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 (“flipases”) 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 phosphatidylinositol bisphosphate. In 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 exhibited a marked decrease in its ability to respond to α-factor, which we could attribute to pronounced reduction in Ste5 stability resulting from an elevated rate of its Cln2 Cdc28-initiated degradation. Similarly, a MATa dnf1Δ dnf3Δ drs2Δ 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 separations in the plane of the membrane, creating microdomains (Lingwood and Simons, 2010), and, anisotropy transversely across the membrane, such that each leaflet of the bilayer has a distinct lipid composition (Fadeel and Xue, 2009). The latter property (referred to as bilayer asymmetry) was first reported for the erythrocyte PM (Gordesky, 1973) but is a property of the PM in every cell type (Devaux, 1991; van Meer, 2011). The outer leaflet of the PM contains predominantly phosphatidylethanolamine and sphingolipids, whereas the inner leaflet is enriched in phosphatidylethanolamine (PtdEth), phosphatidylserine (PtdSer), and phosphatidylinositol (PtdIns) and its phospho-rylated derivatives (especially phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate). Thus proper remodeling of plasma membrane aminoglycerolipids and phosphoinositides is necessary for efficient recruitment, stability, and function of the pheromone signaling apparatus.

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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 Discoma 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; PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns(4,5)P2, 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 (Di Nitto et al., 2003; Hurley, 2006; Moravec 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 that 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 (Fain 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).

Bilater asymmetry, in general, and the amount of any given inner-leaflet lipid, in particular, are maintained by active inward translocation (“flopping”) by 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 flippases (Daleke, 2007; Tanaka et al., 2011; Sebastian et al., 2012; Lopez-Marques et al., 2013). The S. cerevisiae genome encodes five flippases: Dnf1, Dnf2, Dnf3, Drs2, and Neo1 (Catty et al., 1997). Paralogues Dnf1 (1571; value in parentheses indicates the 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, Lem3/Ros3 (414; Kato et al., 2004), in-frame to the C-terminus of the chromosomally encoded gene product (crf1) (Saito et al., 2004) and Cdc50 (391; Misu et al., 2003; Takahashi et al., 2011) 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 flippases (López-Marqués et al., 2011) are causes of several inherited diseases (Folmer et al., 2009; van der Mark et al., 2013).

The role of flippases in polarized growth is particularly intriguing because the PM undergoes rapid and highly directional expansion. One known control on PM flippase 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 flippase Drs2), suggesting that Fpk1 action is needed for optimal activity of the remaining flippases. Indeed, although yeast cells lacking Fpk1 (and its parologue, Fpk2/Kin82) are viable and did not have any change in flippase 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 flippases (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 ( lwamoto et al., 2004) and PtdSer become concentrated in the inner leaflet (Fain 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 flippases necessary to maintain it have any role in these processes. We examined the localization of the flippases and the flippase-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 flippase action contributes to signal propagation.

RESULTS

Flippase localization during pheromone response

As an initial means to determine whether any flippase 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 flippase 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 flippase, Neo1, resides exclusively in internal 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 dnf1Δ dnf2Δ dnf3Δ drs2Δ 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 dnf1Δ, dnf2Δ, dnf3Δ, and drs2Δ null alleles. As anticipated, no single deletion mutant displayed any significant defect in its efficiency of shmoo formation upon α-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 dnf3Δ drs2Δ cells (Figure 2A). In contrast, and in agreement with a largely shared function, we found that two triple mutants, dnf1Δ dnf3Δ drs2Δ and especially dnf1Δ dnf2Δ dnf3Δ, 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 dnf1Δ dnf3Δ drs2Δ cells and especially the dnf1Δ dnf2Δ dnf3Δ showed a dramatic reduction in reporter gene expression (Figure 2B). Thus the defect in shmoo formation was attributable to a lack of signaling, regardless of whether the flippases may also have some role in the PM remodeling that may accompany highly polarized growth.

