpressing cells. Cells were grown in YPDA or SDA-U medium, followed by extraction of total cellular lipids. Ergosterol contents were analyzed by TLC as described under *Materials and Methods*. Percentage average ergosterol levels relative to wild-type or wild-type carrying YEp24 vector are presented, with SD of six independent experiments (*$p < 0.05$, **$p < 0.01$; Tukey's test). Top panel shows the scanned images of HPTLC plates.

was induced by other stresses, including high or low pH of medium (pH 8.0 and 4.4), high or low growth temperature (37° and 18°C), and high osmolarity (1 M sorbitol). We found that Sfk1p-EGFP was highly induced when low pH medium was combined with a low growth temperature (Figure 9, C and D). This induction was additive with the effect of 1 M NaCl, as confirmed by Western blotting (Figure 9, C–E). We examined whether the up-regulation of Sfk1p by

stresses is mediated by the Hog1p and/or Rim101p pathways, which are involved in the response to high osmolarity and high salinity, respectively (Brewster *et al.*, 1993; Marques *et al.*, 2015). Although 1 M sorbitol had little effect on the expression of Sfk1p-EGFP in our microscopic examination (Figure 9D), the response to 1 M NaCl stress, but not to low pH and low temperature, was dependent on the Hog1p pathway (Figure 9F). Then we tested expression of Sfk1p in 1 M sorbitol and 0.5 M NaCl, which is equivalent to 1 M sorbitol in osmolarity. Sfk1p was induced in 1 M sorbitol as well as in 0.5 M NaCl in a Hog1p-dependent manner, but the induction level was lower compared with 1 M NaCl (Figure 9G).

We monitored growth of the *lem3Δ sfk1Δ* cells at 18°C and pH 4.4, but they grew normally, implying that *SFK1* might be redundant with a gene other than *LEM3* that is required for growth under this condition. Although we do not know how harmful NaCl, low pH, and low temperature are to the cell membrane, Sfk1p may counteract the effects of these stresses by down-regulating phospholipid translocation.

DISCUSSION
Sfk1p is a novel regulator of phospholipid asymmetry in the plasma membrane
We identified Sfk1p as a novel regulator of phospholipid asymmetry in the plasma membrane. Overexpression of *SFK1* repressed phospholipid flipping as well as PS/PE exposure in *lem3Δ*, but deletion of *SFK1* exhibited opposite effects. Although we were unable to measure the flop rate due to a technical difficulty, our results are most consistent with the notion that Sfk1p represses both flip and flop. A model for Sfk1p action is depicted in Figure 10. In the presence of Lem3p-Dnf1/2p, neither deletion nor overexpression of *SFK1* influences phospholipid asymmetry, because the rate of flip by Lem3p-Dnf1/2p always exceeds the rate of flop, resulting in maintenance of normal phospholipid asymmetry. On the other hand, in the absence of Lem3p-Dnf1/2p, PS and PE are exposed on the cell surface due to the reversal of flip/flop rates. This PS/PE exposure is suppressed by overexpression of *SFK1* and enhanced by deletion of *SFK1* through repression and disinhibition of flip and flop, respectively.

