Plasma membrane aminoglycerolipid flippase function is required for signaling competence in the yeast mating pheromone response pathway

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ABSTRACT The class 4 P-type ATPases (“flippases”) maintain membrane asymmetry by translocating phosphatidylethanolamine and phosphatidylserine from the outer leaflet to the cytosolic leaflet of the plasma membrane. In Saccharomyces cerevisiae, five related gene products (Dnf1, Dnf2, Dnf3, Drs2, and Neo1) are implicated in flipping of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. In MATa cells responding to α-factor, we found that Dnf1, Dnf2, and Dnf3, as well as the flippase-activating protein kinase Fpk1, localize at the projection (“shmoo”) tip where polarized growth is occurring and where Ste5 (the central scaffold protein of the pheromone-initiated MAPK cascade) is recruited. Although viable, a MATa dnf1Δ dnf2Δ dnf3Δ triple mutant also displayed marked reduction in its ability to respond to α-factor, which we could attribute to inefficient recruitment of Ste5 to the plasma membrane due to severe mislocalization of the cellular phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate pools. Thus proper remodeling of plasma membrane aminoglycerolipids and phosphoinositides is necessary for efficient recruitment, stability, and function of the pheromone signaling apparatus.

INTRODUCTION

In eukaryotic cells, the plasma membrane (PM) is a complex structure containing a plethora of lipid species (Harkewicz and Dennis, 2011). The lipids appear to be organized spatially in two major ways: phase

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Abbreviations used: GFP, green fluorescent protein; KD, kinase-dead mutant; Lact, mammalian lactadherin; MAPK, mitogen-activated protein kinase; mCherry, color variant of monomeric Discosoma red fluorescent protein; PH, pleckstrin homology domain; PM, plasma membrane; PtdEth, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdIns(4,5)P2, phosphatidylinositol-4,5-bisphosphate; PtdIns(4)P, phosphatidylinositol-4-phosphate; PtdIns(4,5)P3, phosphatidylinositol-4,5-bisphosphate; PtdSer, phosphatidylserine.

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and signal transduction (Toker, 2002). The head groups of inner-leaflet glycerophospholipids recruit proteins that contain lipid-binding domains of the appropriate specificity (DiNitto et al., 2003; Hurley, 2006; Moravcevic et al., 2012; Stahelin et al., 2014), as well as other classes of proteins containing polybasic elements (Heo et al., 2006; Yeung et al., 2008), membrane-insertion motifs (Antonny, 2011), or curvature-inducing scaffolds (Kozlov et al., 2014).

Because of the influence of the PM lipids exert on other cellular processes, how the localization and dynamics of inner-leaflet lipids are controlled is a biological question of substantial interest. In budding yeast (Saccharomyces cerevisiae), genetic analysis has implicated PM lipid asymmetry in several aspects of vesicle-mediated protein trafficking (Gall et al., 2002; Hua et al., 2002; Pomorski et al., 2003; Hachiro et al., 2013). Moreover, PtdIns(4,5)P2 is enriched at highly localized sites, such as at the bud neck (Bertin et al., 2010) and endocytic patches (Sun and Drubin, 2012). Similarly, a concentration of PtdSer appears to be important for proper localization of the small GTPase Cdc42 and its role in the development of cell polarity (Fairn et al., 2011). Conversely, locally amassed PtdEth seems to be required for activation of the GTPase-activating proteins (GAPs) that down-regulate Cdc42-GTP (Saito et al., 2007) and/or for the guanine nucleotide dissociation inhibitor (GDI)–mediated dissociation of Cdc42 from the PM (Das et al., 2012).

Bilayer asymmetry, in general, and the amount of any given inner-leaflet lipid, in particular, are maintained by active inward translocation (“flopping”) concomitant with similar translocation outward of exoleaflet lipids (“flopping”; Daleke, 2003; van Meer, 2011). These movements are necessary in the face of continual exocytic vesicle insertion and endocytic vesicle removal, which act to scramble leaflet lipid content. In eukaryotes, inward translocation of PtdEth and PtdSer is catalyzed by members of a subfamily (class 4) of the P-type ATPases dubbed flipases (Daleke, 2007; Tanaka et al., 2011; Sebastian et al., 2012; Lopez-Marques et al., 2013). The S. cerevisiae genome encodes five flipases: Dnf1, Dnf2, Dnf3, Drs2, and Neo1 (Catty et al., 1997). Paralogues Dnf1 (1571; value in parentheses indicates number of residues) and Dnf2 (1612) localize primarily in the PM, whereas Dnf3 (1656), Drs2 (1355), and Neo1 (1151) are confined mainly to intracellular membranes (Daleke, 2007; Muthusamy et al., 2009). Exit of Dnf1 and Dnf2 from the ER and their insertion and function in the PM require their association with a smaller escort protein, Lmn3/Ros3 (414; Kato et al., 2002; Noji et al., 2006); related proteins Crf1 (393; Saito et al., 2004) and Cdc50 (391; Misu et al., 2003; Takahashi et al., 2012) serve the same function for Dnf3 and Drs2, respectively. Such a factor has not yet been identified for Neo1 (Barbosa et al., 2010). Mutations in certain of the 14 human homologues of the yeast flipases (Lopez-Marques et al., 2011) are causes of several inherited diseases (Folmer et al., 2009; van der Mark et al., 2013).

The role of flipases in polarized growth is particularly intriguing because the PM undergoes rapid and highly directional expansion. One known control on PM flipase function is exerted by stimulatory phosphorylation by the Ser/Thr protein kinase Fpk1. The FPK1 gene was first identified by loss-of-function mutations synthetically lethal with a cdc50Δ mutation (which inactivates flipase Drs2), suggesting that Fpk1 action is needed for optimal activity of the remaining flipases. Indeed, although yeast cells lacking Fpk1 (and its parologue, Fpk2/Kin82) are viable and did not have any change in flipase abundance or localization, they had a decreased ability to internalize fluorescently labeled PtdEth and PtdSer (Nakano et al., 2008). In vitro purified Fpk1 directly phosphorylates four of the five flipases (not Neo1), with a marked preference for Dnf1 and Dnf2 (Nakano et al., 2008). Fpk1 is, in turn, phosphorylated and inactivated by Ypk1 (Roelants et al., 2010), a protein kinase whose function is up-regulated in response to membrane stress (Roelants et al., 2011).

One biological stimulus in yeast that elicits highly polarized growth in haploid cells is exposure to mating pheromone (Segall, 1993). It was reported that PtdEth becomes detectable in the outer leaflet (Iwamoto et al., 2004) and PtdSer becomes concentrated in the inner leaflet (Fairn et al., 2011) at the leading edge of the projection (shmoo tip) that forms in pheromone-treated cells. Similarly, we demonstrated that PtdIns(4,5)P2 becomes enriched at the same location and is required for efficient recruitment of the mitogen-activated protein kinase (MAPK) scaffold protein Ste5 and maximal MAPK signaling (Garrenton et al., 2010). Hence we sought to determine whether plasma membrane lipid asymmetry and the flipases necessary to maintain it have any role in these processes. We examined the localization of the flipases and the flipase-regulating protein kinase Fpk1 upon pheromone exposure, used genetic analysis to determine that these proteins are indeed necessary for optimal pheromone response, and interrogated both wild-type and mutant cells using genetic, biochemical, and cell biological methods to determine how flipase action contributes to signal propagation.

RESULTS
Flipase localization during pheromone response
As an initial means to determine whether any flipase might contribute to PM lipid dynamics necessary for cell morphogenesis and/or signaling during pheromone response, we examined the subcellular location of these enzymes. Toward this end, we successfully fused a fluorescent marker, either green fluorescent protein (GFP; Tsien, 1998) or mCherry (Shaner et al., 2004), in-frame to the C-terminal end of the chromosomal open reading frame for each of the four members of the yeast flipase family believed to reside in, or be trafficked into and out of, the PM (Daleke, 2007; Tanaka et al., 2011; Sebastian et al., 2012). Using appropriate complementation tests, we found that these constructs, each of which is expressed at its endogenous level from its native promoter, retained full biological function (Rockwell et al., 2009; Rockwell and Thorner, unpublished results). The fifth flipase, Neo1, resides exclusively in intracellular membranes (Wicky et al., 2004), and we found that its distribution (Supplemental Figure S1A) and level (Supplemental Figure S1B) were unaffected by pheromone treatment.