Figure 1: Flippase and flippase kinase localization in pheromone-treated cells. (A) MATa cells expressing the indicated flippase from its native promoter at its normal chromosomal locus as the sole source of each protein, Dnf1-GFP (NRY921), Dnf2-GFP (NRY923), Dnf3-GFP (YEB1), and Drs2-mCherry (YEB2), were grown to mid–exponential phase, exposed to α-factor (10 μM final concentration) for the indicated time, and viewed by fluorescence microscopy. (B) Cells (BY4741) expressing Fpk1-GFP from the TPI1 promoter on a CEN plasmid (pFR150) were grown to mid–exponential phase in SCGlc-Leu-Trp and treated as in A. Scale bars, 5 μm.

These findings suggest that the marked relocalization exhibited by Dnf1, Dnf2, and Dnf3 places them in a position that could allow them to participate directly in membrane remodeling at the shmoo tip, the site from which signaling emanates (Garrenton et al., 2010) and at which polarized growth occurs (Madden and Snyder, 1998). The function of the flippases appears to be stimulated by the action of the protein kinase Fpk1 (and its paralogue Fpk2; Nakano et al., 2008; Roelants et al., 2010). Hence we used the same approach to monitor localization of Fpk1 and found that it too becomes markedly concentrated at the shmoo tip in pheromone-treated cells (Figure 1B).
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 otherwise wild-type cells (i.e., with a full complement of flippases) may have a defect in pheromone response if they lack these flippase-activating protein kinases. Consistent with this view, loss of either Fpk1 alone or 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 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.

### 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 MATα 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., 2003). Hence lack of flippase function is critical for induction of pheromone response (unpublished data) when Gβγ complex described 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 (Figure 4A) or dnf1Δ dnf3Δ dns2Δ cells (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.
the steady-state level of Ste5 was markedly lower in the dnf1Δ dnf2Δ dnf3Δ cells. (A) WT (YELO17) and dnf1Δ dnf2Δ dnf3Δ (YELO18) 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) 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.

Paramount among these factors is the MAPK cascade scaffold protein Ste5, whose signaling function requires its efficient recruitment to and stable association with the PM via insertion of an N-terminal amphipathic helix (Winters et al., 2005), interaction of its RING domain with Gβγ (Inouye et al., 1997), and binding of its PH domain to PtdIns(4,5)P2 (Garrenton et al., 2006). Moreover, ample evidence implicates Ste5 as the rate-limiting component for initiation and maintenance of pheromone-evoked MAPK signaling (Takahashi and Pryciak, 2008; Garrenton et al., 2009; Thomson et al., 2011). Strikingly, immunoblot analysis (Figure 4C, left) revealed that the steady-state level of Ste5 was markedly lower in the dnf1Δ dnf2Δ dnf3Δ mutant than in otherwise isogenic control cells and presumably below the threshold adequate for most cells in the population to mount an effective pheromone response. The decrease in Ste5 could be due either to a lower level of expression or to a higher rate of degradation (or both). Analysis of the level of Ste5 mRNA by quantitative reverse transcriptase-PCR showed that the amount of Ste5 transcript in dnf1Δ dnf2Δ dnf3Δ cells was indistinguishable from that in WT cells (unpublished data), indicating that the reduction in Ste5 protein was likely due to an increase in its rate of turnover.

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 (pAM75), 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) SCFCdc4 (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 Cdc1/Cdc28 phosphorolates 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 dnf1Δ dnf2A dnf3Δ 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 mutationaly crippled, the steady-state level of Ste5 in dnf1Δ dnf2A dnf3Δ 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 dnf1Δ dnf2Δ dnf3Δ 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 dnf1Δ dnf2Δ dnf3Δ 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 dnf1Δ dnf2Δ dnf3Δ 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 dnf1Δ dnf2Δ dnf3Δ cells (Figure 5B) and, consistent 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 dnf1Δ dnf2Δ dnf3Δ 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 cyclin B (Clb)–bound form of Cdk1/Cdc28 is compromised (Howell and Lew, 2012). Moreover, because Clb2-Cdk1 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 Clb2-Cdk1 might explain the higher level of Cln2 we observed in dnf1Δ dnf2A dnf3Δ cells. Consistent with this view, we found a modest but reproducible reduction in the cellular content of Clb2 in the dnf1Δ dnf2A dnf3Δ 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 Rd1-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 dnf1Δ dnf2Δ dnf3Δ cells is even greater than of lem3Δ 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 (cho1Δ/ps1Δ) mutant has impaired polarization of Cdc42, causing a delay in bud emergence and defective mating (Fairn et al., 2011).