It remains to be elucidated how Sfk1p represses flip and flop. Importantly, the increase in NBD-lipid uptake on deletion of *SFK1* occurred in an ATP-independent manner (Figure 4C). Sfk1p could affect flip/flop by inhibiting a phospholipid scramblase, but no scramblase has yet been identified in yeast. One possibility is that Sfk1p is a novel regulatory factor of spontaneous flip and flop, which are thought to occur rarely in the plasma membrane due to
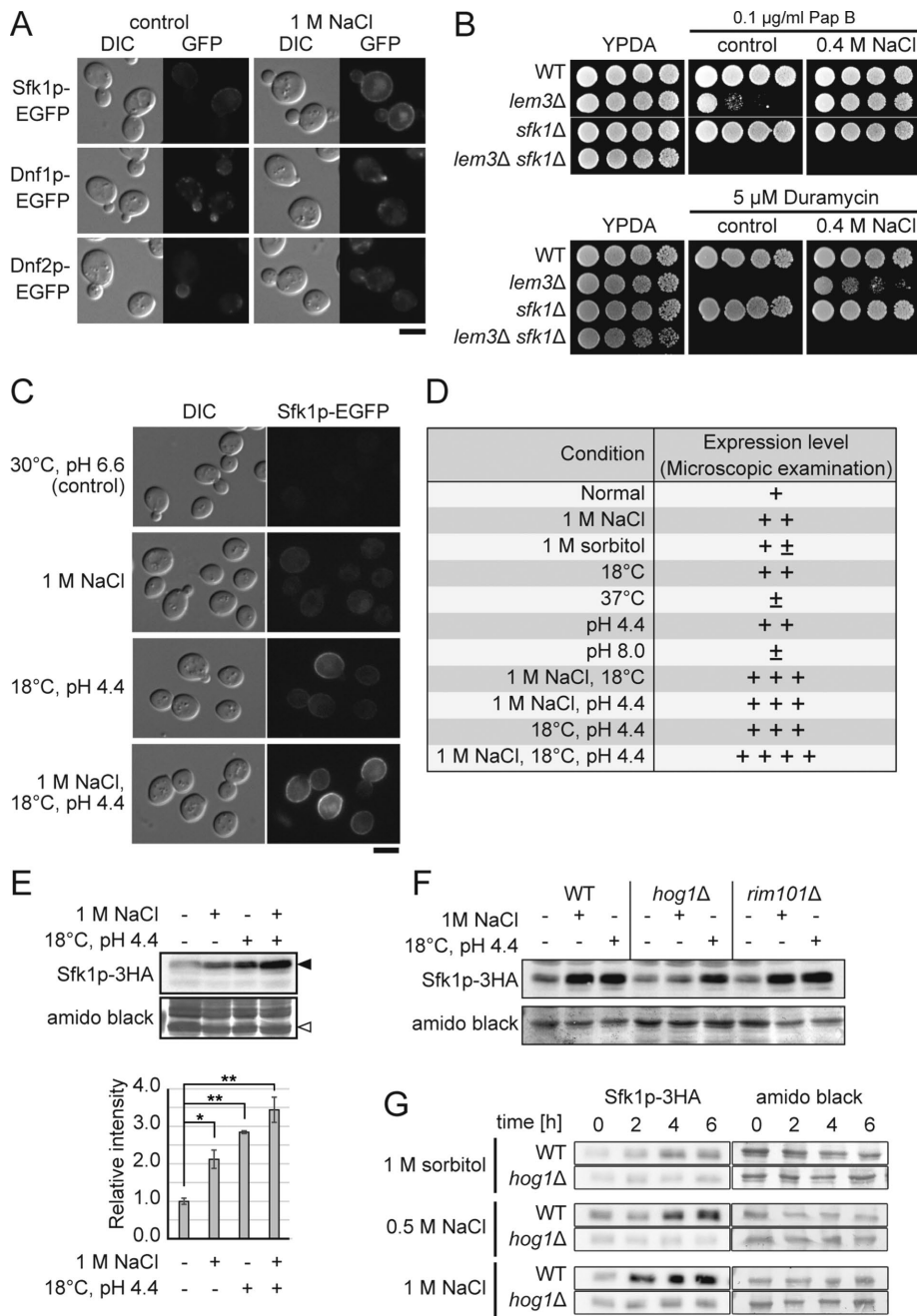
FIGURE 9: Expression of *SFK1* is up-regulated by several stresses. (A) Up-regulation of Sfk1p-EGFP by 1M NaCl. Sfk1p-EGFP-, Dnf1p-EGFP-, or Dnf2p-EGFP-expressing wild-type cells were cultured in YPDA medium with or without 1 M NaCl at 30°C for 6 h in the exponential phase and subjected to fluorescence microscopy. Scale bar: 5 μm. (B) Suppression of sensitivity to pap B and duramycin in *lem3Δ* by NaCl. Cells were cultured in YPDA medium at 30°C, serially diluted, and spotted onto YPDA plates containing 0.1 μg/ml pap B or 5 μM duramycin with or without 0.4 M NaCl, followed by incubation for 2 d at 30°C. (C–E) Up-regulation of Sfk1p by cold temperature and low pH. (C) Sfk1p-EGFP was induced by low pH and cold temperature. Wild-type cells expressing *SFK1*-EGFP were cultured in YPDA medium under the indicated conditions for >12 h in the exponential phase and then subjected to fluorescence microscopy. Scale bar: 5 μm. (D) Expression of Sfk1p-EGFP was microscopically examined under various conditions, and relative intensity of GFP fluorescence is shown. (E) Wild-type cells expressing *SFK1*-3HA were cultured in YPDA as in C. Total lysates were prepared and separated by SDS–PAGE, followed by immunoblotting with antibody against HA. Amido black-stained membrane is shown as a loading control. Expression levels were determined by the ratio of Sfk1p-3HA (closed arrowhead) to a band in amido black control (open arrowhead). The band intensity was quantified with ImageJ. The data present average expression levels relative to control with SD of three independent experiments ($*p < 0.05$, $**p < 0.01$; Tukey's test).