In naive cells, Dnf1-GFP and Dnf2-GFP resided in small puncta congruent with the PM disbursed reasonably uniformly around the cell periphery, especially in the PM of the bud (and, occasionally, at the bud neck), whereas the bulk of the Dnf3-GFP and Drs2-mCherry appeared to reside in cortical vesicles, in agreement with prior work indicating that Dnf3 mainly localizes to post-Golgi secretory vesicles and Drs2 in the trans-Golgi cisternae (Hua et al., 2002; Natarajan et al., 2004; Hanamatsu et al., 2014; Figure 1A, left). Strikingly, within 1 h after exposure to pheromone, Dnf1-GFP, Dnf2-GFP and Dnf3-GFP were highly concentrated in the PM at the shmoo tip, whereas Drs2-mCherry remained in the Golgi compartment (Figure 1A, middle). By 90 min after exposure to pheromone, although Dnf3-GFP still showed a bias at the shmoo tip, it seemed to reside mainly in endocytic vesicles, whereas Dnf1-GFP and Dnf2-GFP persisted in the PM at the shmoo tip and Drs2-mCherry remained in the Golgi body (Figure 1A, right). These conclusions derived from standard epifluorescence microscopy were confirmed using confocal fluorescence microscopy (Supplemental Figure S2). Furthermore, immunoblot analysis of these proteins (or corresponding integrated constructs C-terminally tagged with a c-myc epitope) indicated that there was no pronounced change in the level of these proteins during the time course of pheromone treatment (Supplemental Figure S3). Thus...
Flippases are essential to induce a pheromone response

To test whether the observed relocalizations are functionally significant and not merely the consequence of the highly directional secretion and cell growth that occurs during projection formation, we tested whether null alleles in one or more of these genes had any effect on the ability of the cells to respond to pheromone. This analysis was possible because, aside from Neo1, which is an essential gene (Prezant et al., 1996), cells carrying complete deletions of any of the other four flippases, and even of any three together, are viable, although a $dnf1\Delta dnf2\Delta dnf3\Delta drs2\Delta$ quadruple mutant is inviable (Hua et al., 2002), indicating a significant degree of overlap in the physiological roles of these proteins. As a first means to assess pheromone responsiveness, we examined the frequency of shmoo formation in cultures of various combinations of $dnf1\Delta$, $dnf2\Delta$, $dnf3\Delta$, and $drs2\Delta$ null alleles. As anticipated, no single deletion mutant displayed any significant defect in its efficiency of shmoo formation upon $\alpha$-factor treatment (Figure 2A), in keeping with the apparent redundancies in localization and function of these flippases (Daleke, 2007; Sebastian et al., 2012). Indeed, even double-mutant combinations exhibited little or no reduction in shmoo formation or only a very modest (twofold) decrease, in the case of $dnf3\Delta drs2\Delta$ cells (Figure 2A). In contrast, and in agreement with a largely shared function, we found that two triple mutants, $dnf1\Delta dnf3\Delta drs2\Delta$ and especially $dnf1\Delta dnf2\Delta dnf3\Delta$, had a marked reduction in their ability to form a shmoo (Figure 2A).

The defect in shmoo formation exhibited by the two triple mutants we examined could arise from a defect in cell morphogenesis or from an inability to mount a pheromone response of any sort. To distinguish between these possibilities, we also monitored pheromone response by an independent assay, namely the ability to induce expression of a pheromone-responsive reporter gene, $FUS1$ (Trueheart et al., 1987). For this purpose, a single copy of a $FUS1$ promoter-driven lacZ construct was integrated at the $FUS1$ locus in the two triple mutants and in otherwise isogenic wild-type cells as a control. As observed for shmoo formation, even 60 min after pheromone treatment, the $dnf1\Delta dnf3\Delta drs2\Delta$ cells and especially the $dnf1\Delta dnf2\Delta dnf3\Delta$ had a marked reduction in their ability to form a shmoo (Figure 2A).

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Ypk1 (Roelants et al., 2010) and Fpk2 are subject to inhibitory phosphorylation by the protein kinase (Figure 3A). In this same regard, we described before that Fpk1 and Fpk2 for their optimal activity (Nakano et al., 2008), then even wild-type cells (i.e., with a full complement of flippases) may have a defect in pheromone response if they lack these flipase-deficient cells to respond to pheromone. Hence we sought to determine what aspect of signal initiation or propagation is impaired in flippase-deficient cells.

**Ste5 level is dramatically reduced in dnf1Δ dnf2Δ dnf3Δ cells**

All the initial steps of the mating pheromone response pathway take place in, or on the cytosolic surface of, the PM (Merlini et al., 2013). Activation of the pathway in MATa cells is triggered by binding of α-factor pheromone to its cognate G protein-coupled receptor, Ste2 (Blumer et al., 1988). It was reported recently in Drosophila that there was a marked reduction in the amount of an olfactory receptor (Or67d) inserted into the PM in the cilia on specific olfactory neurons that sense a male-specific pheromone in a mutant lacking the apparent fly orthologue (dATP8B) of mammalian flippase ATP8B1 (Ha et al., 2014), whose apparent homologues in S. cerevisiae are Dnf1 and Dnf2 (Folmer et al., 2004; van der Mark et al., 2013). To test whether the amount of Ste2 delivered to the PM was affected in either of the yeast flippase triple mutants, we examined Ste2 localization in cells expressing a C-terminally mCherry-tagged version of the Ste2(7K-to-R) allele, which we demonstrated previously is functional but has markedly retarded ubiquitin-dependent endocytosis (Ballon et al., 2006), making it easier to score the steady-state level of receptor in the PM. We found that delivery of Ste2(7K-to-R)-mCherry to the PM was not defective in either dnf1Δ dnf2Δ dnf3Δ mutant compared to Ste4–Ste18 overexpression (unpublished data); in fact, the level of Ste2(7K-to-R)-mCherry appeared to be somewhat higher in the dnf1Δ dnf2Δ dnf3Δ mutant than in the corresponding control, consistent with the retardation of endocytosis described previously for cells deficient in both Dnf1 and Dnf2, especially at lower temperatures (Pomorski et al., 2003). Hence lack of PM-localized Ste2 cannot account for the inability of the multiply flippase-deficient cells to respond to pheromone.

Focusing here, first, on the underlying cause of the inability of the dnf1Δ dnf2Δ dnf3Δ cells to respond to pheromone, we took advantage of the fact that ectopic overexpression of the pheromone-receptor-associated Gβγ complex (Ste4–Ste18) is known to induce all pathway outputs even in the absence of pheromone (Cole et al., 1990; Whiteway et al., 1990). Hence, if the signaling defect lies at or downstream of Gβγ action, then the dnf1Δ dnf2Δ dnf3Δ mutant should be unresponsive to Ste4–Ste18 overexpression. However, we found no statistically significant difference between the dnf1Δ dnf2Δ dnf3Δ cells and otherwise isogenic controls cells in shmoo formation (Figure 4B) or any other readout of pheromone response (unpublished data) when Gβγ was overexpressed. Therefore we concentrated our attention on the factors that act at the nexus between receptor activation and the function of Gβγ.