**FIGURE 5:** Reduction in G1 cyclin activity partially rescues Ste5 protein level and pheromone responsiveness in dnf1Δ dnf2Δ dnf3Δ cells. (A) WT (BY4741) and derived dnf1Δ dnf2A dnf3Δ cells (PFY3272C) were grown to mid–exponential phase in YPD, collected, and lysed, and equal amounts of protein from the whole-cell extracts were resolved by SDS–PAGE and analyzed by immunoblotting with appropriate antibodies. Representative blot from three independent trials. (B) As in A, for WT, cln1Δ cln3Δ (YELO38), dnf1Δ dnf2A dnf3Δ (PFY3272C), and cln1Δ cln3Δ dnf2A dnf3Δ (YELO39) cells. (C) WT (BY4741) and cln1Δ cln3Δ (YELO38), dnf1Δ dnf2A dnf3Δ (PFY3272C), and cln1Δ cln3Δ dnf1Δ dnf2A dnf3Δ (YELO39) cells were incubated with 10 μM α-factor for 1.5 h and examined by microscopy. Values are mean ± SD from three independent experiments.
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., 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 both before and 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-C2Lact 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-C2Lact.

**FIGURE 6:** Flippase function increases inner-leaflet PtdSer and lowers outer-leaflet PtdEth. (A) WT (BY4741) and isogenic dnf1Δ dnf2Δ dnf3Δ (PFY3272C) cells carrying a URA3-marked CEN plasmid expressing GFP-C2Lact from the TDH3 (GAPDH/GPD) promoter were grown to mid-exponential phase in SC-Glc-Ura medium, treated with 10 mM α-factor, and examined by fluorescence microscopy at the indicated time. (B) Cells shown in A were lysed, and equivalent amounts of protein from the whole-cell extracts were resolved by SDS–PAGE and analyzed with anti-GFP antibodies to detect the GFP-C2Lact fusion protein and anti-Pgk1 antibodies as the loading control. (C) Equivalent numbers of WT (BY4741), isogenic dnf1Δ dnf2Δ dnf3Δ (PFY3272C), dnf1Δ dnf2Δ (YEL09), dnf1Δ dnf3Δ (YEL010), and dnf2Δ dnf3Δ (YEL013) cells were each plated as a lawn in top agar on YPD plates, and sterile filter paper disks onto which 10 μl of a stock solution (8 mM) of duramycin had been spotted were immediately placed and incubated for 2 d at 30°C. After incubation, the plates were photographed. Response of dnf2Δ dnf3Δ cells (not shown) closely resembled that of dnf1Δ dnf3Δ cells.

**Reruitment 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 shmooes 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 flippases creates a PM milieu that is less conducive to Ste5 recruitment. However, when overexpressed, the GFP-tagged versions of both...
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)P2 (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).
of endogenous Ste5, overexpression of a Ste5 mutant, Ste5(R407S K411S), unable to interact stably with the PM due to lack of a functional PH domain (Garrenton et al., 2006, 2010), was unable to support robust shmoo formation in either wild-type or dnf1Δ dnf2Δ dnf3Δ cells (Figure 7A, right). As one means to assess PM PtdIns(4,5)P2 content and distribution, we first examined localization of a fluorescence probe (GST-GFP-PH(123)) that contains the PH domain derived from mammalian PLCδ1, which is highly specific for recognition of PtdIns(4,5)P2 (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 of 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).