the tight packing of lipid bilayer and the amphipathic nature of phospholipids. In model membranes, packing defects at the phase-transition temperature were implicated in rapid flip and flop (John *et al.*, 2002). Sterols, sphingolipids, and saturated acyl chains, all of which are abundant in the plasma membrane, are key components of tightly packed membranes (Bigay and Antonny, 2012). Sfk1p might be involved in the maintenance of packing order via regulation of the distribution, content, or interactions of these key lipids. The decrease of ergosterol in the *lem3Δ sfk1Δ* mutant may suggest that Sfk1p is involved in the regulation of ergosterol levels, but neither the *sfk1Δ* single mutation nor overexpression of *SFK1* affected ergosterol contents, suggesting that the decrease in ergosterol is rather caused by the defects in phospholipid asymmetry.

Our results suggested that the effect of *SFK1* on the phospholipid asymmetry is *STT4* independent (Figure 2, A and E–G), but the interaction between these two proteins implies that the regulation of phospholipid asymmetry by Sfk1p is functionally relevant to PI4P signaling. Recent work showed that the C-terminal region of TMEM150A, a mammalian homologue of Sfk1p, also binds to PI4-kinase type IIIα (Chung *et al.*, 2015). The conserved motif in the TMEM150/FRAG1/DRAM family is located in the region including six transmembrane domains except for the C-tail (Chung *et al.*, 2015), suggesting that the interactions with PI4 kinases are specific to Sfk1p/TMEM150A.

The role of phospholipid asymmetry in passive membrane permeability of the plasma membrane

Low membrane permeability is a very important property for the barrier function of the plasma membrane, specifically, the entry of exogenous toxic molecules. Our results revealed that normal phospholipid asymmetry is required for the impermeability of the plasma membrane; this is a novel
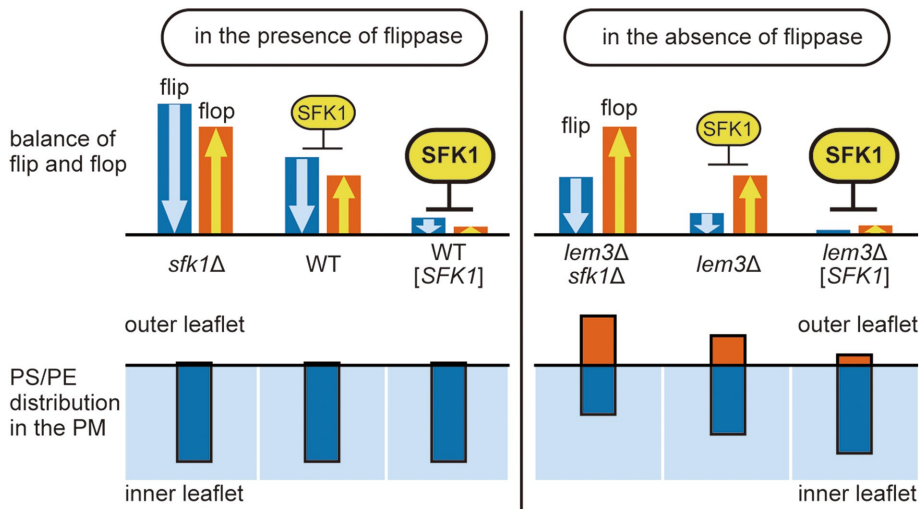
FIGURE 10: Model for the Sfk1p action. Top panel, Blue and orange bars represent the rate of flip and flop, respectively. In this model, Sfk1p represses both flip and flop in a dose-dependent manner. Bottom panel, Blue and orange bars represent the distribution of PS/PE in the inner and outer leaflet of the plasma membrane, respectively. Deletion or overexpression of *SFK1* affects phospholipid asymmetry in the absence of flippase (right) but not in the presence of flippase (left).

located in the plasma membrane, preferentially flips NBD-PC (Takatsu *et al.*, 2014). Mutations in ATP8B1 cause progressive familial intrahepatic cholestasis type 1 (PFIC1), a severe liver disease (Bull *et al.*, 1998; Klomp *et al.*, 2004). Interestingly, a PFIC1-type mutation in ATP8B1 leads to high sensitivity to the detergent action of hydrophobic bile salts in mouse (Paulusma *et al.*, 2006), as well as a reduction in NBD-PC flipping (Takatsu *et al.*, 2014). Thus, the perturbation of PC asymmetry may also bring about imbalance of ergosterol homeostasis, resulting in high membrane permeability and high detergent sensitivity in *lem3Δ sfk1Δ* cells.