If flippase activity is critical for induction of pheromone response and the flippases require phosphorylation and activation by Fpk1 and Fpk2 for their optimal activity (Nakano et al., 2008), then even wild-type cells (i.e., with a full complement of flippases) may have a defect in pheromone response if they lack these flipase-activating protein kinases. Consistent with this view, loss of either Fpk1 alone nor Fpk2 alone had any significant effect on the efficiency of shmoo formation by otherwise wild-type cells, whereas an fpk1Δ fpk2Δ double mutant exhibited a pronounced decrease (Figure 3A). In this same regard, we described before that Fpk1 and Fpk2 are subject to inhibitory phosphorylation by the protein kinase Ypk1 (Roelants et al., 2010). Hence we used high-level overexpression of Ypk1 as an independent means to impede Fpk1 and Fpk2 activity in otherwise wild-type cells and found that this tactic also caused a statistically significant drop in the frequency of shmoo formation, whereas neither empty vector nor a catalytically crippled mutant Ypk1(K376A; Roelants et al., 2004) had any obvious effect (Figure 3B). Collectively these findings indicated that flippase activation contributes in some way to the competence of the cells to respond to pheromone. Hence we sought to determine what aspect of signal initiation or propagation is impaired in flippase-deficient cells.

**FIGURE 2**: Deletion of multiple flippases with overlapping function impairs pheromone response. (A) Exponentially growing cultures of cells of the indicated genotype (see Table 1) were exposed to 10 μM α-factor for 1.5 h and examined by microscopy. Values are the mean ± SD of three independent experiments. (B) WT (YLG32), dnf1Δ dnf3Δ dnf2Δ (YEL04), and dnf1Δ dnf2Δ (YEL03) cells expressing a copy of a FUS1prom-lacZ::URA3 reporter (pSB286) integrated at the FUS1 locus were grown to mid-exponential phase, collected, resuspended in either YPD or YPD plus 10 μM α-factor, and, after 60 min, assayed for galactosidase activity. Values are the mean ± SD from three independent experiments.
the steady-state level of Ste5 was markedly lower in the dnf1Δ dnf2Δ dnf3Δ cells. (A) WT (YELO17) and dnf1Δ dnf2Δ dnf3Δ cells expressing Ste2(7K-to-R)-mCherry from the STE2 promoter at the STE2 locus were grown to mid–exponential phase in YPD and examined by fluorescence microscopy. Scale bar, 10 μm. (B) WT (BY4741) and dnf1Δ dnf2Δ dnf3Δ cells (PFY3272C) carrying plasmid pRS316-GAL-STE4/STE18 were grown to mid–exponential phase in SC-Ura+Raf/Suc medium, collected, resuspended in SC-Ura+Gal, and examined after 8 h by microscopy. Values are the mean ± SD from three independent trials. (C) Cultures of ste5Δ (YELO23) and ste5Δ dnf1Δ dnf2Δ dnf3Δ (YELO24) expressing either Ste5-myc from plasmid pSTESprom-STE5-myc13 or Ste5(NLSm)-myc from plasmid pSTESprom-ste5(NLSm)-myc13 were grown to mid–exponential phase in SCGlc-Ura medium, collected, and lysed, and equal amounts of protein of the resulting whole-cell extracts were resolved by SDS–PAGE and analyzed by blotting with anti-myc monoclonal antibody and anti-Pgk1 as a loading control. Left, the lanes shown were separated on the original gel and have been spliced together here for clarity.

FIGURE 4: Steady-level of Ste5 is markedly decreased in dnf1Δ dnf2Δ dnf3Δ cells. (A) Cultures of WT (BY4741), fpk1Δ (YFR191), fpk2Δ (YFR222), and fpk1Δ fpk2Δ (YFR209) cells were grown to mid–exponential phase in YPD medium, treated with 10 μM α-factor for 1.5 h, and examined by microscopy. (B) WT cells (BY4741) carrying empty vector (YEp352GAL) or the same vector overexpressing Ypk1 (pAM76), or ypk1Δ cells (JTY6142) carrying YEp352GAL or the same vector overexpressing a KD mutant, Ypk1(K376), were grown to mid–exponential phase in SC-Ura+Raf/Suc medium, collected, and resuspended in SC-Ura+Gal medium, grown for an additional 3 h, incubated in the absence and presence of 10 μM α-factor for 1.5 h, and examined by microscopy. Values are the mean ± SD from three independent experiments.

FIGURE 3: Fpk1 and Fpk2 function are required for optimal pheromone response. (A) Cultures of WT (BY4741), fpk1Δ (YFR191), fpk2Δ (YFR222), and fpk1Δ fpk2Δ (YFR209) cells were grown to mid–exponential phase in YPD medium, treated with 10 μM α-factor for 1.5 h, and examined by microscopy. (B) WT cells (BY4741) carrying empty vector (YEp352GAL) or the same vector overexpressing Ypk1 (pAM76), or ypk1Δ cells (JTY6142) carrying YEp352GAL or the same vector overexpressing a KD mutant, Ypk1(K376), were grown to mid–exponential phase in SC-Ura+Raf/Suc medium, collected, and resuspended in SC-Ura+Gal medium, grown for an additional 3 h, incubated in the absence and presence of 10 μM α-factor for 1.5 h, and examined by microscopy. Values are the mean ± SD from three independent experiments.
Ste5, which undergoes robust nucleocytoplasmic shuttling (Pyciak and Huntress, 1998; Mahanty et al., 1999; Künzler et al., 2001), is targeted for degradation exclusively in the nucleus by the nuclearly localized ubiquitin–protein ligase (E3) SCF\(^{\text{Cdc25}}\) (Garrenton et al., 2009). Once sufficient Cln2 has built up to initiate the cell cycle, the cell is no longer susceptible to pheromone because Cln2-bound Cdk1/ Cdc28 phosphorylates any PM-associated Ste5, ejecting it from the PM (Strickfaden et al., 2007), thus allowing it to translocate into the nucleus and be degraded (Garrenton et al., 2009). We reasoned that if the lower steady-state level of Ste5 displayed by \(\text{dnf1}^{\Delta} \text{dnf2}^{\Delta} \text{dnf3}^{\Delta}\) cells was due to an enhanced rate of its degradation in the nucleus, then point mutations that eliminate the major nuclear localization signal (NLS) in Ste5 (Strickfaden et al., 2007) might suppress the effect on Ste5 level observed in the multiple flippase–deficient cells. Indeed, when its major NLS was mutated, the steady-state level of Ste5 in \(\text{dnf1}^{\Delta} \text{dnf2}^{\Delta} \text{dnf3}^{\Delta}\) cells was restored to that seen in control cells (Figure 4C, right). Therefore our attention turned to finding some mechanistic explanation for how loss of Dnf1, Dnf2, and Dnf3 might influence the processes that dictate Ste5 stability.

**Factors contributing to Ste5 instability in \(\text{dnf1}^{\Delta} \text{dnf2}^{\Delta} \text{dnf3}^{\Delta}\) cells**

Because the action of G1 cyclin-bound Cdk1/Cdc28 displaces Ste5 from the PM, permitting its nuclear entry and degradation, we first asked whether there was any effect of the loss of Dnf1, Dnf2, and Dnf3 on the level of Cln2. Unexpectedly, we observed even in asynchronous cultures a modest but reproducible increase in the steady-state level of this G1 cyclin in \(\text{dnf1}^{\Delta} \text{dnf2}^{\Delta} \text{dnf3}^{\Delta}\) cells compared with the WT control (Figure 5A). As an independent means to determine whether this moderate increase was enough to contribute to the lower level of Ste5 seen in \(\text{dnf1}^{\Delta} \text{dnf2}^{\Delta} \text{dnf3}^{\Delta}\) cells, we reduced the total G1 cyclin-producing capacity of the cell by deleting both the CLN1 and CLN3 genes. This tactic more than doubled the relative level of Ste5 in \(\text{dnf1}^{\Delta} \text{dnf2}^{\Delta} \text{dnf3}^{\Delta}\) cells (Figure 5B) and, consistently with the elevation in Ste5 content, partially restored (a threefold increase) the ability of the cell population to respond to pheromone (Figure 5C).