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

PtdIns4P is converted to PtdIns(4,5)P2 by the sole Ptdlns4P 5-kinase in yeast Mss4 (Audhya and Emr, 2003; Strahl and Thorner, 2007), and an mss4Δ mutant fails to recruit Ste5 to the PM and is unable to respond to pheromone at the nonpermissive temperature (Garrenton et al., 2010). When Ste5 cannot be stably tethered at the PM, it is subject to rampant degradation in the nucleus (Garrenton et al., 2009). Hence inefficient generation of PM PtdIns(4,5)P2 could explain both the lower level of Ste5 and the signaling defect observed in dnf1Δ dnf2Δ dnf3Δ cells. For these reasons, we examined the subcellular localization of Mss4.

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 \text{ cells}] \(WT, 2.6 \pm 0.5; \text{dnf1Δ dnf2Δ dnf3ΔA, } 0.7 \pm 0.2; \text{Figure 8A, left, top})

Immunoblotting demonstrated that this difference in PM decoration by Mss4-GFP was not due to any difference in expression...
microscopy. Scale bar, 5 μm.

SCGal-Ura medium for 3 h, and then examined by fluorescence microscopy.

The 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.

FIGURE 9: Phosphoinositide distribution is markedly perturbed in dnf1A dnf3A dnf2A cells. URA3-marked plasmids expressing under the GAL1 promoter either GST-GFP-PH\(^{\text{Gala}}\) (top), (PH\(^{\text{Gala}}\))\(^2\)-GFP (middle), or Mss4-GFP (bottom) were introduced into wild-type cells BY4741 or an otherwise isogenic dnf1A dnf3A dnf2A (ZHY708) triple mutant, and the resulting transformants were grown to mid–exponential phase in SCGal-Ura, collected, resuspended in SCGal-Ura medium for 3 h, and then examined by fluorescence microscopy. Scale bar, 5 μm.

We found that another flippase triple mutant, dnf1A dnf3A dnf2A cells, also was defective in pheromone response (Figure 2, A and B), although not quite as severe as dnf1A dnf2A dnf3A cells.

However, dnf1A dnf3A dnf2A 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 decoration and nuclear localization, we examined the distribution of Mss4-GFP in dnf1A dnf2A dnf3A cells, which appeared quite comparable to those in control cells (Supplemental Figure S8). Hence the failure of shmoo formation in dnf1A dnf2A dnf3A cells is not likely an indirect consequence of defects in the actin cytoskeleton.

Distribution of phosphoinositides is grossly aberrant in dnf1A dnf3A dnf2A cells

Through its generation of PtdIns(4,5)\(^2\)P, Mss4 has been implicated in the establishment of cell polarity (Orlando et al., 2008; Yakir-Tamang and Gerst, 2009). Given the aberrations in Mss4 localization we observed in dnf1A dnf2A dnf3A 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 was observed in wild-type cells, as also observed previously (Audhya et al., 2005). Indeed, consistent with the lower level of inner-leaflet PtdSer in dnf1A dnf2A dnf3A cells being responsible for the poor PM recruitment and mislocalization of Mss4, we found that, in cho1A 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; cho1A, 0.74 ± 0.11) and very prominently to the same internal, apparently vesicular compartment seen in the dnf1A dnf2A dnf3A mutant (Figure 8C).

We overexpressed GST-GFP-PH\(^{\text{Gala}}\) on a multiple-copy plasmid in dnf1A dnf2A dnf3A cells, and the resulting transformants were grown to mid–exponential phase in SCGal-Ura medium for 3 h, and then examined by fluorescence microscopy. Scale bar, 5 μm.

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

pheromone was now close to 90% of that of the wild-type cells carrying empty vector (Figure 8B).

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PM and hence readily explain the signaling defect observed in \( \Delta \text{dnf1}\Delta \text{dnf3}\Delta \text{drs2}\Delta \) cells.