Sfk1p is up-regulated by various stresses

We found that the protein level of Sfk1p was up-regulated by high osmolarity, low temperature, and low pH (Figure 9). The additive effects of these stresses on *SFK1* expression suggest multiple regulatory pathways for *SFK1*, including the Hog1p pathway. Meanwhile, the protein level of Dnf1/2p was not altered by these stresses (Figure 9A and Supplemental Figure S4). The flippase activity of Dnf1/2p is regulated by serine/threonine kinases Fpk1/2p (Nakano *et al.*, 2008), and Fpk1/2p are regulated in turn by Ypk1p and Gin4p kinases and sphingolipids (Roelants *et al.*, 2010, 2015). Therefore, Dnf1/2p may be regulated at the level of flippase activity in response to environmental stresses.

Changes in pH and salinity of medium alter the lipid composition of the plasma membrane (Turk *et al.*, 2007). Growth temperature and carbon source also influence the composition of membrane lipid and fatty acid (Klose *et al.*, 2012). Our findings suggest that the inhibition of phospholipid transbilayer movement, and thus regulation of phospholipid asymmetry, plays a role in adaptation to stress conditions (Figure 11B). Investigation of the mechanisms the cell uses to adapt to these stresses will reveal novel function of phospholipid asymmetry, beyond the maintenance of low membrane permeability.
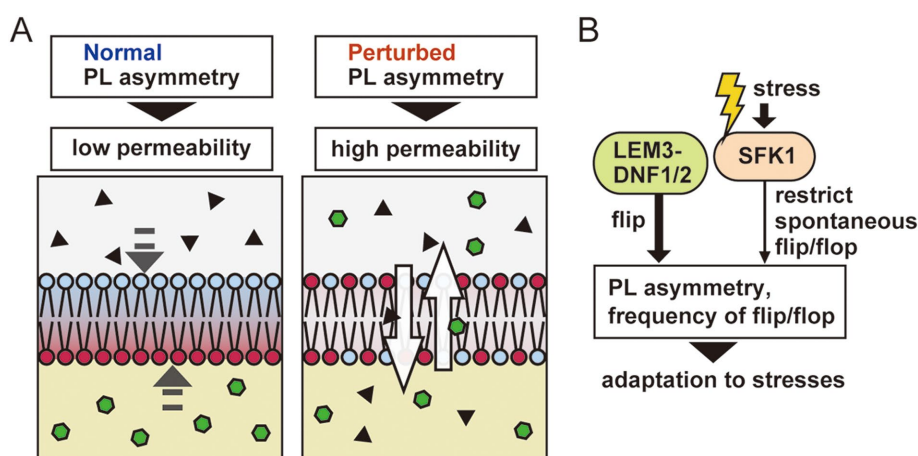
finding regarding the physiological significance of the phospholipid asymmetry (Figure 11A). Loss of phospholipid asymmetry leads to a decreased level of ergosterol, which may account for the increased permeability of the plasma membrane in the *lem3Δ sfk1Δ* mutant.

Phospholipid asymmetry of the plasma membrane seems to play an important role for ergosterol homeostasis. In the *lem3Δ sfk1Δ* mutant, free ergosterol may be decreased due to increased esterification for storage or decreased synthesis. A recent study reported an association between PS and cholesterol: PS is required for retaining cholesterol in the cytosolic leaflet of the plasma membrane (Maekawa and Fairn, 2015). Although a similar interaction of PS with ergosterol has yet to be demonstrated, the abnormal distribution of ergosterol caused by the perturbation of PS asymmetry may trigger imbalance of ergosterol homeostasis in *lem3Δ sfk1Δ* cells. On the other hand, PC is also a potential substrate of Lem3p-Dnf1/2p (Kato *et al.*, 2002; Pomorski *et al.*, 2003). ATP8B1, a mammalian P4-ATPase

FIGURE 11: Physiological role and regulation of phospholipid asymmetry. (A) Contribution of phospholipid asymmetry to the barrier function of the plasma membrane. Perturbation of phospholipid asymmetry causes high permeability in the plasma membrane. (B) Sfk1p may be involved in the adaptation to environmental stresses by regulating spontaneous flip and flop.