We noted that a majority of the \(\text{dnf1}^{\Delta} \text{dnf2}^{\Delta} \text{dnf3}^{\Delta}\) cells exhibited buds whose axial ratio was somewhat more elongated than in the corresponding wild-type cells (Supplemental Figure S4; see also, e.g., Figure 6A). This morphological response can arise when the amount or activity of the Cdc28 is compromised (Howell and Lew, 2012). Moreover, because Cdc28 is known to phosphorylate and inhibit the SBF transcription factor required for expression of the G1 cyclin genes (Amon et al., 1993), a lower level of Cln2-bound Cdk1 might explain the higher level of Cln2 we observed in \(\text{dnf1}^{\Delta} \text{dnf2}^{\Delta} \text{dnf3}^{\Delta}\) cells. Consistent with this view, we found a modest but reproducible reduction in the cellular content of Cln2 in the \(\text{dnf1}^{\Delta} \text{dnf2}^{\Delta} \text{dnf3}^{\Delta}\) cells as compared with the cognate control cells (Supplemental Figure S5).

One of the components required for bud emergence and timely progression through the apical-to-isotropic switch in bud expansion is the small Rho-family GTPase Cdc42 (Bi and Park, 2012). It has been shown that Dnf1- and Dnf2-catalyzed PtdEth flipping to the inner leaflet is required for activation of the GAPS Rga1 and Rga2 that down-regulate Cdc42-GTP (Saito et al., 2007; and/or for the Rdi1-mediated dissociation of Cdc42 from the PM; Das et al., 2012). In cells lacking Lem3 (the required escort protein and cofactor for Dnf1 and Dnf2), polarized Cdc42 persists, leading to continued apical bud growth at low temperatures, resulting in an elongated bud (Saito et al., 2007). Presumably the flippase defect of \(\text{dnf1}^{\Delta} \text{dnf2}^{\Delta} \text{dnf3}^{\Delta}\) cells is even greater than of \(\text{lem3}^{\Delta}\) cells, given that these cells manifest somewhat enlarged and elongated buds even at 30°C (Figure 6A, left). It has also been reported that another flippase substrate, PtdSer, accumulates at bud necks, in the bud cortex, and at the tips of mating projections and that a PtdSer synthase (cho1\(^{\Delta}\) pss1\(^{\Delta}\)) mutant has impaired polarization of Cdc42, causing a delay in bud emergence and defective mating (Fairn et al., 2011).
after pheromone treatment (Figure 6A; average PM pixel intensity per unit area [n = 100 cells]: WT, 3.2 ± 0.8; dnf1Δ dnf2Δ dnf3Δ, 1.4 ± 0.4). Immunoblotting demonstrated that the difference in PM decoration by GFP-C2Lact was not due to any difference in expression of this probe (Figure 6B). Thus, in the triple mutant, inward movement of PtdSer appears to be highly defective.

There is no corresponding genetically encoded probe to assess inner-leaflet PtdEth. Hence we used an indirect measure of the efficiency of PtdEth translocation from the outer to the inner leaflet. The killing action of the antibiotic duramycin involves its specific binding to PtdEth on the exocellular surface of the PM (Zhao, 2011). Hence the more PtdEth in the outer leaflet, the more sensitive a yeast cell is to duramycin (Roelants et al., 2010). Using an agar diffusion bioassay (Figure 6C), we indeed found that, at a concentration of duramycin that has no effect on wild-type cells, the dnf1Δ dnf2Δ dnf3Δ triple mutant displayed a large zone of cell death, and analysis of double mutants indicated that Dnf1 and Dnf2 are the flippases primarily responsible for the inward movement of PtdEth.

Presumably, in the absence of sufficient inner-leaflet PtdSer, Cdc42 is less efficiently recruited to the PM; however, whatever PM-associated Cdc42-GTP is there will have a more protracted lifetime because, in the absence of sufficient inner-leaflet PtdEth, GAP and GDI activity will be less efficient. As one means to assess which of these two effects is the more dominant, we examined the subcellular localization of the Cdc42 GEF (Cdc24) because there is ample evidence that Cdc42-GTP participates in a self-reinforcing positive feedback loop to stably recruit Cdc24 to the site of apical bud growth (Bose et al., 2001; Gulli and Peter, 2001; Butty et al., 2002). Indeed, we found (Supplemental Figure S6) that, compared with the control, in the dnf1Δ dnf2Δ dnf3Δ cultures a much larger fraction of the cells with medium or large buds had readily detectable GFP-Cdc24 at the site of apical bud growth, which suggests that GAP- and/or GDI-mediated down-regulation of Cdc42-GTP is impaired. At this time, however, how this mild morphogenetic anomaly may be connected to the slightly elevated Cln2 and slightly reduced Cbl2 level observed in dnf1Δ dnf2Δ dnf3Δ cells is unclear, aside from the fact that perturbations of Cdc42 function might be expected to delay cell cycle progression.

**Recruitment of Ste5 to the plasma membrane is impaired in dnf1Δ dnf2Δ dnf3Δ cells**

If the primary defect in mounting an efficacious pheromone response in dnf1Δ dnf2Δ dnf3Δ cells is due to the low level of Ste5, then ectopic overexpression from a strong promoter should ameliorate the problem. Toward this end, we overexpressed STE5 from the GAL1 promoter on a multiple-copy plasmid in dnf1Δ dnf2Δ dnf3Δ cells, and indeed found that the fraction of the population competent to form robust shmooos in response to α-factor treatment was increased by an order of magnitude, from 4% (Figure 2A) to >40% (Figure 7A, left). However, we noted that the frequency of shmoo formation was still not at the level displayed by wild-type cells in either the absence (Figure 2A) or presence of overexpressed STE5 (Figure 7A, left). Hence we overexpressed in the same manner a mutant allele of Ste5, Ste5(P44L) (Sette et al., 2000), which has been shown to enhance association of Ste5 with the PM (Winters et al., 2005). Although this construct was slightly toxic to the cells, the efficiency of shmoo formation by the dnf1Δ dnf2Δ dnf3Δ cells was now equivalent to that exhibited by the wild-type cells (Figure 7A, middle), suggesting that, in addition to the lower steady-state level of Ste5 in dnf1Δ dnf2Δ dnf3Δ mutants, the lack of these flippase creates a PM milieu that is less conducive to Ste5 recruitment. However, when overexpressed, the GFP-tagged versions of both

To examine inner-leaflet PtdSer, we expressed, as a fusion to GFP, the C2 domain of the mammalian protein lactadherin (also known as MFG-E8), which is specific for binding to the head group of PtdSer (Shao et al., 2008; Yeung et al., 2008; Ye et al., 2013). As expected, when compared with otherwise isogenic control cells, there was a marked decrease (nearly 60%) in the intensity of PM labeling with this probe in dnf1Δ dnf2Δ dnf3Δ cells, both before and
To initiate and maintain a signal in response to pheromone, recruitment of Ste5 to the PM requires binding of its PH domain to PtdIns(4,5)P$_2$ (Garrenton et al., 2006, 2010). Indeed, in the absence of wild-type Ste5 and the constitutively active Ste5(P44L) allele were recruited to the shmoo tip in the pheromone-treated cells (Supplemental Figure S7).