Similarly, localization of the Ptdlns4P-specific probe in \( \Delta \text{dnf1}\Delta \text{dnf3}\Delta \text{drs2}\Delta \) 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 \( \Delta \text{dnf1}\Delta \text{dnf3}\Delta \text{drs2}\Delta \) cells, vesicle-mediated outbound 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 Dnf3, causes defects in secretory vesicle formation and trafficking from the Golgi 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 lipid flippase family in yeast (Daleke, 2007; Tanaka et al., 2011; Lopez-Marques et al., 2013) 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 \( \Delta \text{dnf1}\Delta \text{dnf2}\Delta \text{dnf3}\Delta \) cells, we traced the primary problem to inefficient PM recruitment of Mss4 and consequent reduction in Ptdlins(4,5)P2. Stable PM recruitment of Ste5 requires its Ptdlins(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 Ptdlins(4,5)P2 explains both the lower level of Ste5 and the signaling defect observed in \( \Delta \text{dnf1}\Delta \text{dnf2}\Delta \text{dnf3}\Delta \) 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 Stt4, including Sfk1 (Audhya and Emr, 2002), Ypp1, and Efr3 (Baird et al., 2008), no such factor has yet been implicated in formation of the PM-associating puncta that contain Mss4, which are distinct from those harboring Stt4 (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 \( \Delta \text{dnf1}\Delta \text{dnf3}\Delta \text{drs2}\Delta \) 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 \( \Delta \text{dnf1}\Delta \text{dnf2}\Delta \text{dnf3}\Delta \) cells. As shown here, the bulk of the Ptdlins(4,5)P2 and of its precursor (Ptdlins4P) remains on internal membranes and thus is not efficiently delivered to the PM. In this regard, Pik1-generated Ptdlins4P 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-derived transport vesicles when Golgi membranes are sufficiently enriched in Ptdlins4P. Similarly, Osh4, which specifically binds Ptdlins4P, 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 Ptdlins(4,5)P2 in \( \Delta \text{dnf1}\Delta \text{dnf3}\Delta \text{drs2}\Delta \) cells may be due, in large measure, to inefficient delivery of its precursor Ptdlins4P, further suggesting that the PM-associated Ptdlins 4-kinase Stt4 is not sufficient to perform this task in these cells.

In any event, Ptdlins(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 preference for interaction with Ptdlins(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 \( \Delta \text{dnf1}\Delta \text{dnf2}\Delta \text{dnf3}\Delta \) cells, the steady-state level of Ste5 in \( \Delta \text{dnf1}\Delta \text{dnf3}\Delta \text{drs2}\Delta \) cells was not markedly reduced. However, because neither Ptdlins(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-associate passenger proteins, especially the next enzyme (MAPKK Ste11) in the MAPK cascade. These considerations likely explain the signaling deficiency of \( \Delta \text{dnf1}\Delta \text{dnf3}\Delta \text{drs2}\Delta \) 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 \( \Delta \text{dnf1}\Delta \text{dnf3}\Delta \text{drs2}\Delta \) 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 relocalization 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 Dns2 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 PtdEth (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 \( \Delta \text{dnf1}\Delta \text{dnf2}\Delta \text{dnf3}\Delta \) cells, as judged by the GFP-C2ααα probe. Our result is also in accord with a study showing no change in total PtdSer content in a \( \Delta \text{dnf1}\Delta \text{dnf2}\Delta \text{dnf3}\Delta \) 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 actin cables that direct highly polarized secretion to support mating projection formation (Bidlingmaier and Snyder, 2004; Pruyne et al., 2004).

In naive cells, Dnf1 and Dnf2 are localized throughout the PM perimeter, especially within daughters once cells have budded and at the bud neck and site of cell separation late in the cell cycle, whereas the majority of Dnf3 is in internal vesicles. The observed changes in localization during cell cycle progression and in response to pheromone (when the cells are arrested in G1) suggest that Dnf1, Dnf2, and Dnf3 localization and perhaps function are under control by the cell cycle machinery. In this regard, Dnf3 was identified among likely targets of Cdk1-Clb2 in an unbiased in vitro screen for novel substrates (Ubersax et al., 2003). In addition, the flipase-activating protein kinase Fpk1 is itself under negative regulation by stress-induced protein kinase Ypk1 (Roelants et al., 2010) and cell cycle–dependent protein kinase Gin4 (Roelants et al., 2015). Whether Dnf1, Dnf2, and/or Dnf3, or Fpk1, Ypk1, or Gin4, are also under direct control of the pheromone-activated MAPK Fus3 remains important to investigate.