MATERIALS AND METHODS

Chemicals, media, and genetic techniques

Chemicals were purchased from Wako Pure Chemicals Industries (Osaka, Japan) unless otherwise indicated. Papuamide B was from the collection of R. Andersen. Duramycin was purchased from Sigma-Aldrich (St. Louis, MO). Standard genetic manipulations and plasmid transformation of yeast were performed as described previously (Elble, 1992; Guthrie and Fink, 2002). Yeast strains were cultured in rich yeast extract peptone dextrose adenine (YPDA) medium containing 1% yeast extract (Difco Laboratories, Detroit, MI), 2% Bacto peptone (Difco), 2% glucose, and 0.01% adenine, or synthetic dextrose (SD) medium containing 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco),

0.5% ammonium sulfate, 2% glucose, and the required amino acids or nucleic acid bases. Strains carrying *URA3*-harboring plasmids were cultured in SD medium containing 0.5% casamino acids (Difco), 0.03% tryptophan, and 0.01% adenine (SDA-U). For serial dilution spot assays, cells were grown to early log phase in appropriate medium and adjusted to a concentration of 1.0×10^7 cells/ml. From 10-fold dilutions, 4-µl drops were spotted onto appropriate plates.

Strains and plasmids

Yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Standard molecular biological techniques (Sambrook and Russell, 2001) were used for the construction of plasmids, PCR amplification, and DNA sequencing. PCR-based procedures were used to construct gene deletions and gene fusions with EGFP, 3HA, and the *GAL1* promoter (Longtine *et al.*, 1998). All constructs produced by the PCR-based procedure were verified by colony PCR to confirm that the replacement occurred at the expected locus. Sequences of PCR primers are available on request.

A multicopy plasmid carrying *SFK1ΔC*, in which the C-terminal 64 amino acids of Sfk1p were deleted, was constructed as follows. Two DNA fragments, which contained the *SFK1* promoter region and *SFK1ΔC* coding region (1374 base pairs) and the *SFK1* terminator region (478 base pairs), respectively, were PCR amplified with the genomic DNA of wild-type cells (YKT38) as a template. These DNA fragments were ligated into the YEplac195 vector plasmid. Schemes detailing the construction of plasmids and DNA sequences of nucleotide primers are available on request.

To create the *lem3Δ abc*[8]*Δ* strain in which all eight known plasma membrane-localized ABC transporters were deleted, the *lem3Δ::HIS3MX6 yor1Δ::HIS3MX6 pdr5Δ::HphMX6 snq2Δ::KanMX6* strain (*lem3Δ abc*[3]*Δ*) was first generated by crossing the *lem3Δ::HIS3MX6 pdr5Δ::HphMX6 snq2Δ::KanMX6* strain with the *yor1Δ::HIS3MX6 pdr5Δ::HphMX6 snq2Δ::KanMX6* strain. *lem3Δ::HIS3MX6* and *yor1Δ::HIS3MX6* were confirmed by colony PCR. Next, *PDR10, PDR11, PDR12, PDR15,* and *AUS1* were individually disrupted in the *lem3Δ abc*[3]*Δ* strain using the *TRP1* disruption cassette. Crosses among these five strains, followed by sporulation and tetrad dissection, yielded strains containing combinations of the disrupted alleles, which were confirmed by colony PCR. This procedure was performed repeatedly, ultimately yielding the *lem3Δ abc*[8]*Δ* strain.

Isolation of multicopy suppressors of papuamide B sensitivity in the *lem3Δ* mutant

The *lem3Δ* strain was transformed with a yeast genomic DNA library constructed in the multicopy plasmid YEp24 (Carlson and Botstein, 1982). A total of 12,000 transformants were selected on SDA-U plates at 30°C and then replica-plated onto YPDA plates containing 0.25 µg/ml pap B. The transformants that contained *LEM3* on the plasmid were identified by colony PCR and eliminated. Plasmids were recovered from the remaining 51 transformants. Restriction enzyme digestion of the plasmids indicated that 21 different clones had been isolated, and they were grouped into seven different genomic regions by DNA sequencing. Fragment subcloning revealed that *SFK1, PDR3, PDR1, PLB3, YLR278C, KIC1,* and *HLR1* were responsible for the suppression. Among these genes, *SFK1* was the strongest suppressor of pap B sensitivity in the *lem3Δ* mutant. *PDR1, YLR278C, KIC1,* and *HLR1* exhibited modest suppression, whereas *PDR3* and *PLB3* exhibited weak suppression (Supplemental Figure S5).