FIGURE 7: Ste5 tethering at the PM is disturbed in $\text{dnf1}^{-}\text{dnf2}^{-}\text{dnf3}^{-}$. (A) Either WT (BY4741) or $\text{dnf1}^{-}\text{dnf2}^{-}\text{dnf3}^{-}$ cells (PFY3272C) overexpressing from the GAL1 promoter either Ste5 (pCJ6) or Ste5(P44L) (pCS7), as indicated, from URA3-marked, YEp352-derived plasmids, and ste5$^{-}$ derivatives of the same strains overexpressing Ste5(R407S K411S) from the same vector, which were grown to mid–exponential phase in SCGlc-Ura, collected, resuspended in SCGal-Ura, propagated for 3–4 h, incubated with 10 μM $\alpha$-factor for 1.5 h, and examined in the microscope. Values are the mean ± SD from three independent trials. (B) Left, a URA3-marked CEN plasmid (pPP1872) expressing the PtdIns(4,5)P$_2$-specific probe GST-GFP-PH$^{\text{PLC5}}$ was introduced into the control and the $\text{dnf1}^{-}\text{dnf2}^{-}\text{dnf3}^{-}$ mutant, which were then treated as in A and viewed by fluorescence microscopy. Scale bar, 5 μm. Right, samples of the same cells were lysed, and equivalent amounts of protein from the whole-cell extracts were resolved by SDS–PAGE and analyzed by immunoblot with appropriate antibodies. (C) As in B, except that the URA3-marked CEN plasmid expressed the PtdIns4P-specific probe 2XPH$^{\text{Osh2}}$-GFP.
FIGURE 8: Mislocalization of Mss4 in *dnf1Δ* *dnf2Δ* *dnf3Δ* cells. (A) Left, URA3-marked CEN plasmids expressing Mss4-GFP from the MSS4 promoter (pCS321; top) or from the GAL1 promoter (pRB2; bottom) were introduced into WT (BY4741) and *dnf1Δ* *dnf2Δ* *dnf3Δ* (PFY3272C) cells. The resulting transformants were grown to mid–exponential phase in SCGlc-Leu, SCGlc-Ura, or SCGlc-Leu-Ura for 3 h, incubated with 10 μM α-Ura, as appropriate, collected, resuspended in SCGal-Leu, SCGal-Ura, or SCGal-Leu-Ura for 3 h, and examined by microscopy. Scale bar, 5 μm. Right, samples of the same cells were lysed, and equivalent amounts of protein from the whole-cell extracts were resolved by SDS–PAGE and analyzed by immunoblot with appropriate antibodies. (B) WT and *dnf1Δ* *dnf2Δ* *dnf3Δ* cells were transformed with an empty LEU2-marked multicopy vector (YEp351GAL) or the same vector carrying a plasmid (pRB2) expressing MSS4 under control of the GAL1 promoter. The resulting transformants were grown to mid–exponential phase in SCGal-Ura and examined by fluorescence microscopy. Scale bar, 5 μm.

Mss4 is mislocalized in *dnf1Δ* *dnf2Δ* *dnf3Δ* cells

PtdIns4P is converted to PtdIns(4,5)P$_2$ by the sole PtdIns4P-specific probe that contains tandem copies of the PH domain derived from mammalian PLCδ1, which is highly specific for recognition of PtdIns(4,5)P$_2$ (Stauffer et al., 1998; Szentpetery et al., 2009). We observed that, compared with control cells, the *dnf1Δ* *dnf2Δ* *dnf3Δ* mutant exhibited both a reduction in overall intensity and much less decoration of the PM in mother cells than in buds (Figure 7B, left). Immunoblotting of the same cells showed that this difference was not attributable to any difference in the level of probe expression (Figure 7B, right). By contrast, using a PtdIns4P-specific probe that contains tandem copies of the PH domain derived from yeast Osh2 (Roy and Levine, 2004), we did not observe any difference in either intensity or pattern between control cells and *dnf1Δ* *dnf2Δ* *dnf3Δ* mutant (Figure 7C).

When expressed at a near-endogenous level (from its native promoter on a CEN plasmid) in wild-type cells, Mss4-GFP decorated the inner perimeter of the PM as a series of bright puncta, as observed before (Audhya and Emr, 2003), whereas in *dnf1Δ* *dnf2Δ* *dnf3Δ* cells, the intensity of the PM decoration was reduced by >70% (average PM pixel intensity per unit area [n = 100 cells]: WT, 2.6 ± 0.5; *dnf1Δ* *dnf2Δ* *dnf3Δ*, 0.7 ± 0.2; Figure 8A, left, top). Immunoblotting demonstrated that this difference in PM decoration by Mss4-GFP was not due to any difference in expression.
Figure 9: Phosphoinositide distribution is markedly perturbed in dnflΔ dnf3Δ dnf2Δ cells. URA3-marked plasmids expressing under the GAL1 promoter either GST-GFP-PH<sup>Δ<sub>MSK4</sub></sup> (top), (PH<sup>GFP</sup>)<sub>Δ<sub>MSK4</sub></sub>-GFP (middle), or Mss4-GFP (bottom) were introduced into wild-type cells (BY4741) or an otherwise isogenic dnflΔ dnf3Δ dnf2Δ (ZHY708) triple mutant, and the resulting transformants were grown to mid–exponential phase in SCGlc-Ura, collected, resuspended in SCGal-Ura medium for 3 h, and then examined by fluorescence microscopy. Scale bar, 5 μm.

If mislocalization of Mss4 and a resulting diminution of PM PtdIns(4,5)P<sub>2</sub> in dnflΔ dnf2Δ dnf3Δ cells contributes to Ste5 instability by preventing its PM recruitment and thereby blocking an efficacious pheromone response, then ectopic overexpression from a strong promoter should ameliorate the problem. Toward this end, we overexpressed Mss4 from the GAL1 promoter on a multiple-copy plasmid in dnflΔ dnf2Δ dnf3Δ cells in the absence and presence of overexpressed STE5. Overexpression of Mss4 alone, but not a catalytically inactive mutant, Mss4(D636A) (Rao et al., 1998; Kobayashi et al., 2005), significantly increased (fourfold) the fraction of the population competent to form robust shmoos in response to α-factor treatment and further enhanced the effect of overproducing Ste5 in a modest but statistically significant manner, such that the frequency of cells in the population competent to respond to pheromone was now close to 90% of that of the wild-type cells carrying empty vector (Figure 8B).

As we demonstrated here, the dnflΔ dnf2Δ dnf3Δ cells have lower levels of inner-leaflet PtdSer and PtdEth (Figure 8). As an independent means to determine whether lower levels of either of these glycerophospholipids might be responsible for lack of efficient Mss4 localization at the PM, we examined the distribution of Mss4-GFP in a cho1Δ mutant that lacks phosphatidylethanolamine synthase and hence is unable to make PtdSer but can still make PtdEth via a salvage pathway (Henry et al., 2012). Indeed, consistent with the lower level of inner-leaflet PtdSer in dnflΔ dnf2Δ dnf3Δ cells being responsible for the poor PM recruitment and mislocalization of Mss4, we found that, in cho1Δ cells, Mss4 localized more weakly to the PM than in wild-type cells (average PM pixel intensity per unit area [n = 100 cells]: WT, 1.4 ± 0.2; cho1Δ, 0.74 ± 0.11) and very prominently to the same internal, apparently vesicular compartment seen in the dnflΔ dnf2Δ dnf3Δ mutant (Figure 8C).

Through its generation of PtdIns(4,5)P<sub>2</sub>, Mss4 has been implicated in the establishment of cell polarity (Audhya and Emr, 2003; Yakir-Tamang and Gerst, 2009). Given the aberrations in Mss4 localization we observed in dnflΔ dnf2Δ dnf3Δ cells, it was possible that there might be corresponding perturbation of the actin cytoskeleton. However, when fixed and stained with Alexa Fluor 488–labeled phalloidin, actin patch distribution, actin cable formation, and overall cytoskeletal organization in dnflΔ dnf2Δ dnf3Δ cells appeared quite comparable to those in control cells (Supplemental Figure S8). Hence the failure of shmoo formation in dnflΔ dnf2Δ dnf3Δ cells is not likely an indirect consequence of defects in the actin cytoskeleton.

Distribution of phosphoinositides is grossly aberrant in dnflΔ dnf3Δ dnf2Δ cells

We found that another flippase triple mutant, dnflΔ dnf3Δ dnf2Δ, also was defective in pheromone response (Figure 2, A and B), although not quite as severe as dnflΔ dnf2Δ dnf3Δ cells.