As shown here, PM lipid remodeling mediated by flipases is important for establishing conditions permissive for activation of the mating pheromone response machinery. Moreover, our studies revealed a previously unappreciated interplay between membrane phosphoinositide composition and the leaftlet distribution of other classes of glycerophospholipids. It is possible that an adequate content of glycerophospholipids like PtdEth and PtdSer is necessary because their amino groups are positioned to serve as counterions and PtdIns(4,5)P2 have been implicated in PM recruitment of pro-

**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′-TACCTCTTATCTTATACACTAAGGAAAGAAA-

**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 flipases 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 20 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-myC (mAb 9E10; Evan et al., 1985); rabbit polyclonal anti-Cln2 (CLN2-9099; gift of Karl Kuchler, Medical University of Vienna, Austria); rabbit polyclonal anti-Cln3 (gift of Doug Kellogg, University of California, Santa Cruz, 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

Routine, 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 \( \alpha \)-factor (10 \( \mu \)M 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 \( \alpha \)-factor (10 \( \mu \)M final concentration) for, typically, 60 min, and then quantifying the level of \( \beta \)-galactosidase activity present using a colorimetric substrate as described previously (Bardwell et al., 1998).

### Table 1: Yeast strains used in this study.

| Strain | Genotype or description | Reference or source | Strain | Genotype or description | Reference or source |
|--------|------------------------|---------------------|--------|------------------------|---------------------|
| BY4741 | MATa his3a1 leu2a0 met15a0 ura3a0 | Brachmann et al. (1998) | YEB1  | BY4741 DNF3-GFP::URA3 | This study |
| ZHY708 | BY4741 drs2a::LEU2 dnf1a::KanMX4 dnf3a::KanMX4 | Hua et al. (2002) | YEB2  | BY4741 DRS2-mCherry::UR3 | This study |
| PFY3272C | BY4741 dnf1a::KanMX4 dnf2a::KanMX4 dnf3a::KanMX4 | Hua et al. (2002) | YEB6  | BY4741 NEO1-GFP::UR3 | This study |
| YLG32  | BY4741 FUS1prom::FUS1-lacZ::URA | Garrenton et al. (2009) | YFR191| BY4741 fpk1a::KanMX4 | Roelants et al. (2010) |
| YEO4   | BY4741 drs2a::LEU2 dnf1a::KanMX4 dnf3a::KanMX4 FUS1prom::FUS1-LacZ::URA | This study | YFR222| BY4741 fpk2a::KanMX4 | Roelants et al. (2010) |
| YEO3   | BY4741 dnf1a::KanMX4 dnf2a::KanMX4 dnf3a::KanMX4 FUS1prom::FUS1-LacZ::URA | This study | YFR205| BY4741 fpk1a::KanMX4 | Roelants et al. (2010) |
| YEO9   | BY4741 dnf1a::KanMX4 dnf2a::KanMX4 dnf3a::KanMX4 | This study | JTY6142| BY4741 ypk1a::KanMX4 | Research Genetics |
| YEO10  | BY4741 dnf1a::KanMX4 dnf2a::KanMX4 dnf3a::KanMX4 | This study | | | |
| YEO13  | BY4741 dnf2a::KanMX4 dnf3a::KanMX4 | This study | | | |
| YEO12  | BY4741 drs2a::KanMX4 dnf1a::KanMX4 dnf3a::KanMX4 | This study | | | |
| YEO11  | BY4741 drs2a::KanMX4 dnf1a::KanMX4 dnf3a::KanMX4 | This study | | | |
| YEO5   | BY4741 dnf1a::KanMX4 dnf2a::KanMX4 dnf3a::KanMX4 | This study | | | |
| YEO6   | BY4741 dnf2a::KanMX4 dnf3a::KanMX4 | This study | | | |
| YEO7   | BY4741 dnf3a::KanMX4 | This study | | | |
| YEO8   | BY4741 dnf2a::LEU2 | This study | | | |
| NRY921 | BY4741 DNF1-GFP::HISMX6 | Rockwell et al. (2009) | | | |
| NRY923 | BY4741 DNF2-GFP::HISMX6 | Rockwell et al. (2009) | | | |