Microscopic observations

Cells were observed using a Nikon ECLIPSE E800 microscope (Nikon Instec, Tokyo, Japan) equipped with an HB-10103AF super-high-pressure mercury lamp and a 1.4 numerical aperture 100× Plan Apo oil immersion objective lens (Nikon Instec) with appropriate fluorescence filter sets (Nikon Instec) or differential interference contrast optics. Images were acquired using a cooled digital charge-coupled devicecamera (C4742-95-12NR; Hamamatsu Photonics, Hamamatsu, Japan) and AQUACOSMOS software (Hamamatsu Photonics). GFP-tagged proteins were observed in living cells, which were grown to early to midlogarithmic phase, harvested, and resuspended in SD medium. For detection of GFP fluorescence, cells were immediately observed using a GFP bandpass filter set. Observations were compiled from the examination of at least 100 cells.

Staining of phosphatidylethanolamine with biotinylated Ro09-0198 peptide

Staining of PE in the outer leaflet of the plasma membrane was performed using the biotinylated Ro09-0198 peptide (Bio-Ro) as described (Iwamoto *et al.*, 2004; Saito *et al.*, 2007), with the following modifications. Bio-Ro, which was prepared essentially as described (Aoki *et al.*, 1994), was a gift from M. Umeda (Kyoto University, Japan). Cells were cultured in YPDA or SDA-U medium at 18°C for 12 h, until reaching midlogarithmic phase. A 1-ml culture of cells at a cell density of OD_{600}/ml = 0.4–0.6 was harvested, resuspended in 20 µl of YPDA containing 40 µM Bio-Ro, and incubated for 3 h on ice. The cells were washed once with phosphate-buffered saline (PBS) and fixed with 5% formaldehyde in PBS for 1 h at room temperature. After two washes with spheroplast buffer (1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.4), cells were resuspended in 100 µl of spheroplast buffer containing 1 mg/ml Zymolyase 100T (Seikagaku Kogyo, Tokyo, Japan) and 0.2 µl of β-mercaptoethanol (from 14.2 M stock solution), followed by incubation for 30 min at 30°C. To visualize Bio-Ro, the cells were washed three times with PBS and then incubated in PBS containing 5 µg/ml Alexa Fluor 488–streptavidin (Invitrogen, Carlsbad, CA) for 1 h at room temperature. After five washes with PBS, cells were observed using a fluorescein isothiocyanate (FITC) filter set.

Lipid preparation

1-Palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (NBD-PC), 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphoethanolamine (NBD-PE), 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphoserine (NBD-PS), 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-*sn*-glycero-3-phospho-L-serine (TopFluor PS), and dioleoylphosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids (Alabaster, AL). To prepare large unilamellar vesicles, lipids were mixed at 40 mol% NBD- or TopFluor-phospholipid and 60 mol% DOPC. Chloroform was removed by evaporation followed by vacuum desiccation. Desiccated phospholipids were solubilized in SD medium; the mixture was passed seven times through a LiposoFast-Basic stabilizer (Avestin, Ottawa, Canada), equipped with 0.1-µm filters to produce evenly sized vesicles, containing a 1 mM total concentration of lipids.

Internalization of fluorescence-labeled phospholipids into yeast cells

Fluorescently labeled phospholipid internalization experiments were performed as described (Kato *et al.*, 2002). Briefly, cells were grown to early logarithmic phase in SD or SDA-U medium at 30°C. After dilution to 0.35 OD_{600}/ml, cells were preincubated in SD with or without