However, dnflΔ dnf3Δ dnf2Δ cells did not exhibit a dramatic reduction in the total level of Ste5 present (Supplemental Figure S9), indicating a different underlying cause for their inability to respond to pheromone. Given the interplay between PM PtdIns(4,5)P<sub>2</sub> level and Ste5 stability and function that we uncovered in the course of analyzing the signaling defect in the dnflΔ dnf2Δ dnf3Δ cells, we used the same probes to examine the distribution of PtdIns(4,5)P<sub>2</sub> in dnflΔ dnf3Δ dnf2Δ cells.

The PtdIns4P that is converted to PtdIns(4,5)P<sub>2</sub> at the PM by the action of Mss4 can be supplied either by direct synthesis at the PM by the essential PtdIns 4-kinase Sst4 or delivered via Golgi compartment–derived secretory vesicles that contain PtdIns4P generated by the Golgi body–associated essential PtdIns 4-kinase Pkh1 (Strahl and Thorner, 2007). We found that, compared with control cells, the distribution of the PtdIns(4,5)P<sub>2</sub>-specific probe was strikingly different in the dnflΔ dnf3Δ dnf2Δ mutant. Although there was faint decoration of the PM, the most prominent fluorescent feature was several bright internal puncta (Figure 9, top). In the dnflΔ dnf3Δ dnf2Δ population, >70% of the cells exhibited such prominent dots, whereas <8% of wild-type cells had any sort of visible internal feature. Similarly, unlike in wild-type cells, where overexpressed Mss4-GFP is confined to the PM but faintly visible in the nucleus, the Mss4-GFP in dnflΔ dnf3Δ dnf2Δ cells was reduced at the PM and most prominent in a number of very bright internal puncta (Figure 9, bottom).

We suspect that the reduction in PM-associated Mss4 and consequent dramatic reduction in PM PtdIns(4,5)P<sub>2</sub> could be sufficient, by themselves, to compromise the efficiency of Ste5 recruitment to the

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flippase function and pheromone response
PM and hence readily explain the signaling defect observed in dnf1Δ dnf2Δ dnf3Δ dnf7Δ cells.

Similarly, localization of the PtdIns4P-specific probe in dnf1Δ dnf3Δ dnf7Δ cells was much less polarized than in the wild-type cells, markedly reduced at the PM, and mainly confined to small vesicles distributed roughly equally between mother and bud (Figure 9, middle). These findings indicate that, in dnf1Δ dnf3Δ dnf7Δ cells, vesicle-mediated outward lipid and protein trafficking is impaired, preventing efficient provision of phosphoinositides to the PM. Although phosphoinositides were not examined in prior work, our results are consistent with previous studies demonstrating that the lack of Drs2, or additional flippases in combination with Drs2, causes defects in secretory vesicle formation and trafficking from the Golgi structure to the PM, as well as in delivery of late and early endosomes to the Golgi (Chen et al., 1999; Gall et al., 2002; Sebastian et al., 2012).

DISCUSSION

In yeast and other eukaryotes, PM bilayer composition undergoes continual remodeling due to insertion of secretory vesicles (Mizuno-Yamasaki et al., 2012), removal of endocytic vesicles (Weinberg and Drubin, 2012), and action of dedicated transporters that catalyze ATP-dependent transfer of lipids from one leaflet to the other (Daleke, 2003; van Meer, 2011). The class 4 P-type ATPases that constitute the PM (Drs2, Dnf1, Dnf2, and Dnf3) are distinct from those harboring Stt4 (Audhya and Emr, 2002). If the ablation of these five flippases cannot be spared Ste5 from degradation in the nucleus (Strickfaden et al., 2012), act on different glycerophospholipids in various cellular compartments (Muthusamy et al., 2009; Sebastian et al., 2012). It seems remarkable, therefore, that a yeast cell missing three of its five flippases can survive and manifests only modest morphological abnormalities despite rather profound dislocations in PM lipids, as documented here. However, flippase dysfunction has its consequences. As shown here, signaling in the mating pheromone response pathway is abrogated, due largely to effects on stability, recruitment, and/or function of MAPK scaffold protein Ste5.

In dnf1Δ dnf2Δ dnf3Δ cells, we traced the primary problem to inefficient PM recruitment of Ste5 and consequent reduction in PtdIns(4,5)P2. Stable PM recruitment of Ste5 requires its PtdIns(4,5)P2-specific PH domain (Garrenton et al., 2006, 2010), and PM tethering spares Ste5 from degradation in the nucleus (Strickfaden et al., 2007; Garrenton et al., 2009). Hence lack of optimal Mss4-generated PtdIns(4,5)P2 spares both the lower level of Ste5 and the signaling defect observed in dnf1Δ dnf2Δ dnf3Δ cells. Indeed, overexpression of both Mss4 and Ste5 largely ameliorated the signaling defect in these cells. Although several proteins are involved in PM localization of Ste4, including Sk1 (Audhya and Emr, 2002), Ypp1, and Efr3 (Baird et al., 2008), no such factor has yet been implicated in the formation of the PM-associated puncta that contain Mss4, which are distinct from those harboring Ste4 (Audhya and Emr, 2002). If there are such proteins for Mss4, then our findings indicate that leaflet lipid composition may be important for their trafficking and delivery to the PM. In Mss4 (779 residues), the catalytic domain is located at its C-terminus (residues 376–769), whereas its N-terminal segment (1–375) contains no recognizable diagnostic folds. Hence perhaps the N-terminal region of Mss4 possesses protein–protein interaction elements and/or lipid-binding motifs or domains important for its PM interaction. There is some evidence that PM sphingolipid content influences Mss4 binding (Kobayashi et al., 2005; Gallego et al., 2010).

In dnf1Δ dnf3Δ dnf7Δ cells, the previously characterized defect these cells exhibit in global trafficking and secretion from the Golgi compartment to the PM (Chen et al., 1999; Gall et al., 2002; Sebastian et al., 2012) could explain its defective pheromone response, which was somewhat less severe than that of dnf1Δ dnf2Δ dnf3Δ cells. As shown here, the bulk of the PtdIns(4,5)P2 and of its precursor (PtdIns4P) remains on internal membranes and thus is not efficiently delivered to the PM. In this regard, Pik1-generated PtdIns4P in the Golgi compartment binds to the C-terminal tail of Drs2 and stimulates its function (Natarajan et al., 2009), suggesting that Drs2 may only be fully operative and hence able to optimally generate Golgi-derives transport vesicles when Golgi membranes are sufficiently enriched in PtdIns4P. Similarly, Osh4, which specifically binds PtdIns4P, facilitates exocyst complex–mediated secretory vesicle docking at sites of polarized growth at the PM (Alfaro et al., 2011; Graham and Burd, 2011). Therefore the substantial defect we observed in PM PtdIns(4,5)P2 in dnf1Δ dnf3Δ dnf7Δ cells may be due, in large measure, to inefficient delivery of its precursor PtdIns4P, further suggesting that the PM-associated PtdIns 4-kinase Stt4 is not sufficient to perform this task in these cells.

In any event, PtdIns(4,5)P2 mislocalization has significant consequences for the capacity of the cell to respond to pheromone. In this situation, because both its PH domain (Garrenton et al., 2006; Garrenton et al., 2010) and PM element (Winters et al., 2005) display marked preferrence for interaction with PtdIns(4,5)P2, any Ste5 that traffics from the nucleus will be recruited to the cytosolic surface of internal membranes rather than to the PM but nonetheless be spared from degradation. Consistent with this prediction, we found that, in contrast to dnf1Δ dnf2Δ dnf3Δ cells, the steady-state level of Ste5 in dnf1Δ dnf3Δ dnf7Δ cells was not markedly reduced. However, because neither PtdIns(4,5)P2 nor, consequently, Ste5 gets efficiently delivered to the PM, this scaffold protein will not encounter the other components of the pheromone-activated signaling apparatus (in particular, MAPKKKK Ste20) at a level sufficient to activate the Ste5–associated passenger proteins, especially the next enzyme (MAPKK Ste11) in the MAPK cascade. These considerations likely explain the signaling deficiency of dnf1Δ dnf3Δ dnf7Δ cells. Consistent with these conclusions, we found that overexpression of Ste5(P44L), an allele that enhances the PM targeting ability of the N-terminal amphipathic helix (PM motif) in Ste5, which can interact with other acidic phospholipids, like PtdSer (Winters et al., 2005), unlike the PH domain of Ste5 (Wu et al., 2012), rescued fully the shmoo formation defect of dnf1Δ dnf3Δ dnf7Δ cells (unpublished data).