**Table 1:** Yeast strains used in this study.
To visualize fusion proteins marked with GFP or mCherry, cells were grown to mid-exponential phase and viewed directly under an epi-fluorescence microscope (Model BH-2; Olympus America, Center Valley, PA) using a 100× objective equipped with appropriate band-pass filters (Chroma Technology, Rockingham, VT). Images were collected using either an Olympus MH-228 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 PlanSApo 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.

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**Fig. S1. Localization and level of flippase Neo1 before and after pheromone treatment.**

(A) Cells (YEB6) expressing Neo1-GFP from its native promoter at its normal chromosomal locus as the sole source of this protein were grown in YPD medium to mid-exponential phase, exposed to α-factor (10 µM final concentration) for the indicated time and viewed by fluorescence microscopy. Scale bar, 5 µm. (B) Cells (YEB7) expressing Neo1-myc13 from its native promoter at its normal chromosomal locus as the sole source of this protein were withdrawn at the indicated times after exposure to 10 µM α-factor and whole-cell extracts were prepared, resolved by SDS-PAGE, and analyzed by immunoblotting with either an anti-myc epitope mAb or by staining of the same region of the gel with Coomassie Blue dye as a loading control (Neo1 is such a large protein that its entry into the gel requires very long running times during which our standard loading control, Pgk1, migrates off the bottom of the gel).
Fig. S2. Flippase redistribution in response to pheromone. To examine flippase localization, cells (BY4741) carrying an integrated copy of Dnf1-GFP (NRY921), Dnf3-GFP (YEB1) or Drs2-mCherry (YEB2) were grown to mid-exponential phase, incubated with 10 µM α-factor for the indicated time, and examined by fluorescence microscopy using a spinning disk confocal microscope (see Supplemental Methods). Representative images (in some panels, collages of representative images) are the sum of the Z-stack analysis after deconvolution.
Fig. S3. Flippase protein level does not change in response to pheromone. Cells (BY4741) expressing Dnf1-myc (YEB4), Dnf2-GFP (NRY923), Dnf3-myc (YEB5) or Drs2-myc (YEB3), as indicated, were grown to mid-exponential phase in YPD medium, incubated with 10 µM α-factor, and harvested at the indicated time. Whole-cell extracts were prepared, resolved by SDS-PAGE, and analyzed by immunoblot with either an anti-myc epitope mAb or polyclonal anti-GFP antibodies, or anti-Pgk1 antibodies as a loading control.
Fig. S4. Comparison of morphological parameters of wild-type and \textit{dnf1}Δ \textit{dnf2}Δ \textit{dnf3}Δ cells. Digital images of fields of WT (n = 100) and \textit{dnf1}Δ \textit{dnf2}Δ \textit{dnf3}Δ (n = 110) cells expressing plasmid pGFP-C2\textsuperscript{Lact} to highlight the plasma membrane were analyzed using ImageJ software (Collins, 2007), which permits measurement of the maximum and minimum dimensions (diameters) of any selected area and calculates the ratio. Selecting mother cells with large buds, a line was drawn around the perimeter of the mother cell and, likewise, its bud. Measurement of the perimeter of the mother cell gives an estimate of its approximate size, measurement of the ratio of its area to its longest axis \(4\times\text{Area}/\pi\times\text{[major axis]}^2\) gives an estimate of its approximate roundness, and measurement of ratio of the longest axis to the shortest axis of the bud (aspect ratio: [major axis]/[minor axis]) gives an estimate of its degree of elongation. The values for each of these parameters was set at 100 for WT cells and the values for \textit{dnf1}Δ \textit{dnf2}Δ \textit{dnf3}Δ cells then normalized accordingly. Error bars represent standard deviation of the mean. Comparing \textit{dnf1}Δ \textit{dnf2}Δ \textit{dnf3}Δ cells to otherwise isogenic WT cells, the mother cells are slightly larger, more elongated (less round), and their buds are also more elongated.
FIG. S5. Flippase-deficient cells have a lower level of Clb2. WT (BY4741) and otherwise isogenic \( dnf1\Delta \ dnf2\Delta \ dnf3\Delta \) cells (PFY3272C) were grown to mid-exponential phase, collected, lysed and equal amounts of protein from the resulting whole-cell extracts were resolved by SDS-PAGE and analyzed by immunoblotting with appropriate antibodies.
**Fig. S6. Cdc24 is sequestered at the bud tip in dnf1Δ dnf2Δ dnf3Δ cells.**