Strain[a]	Mating type	Genotype	Reference or source
YKT38	a	Wild-type (*lys2-801 ura3-52 his3Δ-200 trp1Δ-63 leu2Δ-1*)	Misu *et al.* (2003)
YKT249	a	*cdc50Δ::HIS3MX6*	Misu *et al.* (2003)
YKT715	a	*lem3Δ::TRP1*	Saito *et al.* (2004)
YKT745	α	*drs2Δ::KanMX6*	Saito *et al.* (2004)
YKT770	α	*lem3Δ::TRP1 DRS2-EGFP::KanMX6*	Saito *et al.* (2004)
YKT771	α	*DNF1-EGFP::KanMX6*	Saito *et al.* (2004)
YKT773	α	*lem3Δ::TRP1 DNF1-EGFP::KanMX6*	Saito *et al.* (2004)
YKT918	a	*dnf1Δ::HphMX6 dnf2Δ::KanMX6*	This study
YKT921	α	*DNF2-EGFP::KanMX6*	Furuta *et al.* (2007)
YKT923	α	*lem3Δ::TRP1 DNF2-EGFP::KanMX6*	Furuta *et al.* (2007)
YKT927	α	*lem3Δ::TRP1 DNF3-EGFP::KanMX6*	This study
YKT930	α	*lem3Δ::TRP1 NEO1-EGFP::KanMX6*	This study
YKT1161	a	*lem3Δ::TRP1 pdr5Δ::KanMX6*	This study
YKT1162	a	*pdr5Δ::KanMX6*	This study
YKT1709	a	*stt4-1 ade8 TRP1*	Yoshida *et al.* (1994)
YKT2000	α	*drs2Δ::HphMX4 TRP1*	This study
YKT2112	a	*cdc50Δ::HphMX4 TRP1*	This study
YKT2117	a	*sfk1Δ::KanMX6*	This study
YKT2118	a	*lem3Δ::HIS3MX6 sfk1Δ::KanMX6*	This study
YKT2119	a	*erg6Δ::KanMX6 TRP1*	This study
YKT2120	α	*SFK1-EGFP::KanMX6*	This study
YKT2121	α	*lem3Δ::CaLEU2 plb1Δ::HIS3MX6 plb2Δ::KanMX6 plb3Δ::HphMX6*	This study
YKT2122	a	*lem3Δ::HIS3MX6 stt4-1 TRP1*	This study
YKT2123	α	*lem3Δ::HIS3MX6 yor1Δ::HIS3MX6 pdr5Δ::HphMX6 snq2Δ::KanMX6 pdr10Δ::TRP1 pdr11Δ::TRP1 pdr12Δ::TRP1 pdr15Δ::TRP1 aus1Δ::TRP1*	This study
YKT2124	a	*yor1Δ::HIS3MX6 pdr5Δ::HphMX6 snq2Δ::KanMX6*	This study
YKT2125	a	*lem3Δ::HIS3MX6 yor1Δ::HIS3MX6 pdr5Δ::HphMX6 snq2Δ::KanMX6*	This study
YKT2126	a	*lem3Δ::TRP1 sfk1Δ::HphMX6*	This study
YKT2129	a	*lem3Δ::TRP1 pdr5Δ::KanMX6 sfk1Δ::HphMX6*	This study
YKT2130	α	*lem3Δ::HISMX6 erg6Δ::TRP1*	This study
YKT2131	a	*sfk1Δ::HphMX6 erg6Δ::TRP1*	This study
YKT2132	α	*lem3Δ::HIS3MX6 sfk1Δ::HphMX6 erg6Δ::TRP1*	This study
YKT2133	a	*SFK1-3HA::KanMX6*	This study
YKT2134	a	*hog1Δ::KanMX6 SFK1-3HA::HIS3MX6*	This study
YKT2135	a	*rim101Δ::KanMX6 SFK1-3HA::HIS3MX6*	This study
YKT2136	a	*HIS3MX6::P$_{GAL1}$-SFK1-3HA:: KanMX6*	This study
YKT2137	a	*HIS3MX6::P$_{GAL1}$-SFK1ΔC-3HA:: KanMX6*	This study
YKT2138	a	*HIS3MX6::P$_{GAL1}$-SFK1-GFP:: KanMX6*	This study
YKT2139	a	*HIS3MX6::P$_{GAL1}$-SFK1ΔC-GFP:: KanMX6*	This study
YKT2140	a	*cwp1Δ::HIS3MX6 cwp2Δ::KanMX6*	This study
YKT2141	α	*lem3Δ::TRP1 cwp1Δ::HIS3MX6 cwp2Δ::KanMX6*	This study
YKT2142	a	*sfk1Δ::hphMX6 cwp1Δ::HIS3MX6 cwp2Δ::KanMX6*	This study
YKT2143	a	*lem3Δ::TRP1 sfk1Δ::hphMX6 cwp1Δ::HIS3MX6 cwp2Δ::KanMX6*	This study
YKT2144	α	*lem3Δ::TRP1 SFK1-EGFP::KanMX6*	This study
YKT2145	a	*lem3Δ::TRP1 SFK1-3HA::KanMX6*	This study

[a]YKT strains are isogenic derivatives of YEF473 (Longtine *et al.*, 1998). Only relevant genotypes are described.

TABLE 1: *Saccharomyces cerevisiae* strains used in this study.

Plasmid	Characteristics	Reference or source
YEp24	*URA3 2μm*	Carlson and Botstein (1982)
pKT1724 [YEp24-*SFK1*]	*SFK1 URA3 2μm*	This study
pKT1721 [YEp352-*STT4*]	*STT4 URA3 2μm*	A gift from Yoshikazu Ohya (The University of Tokyo)
pKT1722 [YEp352-*MSS4*]	*MSS4 URA3 2μm*	A gift from Yoshikazu Ohya
pKT2193 [YEplac195-*SFK1*]	*SFK1 URA3 2μm*	This study
pKT2194 [YEplac195-*SFK1ΔC*]	*SFK1ΔC URA3 2μm*	This study

TABLE 2: Plasmids used in this study.