In wild-type cells responding to pheromone, Dnf1, Dnf2, and Dnf3, as well as the flippase-activating protein kinase Fpk1, exhibited dramatic relocation to the shmoo tip. For Dnf1 and Dnf2, appearance at the tip was much more concentrated and sustained than that exhibited by Dnf3. In contrast to the other three flippases, Drs2 was visualized only in Golgi structures, with no difference in localization between vegetative and pheromone-treated cells. However, cells lacking Drs2 show a significant defect in protein transport from the trans-Golgi network and a reduction in the amount of Dnf1 at the cell surface (Chen et al., 1999; Hua et al., 2002). Hence Drs2 activity could indirectly influence Dnf1 function (and, likewise, Dnf2 and Dnf3).

Pheromone-evoked changes result in higher inner-leaflet PtdSer (Fairn et al., 2011) and lower inner-leaflet PtdEt (Iwamoto et al., 2004) at the shmoo tip. Thus, somehow, coordination among the three Fpk1-regulated flippases enhances the rate of inward PtdSer translocation at the expense of inward PtdEth movement. Consistent with this conclusion, the amount of PtdSer in the inner leaflet of the PM was highly reduced in dnf1Δ dnf2Δ dnf3Δ cells, as judged by the GFP-C2–act probe. Our result is also in accord with a study showing no change in total PtdSer content in a dnf1Δ dnf2Δ dnf3Δ mutant compared with wild type, yet enhanced binding of another
PtdSer-specific probe, annexin V, to the exocellular surface of the PM in the mutant cells relative to wild type (Chen et al., 2006).

Elevated inner-leaflet PtdSer enhances PM localization of Cdc42-GTP (Fairn et al., 2011), and reduced inner-leaflet PtdSer compromises the action of Cdc42-specific GAPs (Saito et al., 2007) and/or the Cdc42-directed GDI (Das et al., 2012) that down-regulate Cdc42-GTP. Thus the membrane environment established in response to pheromone is conducive to maintaining a highly localized, PM-associated pool of Cdc42 in its active (GTP-bound) form, which, in turn, can stimulate the tip-associated formin Bni1 to generate a previously unappreciated interplay between membrane phosphoinositide composition and the leaflet distribution of other Lipid species. This reveals a previously unappreciated interplay between membrane phosphoinositide composition and the leaflet distribution of other Lipid species, such as PtdIns4P (pES4) as the template, a pair of primers Ste2-′TTTGTTAATTTGCATT-CATGACAT-3′ and 5′-GCAAGCAA TTC-3′, the resulting construct was confirmed by direct nucleotide sequencing.

### MATERIALS AND METHODS

#### Strains and growth conditions

Yeast strains used in this study are listed in Table 1 and were grown routinely at 30°C. Yeast cells were cultivated in either standard rich (YP) or defined minimal (SC) medium (Sherman et al., 1986) containing either 2% glucose (Glc), 2% raffinose and 0.2% sucrose (Raf-Suc), or 2% galactose (Gal) as the carbon source and, where necessary, supplemented with appropriate nutrients to maintain selection for plasmids. For induction of GAL1 promoter-driven expression, cells were pregrown to mid–exponential phase in SCRaf-Suc medium, collected and resuspended in Gal-containing medium (2% final concentration), and incubation was continued for 3 h. Standard yeast genetic techniques were performed according to Sherman et al. (1986). Strains YELO17 and YELO18 expressing Ste2(7K-to-R)-mCherry from the STE2 promoter at the STE2 locus were constructed by amplifying the STE2(7K-to-R)-mCherry-CAURA3 cassette from genomic DNA of strain YDB119 (Ballon et al., 2006) using primers Ste2-Ndel 5′-GGGTAAGTACATGATGAAACACA-TATGAA-GAAA-3′, and Ste2-EcoRI, TTGTAGAGCATCATCACCACATCT- TAAGCCG-3′. The resulting product was introduced by DNA-mediated transformation into BY4741 and dnf1Δ dnf2Δ dnf3Δ cells selecting for Ura+ clones. Successful integration at the STE2 locus was verified by colony PCR (Ward, 1992). Integration of the FUS-1prom- lacZ reporter gene was accomplished by linearizing pS8286 (Trueheart et al., 1987) with Spel, followed by selection for Ura+ transformants, which were then confirmed by colony PCR.

### Plasmids and recombinant DNA methods

Plasmids used in this study are listed in Table 2. Plasmids were constructed using standard procedures (Sambrook et al., 1989) in Escherichia coli strain DH5α. Fidelity of all constructs was verified by nucleotide sequence analysis. Plasmid pRB2 (pGAL-MSS4-GFP) was constructed using in vivo gap repair (Kitazono, 2009). The MSS4-GFP sequence was amplified by PCR from genomic DNA of a yeast strain containing a copy of MSS4-GFP integrated at the MSS4 locus (Audhya and Emr, 2003) using synthetic oligonucleotide primers (Mss4-1, 5′-TACCTCTATATTTAAGCAGGAAA- AACCCCATGTCACTTGCTGACCTACACTCT3′, and Mss4-2, 5′-ATGTTGACCTTCTGATGTAGTACTGTGTTGCGACC-CGCCGGAGATCTGTGATC-3). The resulting product and URA3-marked plasmid pRS316-GAL1-10, which had been digested with BamHI and Spel restriction enzymes, were introduced by DNA-mediated transformation (Pham et al., 2011) into BY4741, and Ura+ transformants were selected on SCGlc-Ura medium. Candidate clones were extracted, plasmid DNA was recovered from E. coli, and presence of the desired insert was assessed by agarose gel electrophoresis and confirmed by nucleotide sequence analysis.

A catalytically defective (′kinase-dead′) (KDI) allele, pGAL1-Mss4(D636A)-GFP (pES20), was constructed via site-directed mutagenesis using pGAL1-Mss4-GFP (pES4) as the template, a pair of phosphorylated synthetic oligonucleotide primers (5′-TTATTCC- TTGTTAATTTGCAATGACAT-3′ and 5′-GCCATTGATTCAA-TTTAGCAAGCCAATTC-3′), and a single-step plasmid amplification method employing Phusion polymerase (Zhang et al. 2009), and the resulting construct was confirmed by direct nucleotide sequencing using a reverse synthetic oligonucleotide primer starting 120 base pairs after the GFP start codon (5′-TAAATTTTCCGTATGTTC-ACTC-3′).

### Preparation of cell extracts

Preparation of yeast cell extracts by rapid alkaline lysis followed by trichloroacetic acid (TCA) precipitation was performed as described previously (Westfall et al., 2008). To extract flippases efficiently from yeast cells, a few modifications were applied. Briefly, cells from samples (3 ml) of mid–exponential–phase cultures were collected and stored at −80°C overnight, and the resulting pellets were resuspended in water (500 μl final volume) and incubated on ice for 10 min with 50 μl of 1.85 M NaOH and 2% β-mercaptoethanol.