*LEU2*marked *CEN* plasmid expressing GFP-Cdc24 from the Met-repressible *MET25* promoter was introduced by DNA-mediated transformation into WT cells (BY4741) (*upper panels*) and otherwise isogenic *dnf1Δ dnf2Δ dnf3Δ* cells (PFY3272C) (*lower panels*), and the resulting transformants were grown to mid-exponential phase in SCGlc-Leu medium, collected, resuspended in SCGlc-Leu-Met medium for 3 h, and then viewed by fluorescence microscopy. Cells representative of the indicated cell cycle stage (*n* = 150) were scored and binned according to the pattern of GFP-Cdc24 displayed; a representative image of the GFP-Cdc24 pattern that was exhibited by the majority of the cells at the indicated cell cycle stage are shown (values represent the fraction of the population that displayed the pattern shown).
Fig. S7. Recruitment of Ste5 and Ste5 mutants to the plasma membrane at the shmoo tip in pheromone-treated cells.

Plasmids expressing from the GAL1 promoter either Ste5-GFP (pC52) or the hyperactive allele Ste5(P44L)-GFP (pCS18) were introduced by DNA-mediated transformation into WT (BY4741) and dnf1Δ dnf2Δ dnf3Δ cells (P FY3272C), as indicated, which were grown to mid-exponential phase in SCGlc-Ura, collected, resuspended in SCGal-Ura, propagated for 3-4 h, then incubated with 10 µM α-factor for 1.5 h, and examined by fluorescence microscopy. Similarly, a plasmid expressing from the GAL1 promoter Ste5(R407S K411S)-GFP (pLG35), which in defective in stable plasma membrane associated due to mutation of its PH domain, was introduced into ste5Δ derivatives of WT cells (YELO23) and dnf1Δ dnf2Δ dnf3Δ cells (YELO24), which were grown to mid-exponential phase in SCGlc-Leu, collected, resuspended in SCGal-Leu, propagated for 3 h, then incubated with 10 µM α-factor for 1.5 h, and examined by fluorescence microscopy. Arrows, Ste5 localization at the shmoo tip.
Fig. S8. Organization of the actin cytoskeleton in WT and dnf1Δ dnf2Δ dnf3Δ cells. WT (BY4741) and otherwise isogenic dnf1Δ dnf2Δ dnf3Δ cells (PFY3272C) were grown to mid-exponential phase in YPD, fixed with formaldehyde for 1.5 h, washed and incubated for 30 min with Alexa Fluor®-488-labelled phalloidin, as described in Materials and Methods, and then examined by fluorescence microscopy.
Fig. S9. Ste5 protein is not unstable in dnf1Δ dnf3Δ drs2Δ cells.
Cultures of WT (BY4741), dnf1Δ dnf2Δ dnf3Δ (PFY3272C) and dnf1Δ dnf3Δ drs2Δ (ZHY708) cells were grown to mid-exponential phase, collected, lysed and samples from these whole-cell extracts containing equivalent amounts of protein were resolved by SDS-PAGE and analyzed by immunoblotting with polyclonal rabbit anti-Ste5 antibody and with polyclonal rabbit anti-Pgk1 antibody as a loading control.