1 mM sodium azide for 30 min at 30°C and incubated for 30 min at 30°C with vesicles containing 40% NBD- or TopFluor-phospholipid and 60% DOPC at a final concentration of 50 μM. Cells were then suspended in SD containing 20 mM sodium azide. Flow cytometry of fluorescently labeled cells was performed on a FACSCalibur cytometer using the CellQuest software (BD Biosciences, San Jose, CA).

Labeling with TMA-DPH and measurement of steady-state anisotropy

Labeling with TMA-DPH was performed as described (Abe *et al.*, 2009). Briefly, cells were grown in SD medium at 25°C at 0.5–1.0 OD_{600}. The cells (2 OD_{600} units) were collected, washed twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0), and labeled with TMA-DPH (Invitrogen, Carlsbad, CA) at 25°C for 10 min in the dark. After washing twice, the cells were subjected to fluorescence microscopy or analysis by spectrophotometry. Before the microscopic observation, TMA-DPH-stained cells were kept on ice for 45 min, and the cells were observed using a UV-1A filter set.

For measurement of the steady-state anisotropy of TMA-DPH, an RF-5300PC spectrofluorometer (Shimadzu Co., Kyoto, Japan) was used. TMA-DPH-stained cells were resuspended in TE buffer at 0.25 OD_{600} and placed on ice until use. The cell suspensions were warmed at 30°C for 5 min prior to measurements. The excitation and emission wavelengths were 361 and 432 nm, respectively. Calculation of the steady-state anisotropy was performed as described (Abe *et al.*, 2009).

Rhodamine uptake assay

Cells were grown to early logarithmic phase in YPDA or SDA-U medium at 30°C. After dilution to 0.5 OD_{600}/ml, cells were preincubated in SD with or without 1 mM sodium azide for 30 min at 30°C and incubated for 1 h at 30°C with rhodamine 6G (Sigma-Aldrich) at a final concentration of 50 μM. Cells were then subjected to flow cytometry analysis on a FACSCalibur cytometer using the CellQuest software.

Lipid analysis

Total lipids were extracted basically by the Bligh and Dyer method (Bligh and Dyer, 1959). Cells were grown in 100–200 ml of YPDA or SDA-U medium to 0.8–1.0 OD_{600}/ml at 30°C. The cells were collected and resuspended in 3.8 ml of chloroform-methanol-0.1 M HCl/0.1 M KCl (1:2:0.8) and lysed by vortexing with glass beads for 1 min. Then 1.0 ml each of chloroform and 0.1 M HCl/0.1 M KCl were added, followed by centrifugation, isolation of the lipid containing phase, and evaporation of the solvent. The extracted lipids were dissolved in appropriate volume of chloroform. Total phospholipids were determined by phosphorus assay (Rouser *et al.*, 1970).

For phospholipid analysis, samples were subjected to thin-layer chromatography (TLC) on a TLC plate (Silica gel 60; Merck Millipore), with the solvent system chloroform/methanol/acetic acid/formic acid (50:30:4.5:6.5). After migration, plates were dried and sprayed with primuline solution (Sigma-Aldrich). Fluorescent spots of PE, PS, PI, and PC were scraped into glass tubes and subjected to lipid extraction and phosphorus assay as described above.

For ergosterol analysis, the samples containing 20 nmol phosphate were subjected to TLC on a HPTLC plate (Silica gel 60; Merck Millipore), with the solvent system hexane/diethyl ether/acetic acid (80:20:1) (Dodge and Phillips, 1967). After migration, plates were dried and sprayed with a 10% (wt/vol) cupric sulfate solution in 8% (wt/vol) orthophosphoric acid. Plates were heated in an oven at 180°C for 20 min. Plates were scanned with a CanoScan 8800F image scanner (Canon, Tokyo, Japan), and the acquired images were quantified with ImageJ (https://imagej.nih.gov/ij/).

Immunoblot analysis

Immunoblot analysis was performed as described previously (Misu *et al.*, 2003). For SDS–PAGE of Sfk1p-3HA, samples were heated at 95°C for 5 min before loading. The mouse anti-HA (HA.11) monoclonal antibody (Babco, Richmond, CA) was used at 1:1000 dilution.

Statistical analysis and data reproducibility

Significance for Figure 5G was determined using a two-sided Student's *t* test. Significance for all other figures was determined by a one-way analysis of variance with a Tukey's test. All data are representatives of at least three independent experiments of biological replicates that gave reproducible results.

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