After this alkaline lysis, protein was precipitated by addition of 50 μl of 50% TCA and, after incubation for 15 min on ice, collected by centrifugation at maximum speed for 5 min at room temperature in a microfuge. The resulting pellets were resuspended in urea-SDS buffer (8 M urea, 5% SDS, 0.1 mM EDTA, 0.1% bromophenol blue, 100 mM dithiothreitol, 200 mM Tris-HCl, pH 6.8), typically 60 μl/1 A600nm, at time of harvest, heated for 10 min at 37°C, and clarified by centrifugation at maximum speed for 2 min in a microfuge to remove any insoluble debris, and samples of the resulting supernatant solution were stored at −20°C before further analysis.
Antibodies and immunoblotting

SDS–PAGE and immunoblotting were performed as described previously (Westfall et al., 2008). Proteins resolved in SDS–polyacrylamide slab gels were transferred to nitrocellulose filter paper, incubated with the appropriate primary antibodies, and then incubated with appropriate infrared dye–conjugated secondary antibodies. The resulting filter-bound immune complexes were then visualized using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) and v2.1 software. Primary antibodies used were polyclonal rabbit anti-Ste5 antisera (Thomson et al., 2011; gift of Kirsten Benjamim, Molecular Sciences Institute, Berkeley, CA); mouse monoclonal anti-GFP (Roche Diagnostics, Indianapolis, IN); mouse monoclonal anti-Clb2 (Clb2-GFP::URA3); rabbit polyclonal anti-Ste5 antiserum (Thomson et al., 2011; gift of Kirsten Benjamim, Molecular Sciences Institute, Berkeley, CA); and rabbit polyclonal anti-Pgk1 (Baum et al., 1978). Secondary antibodies used were Alexa Fluor 680–conjugated goat anti-rabbit immunoglobulin (IgG; Molecular Probes, Waltham, MA) and IRDye 800–conjugated goat anti-mouse IgG (Rockland Immunochemicals, Limerick, PA). Protein amounts were quantified using ImageJ software (National Institutes of Health, Bethesda, MD) and normalized levels determined as a ratio relative to the loading control (Pgk1).

Quantification of pheromone response pathway

Routinely, to gauge the ability of a given strain to respond to pheromone, the percentage of the cells in a population that were converted to unequivocally recognizable shmoos was assessed after growing the culture to mid–exponential phase, treating it with α-factor (10 μM final concentration) for, typically, 1.5 h, and then examining samples of such cultures by microscopy. As an independent measure of the capacity of cells to respond to pheromone, we used the level of induction of an integrated pheromone-responsive reporter gene, FUS1-lacZ (derived from plasmid pSB286), after growing cultures to mid–exponential phase, treating them with α-factor (10 μM final concentration) for, typically, 60 min, and then quantifying the level of β-galactosidase activity present using a colorimetric substrate as described previously (Bardwell et al., 1998).

TABLE 1: Yeast strains used in this study.

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<td>BY4741 ste5Δ::HIS3 dnf1Δ::KanMX4</td>
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To visualize actin organization, cultures (4.5 ml) were grown to mid-exponential phase and fixed by addition of a formaldehyde solution (670 μl of fresh 37% formaldehyde stock in 0.5 ml of potassium phosphate, pH 6.5) for 1.5 h at room temperature, and the cells were collected by brief centrifugation. After three washes with 0.5 ml of PBS and resuspension in 0.5 ml of PBS, samples (0.2 ml) were incubated in the dark with constant agitation on a roller drum for 30 min with 45 μl of a solution containing Alexa Fluor 488–phalloidin (Life Technologies, Grand Island, NY; 3.3 μM Alexa Fluor 488–phalloidin) for 30 min with 45 μl of a solution containing Alexa Fluor 488–phalloidin (Life Technologies, Grand Island, NY; 3.3 μM Alexa Fluor 488–phalloidin) and Alexa Fluor 561–cytochrome c oxidase inhibitor (Life Technologies, Grand Island, NY; 3 μM Alexa Fluor 561–cytochrome c oxidase inhibitor). After three washes with 0.5 ml of PBS, the final cell pellets were resuspended in 15 μl of Fluoroshield mounting buffer (Sigma-Aldrich, St. Louis, MO) and examined by fluorescence microscopy.

ACKNOWLEDGMENTS
This work was supported by National Institutes of Health R01 Research Grant GM21841 and France-Berkeley Fund Research Grant 2012-0056 (to J.T.). We thank Todd R. Graham (Vanderbilt University School of Medicine, Nashville, TN) for the gift of a dnf1Δ dnf3Δ strain and for many useful discussions, Kurt Toenjes (Montana State University, Bozeman, MT) for providing plasmid p415Met25-GFP8A-Cdc24, Steven E. Ruzin of the College of Natural Resources Biological Imaging Facility (University of California, Berkeley, CA) for assistance with confocal fluorescence microscopy, Robert A. Arkowitz and Vikram Ghugtyal (University of Nice, Nice, France) for their hospitality and stimulating discussions, Nathan R. Rockwell (University of California, Davis, CA; formerly of this laboratory) for several gene constructs and unpublished results, and other members of the Thorner lab for additional research reagents, helpful advice, and enthusiastic encouragement.

REFERENCES

**TABLE 2: Plasmids used in this study.**

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<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<td>pSB286</td>
<td>FUS1prom::FUS1-LacZ</td>
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<td>pFR150</td>
<td>pRC181-TPI1prom::FPK1-GFP::LEU2</td>
<td>Roelants et al. (2010)</td>
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<td>YEp352GAL</td>
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<td>Benton et al. (1994)</td>
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<td>pAM76</td>
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<td>pPP1872</td>
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<td>pCdc24</td>
<td>p415-MET25-prom::GFP-8A-CDC24</td>
<td>Toenjes et al. (1999)</td>
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**Epifluorescence and confocal fluorescence microscopy**

To visualize fusion proteins marked with GFP or mCherry, cells were grown to mid-exponential phase and viewed directly under an epifluorescence microscope (Model BH-2; Olympus America, Center Valley, PA) using a 100× objective equipped with appropriate bandpass filters (Chroma Technology, Rockingham, VT). Images were collected using either an Olympus MH-Z28 charge-coupled device camera (Olympus America) and processed with Magnafire SP imaging software (Optronics, Goleta, CA) or a CoolSnap MYO charge-coupled device camera (Photometrics, Tucson, AZ) and processed with Micro-Manager open source microscopy software (www.micro-manager.org/). For preparation of figures, images were reproduced using Photoshop (Adobe, San Jose, CA). Quantification of the fluorescence in cells was carried out using ImageJ (Collins, 2007). For each cell, its corrected total PM fluorescence was measured by determining the pixel count within an area delineated by free-form lines drawn around the inner and outer perimeter of the PM and subtracting, as background, the pixel count of an equivalent area in an immediately adjacent cell-free portion of the field. The average PM pixel intensity per unit area is the mean of such measurements performed on at least 100 cells.

Visualization of the subcellular distribution of flippases was also performed using a spinning-disk laser confocal microscopy system (Revolution XD; Andor Technology, South Windsor, CT) comprising an inverted microscope (Nikon TE 2000), a confocal spinning disk unit (model CSU-X1™; Yokogawa Electric Corp., Newman, GA), a piezo-controlled motorized XYZ stage, and two charge-coupled device cameras. A PlanApo 1.4 numerical aperture/100× objective was used with 488-nm or 561-nm laser excitation. The z-stacks were deconvolved using Huygens Professional software (version 3.7; Scientific Volume Imaging, Amsterdam, Netherlands). Sum projections were quantified using ImageJ. All samples were imaged in aqueous media, either growth medium, or collected by brief centrifugation and resuspended in phosphate-buffered saline (PBS).

To visualize actin organization, cultures (4.5 ml) were grown to mid-exponential phase and fixed by addition of a formaldehyde solution (670 μl of fresh 37% formaldehyde stock in 0.5 ml of potassium phosphate, pH 6.5) for 1.5 h at room temperature, and the cells were collected by brief centrifugation. After three washes with 0.5 ml of PBS and resuspension in 0.5 ml of PBS, samples (0.2 ml) were incubated in the dark with constant agitation on a roller drum for 30 min with 45 μl of a solution containing Alexa Fluor 488–phalloidin (Life Technologies, Grand Island, NY; 3.3 μM Alexa Fluor 488–phalloidin and 0.1% Triton X-100 in PBS). After three washes with 0.5 ml of PBS, the final cell pellets were resuspended in 15 μl of Fluoroshield mounting buffer (Sigma-Aldrich, St. Louis, MO) and examined by fluorescence microscopy.


