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Journal

Molecular Biology of the Cell, 26(1)

ISSN

1059-1524

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Publication Date

2015

DOI

10.1091/mbc.e14-07-1193

Peer reviewed

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 *dnf1 Δ dnf2 Δ dnf3 Δ* 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 *dnf1 Δ dnf3 Δ drs2 Δ* 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.

Monitoring Editor

Benjamin S. Glick
University of Chicago

Received: Jul 7, 2014

Revised: Oct 17, 2014

Accepted: Oct 28, 2014

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 phosphatidylcholine and sphingolipids, whereas the inner leaflet is enriched in phosphatidylethanolamine (PtdEth), phosphatidylserine (PtdSer), and phosphatidylinositol (PtdIns) and its phosphorylated derivatives (especially phosphatidylinositol 4-phosphate [PtdIns4P] and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] (Devaux, 1991; Vance and Steenbergen, 2005; Fadeel and Xue, 2009). Moreover, this lipid constitution is important for many PM functions, including nutrient transport (Divito and Amara, 2009), endocytosis (Platta and Stenmark, 2011), cell signaling (Groves and Kuriyan, 2010), and cytokinesis (Emoto *et al.*, 2001; Luo *et al.*, 2009). Phosphoinositides, in particular, have been implicated in polarity establishment (Shewan *et al.*, 2011), cytoskeletal dynamics (Kapus and Janmey, 2013), membrane trafficking (Di Paolo and De Camilli, 2006),

This article was published online ahead of print in MBoc in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E14-07-1193>) on November 5, 2014.

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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; PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol-4,5-bisphosphate; PtdSer, phosphatidylserine.

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Supplemental Material can be found at:
<http://www.molbiolcell.org/content/suppl/2014/11/03/mbc.E14-07-1193v1.DC1.html>

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 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 (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 ("flipping") 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 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.*, 2002; Noji *et al.*, 2006); related proteins Crf1 (393; 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 paralogue, 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 (Iwamoto *et al.*, 2004) and PtdSer become 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 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 Thorne, 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

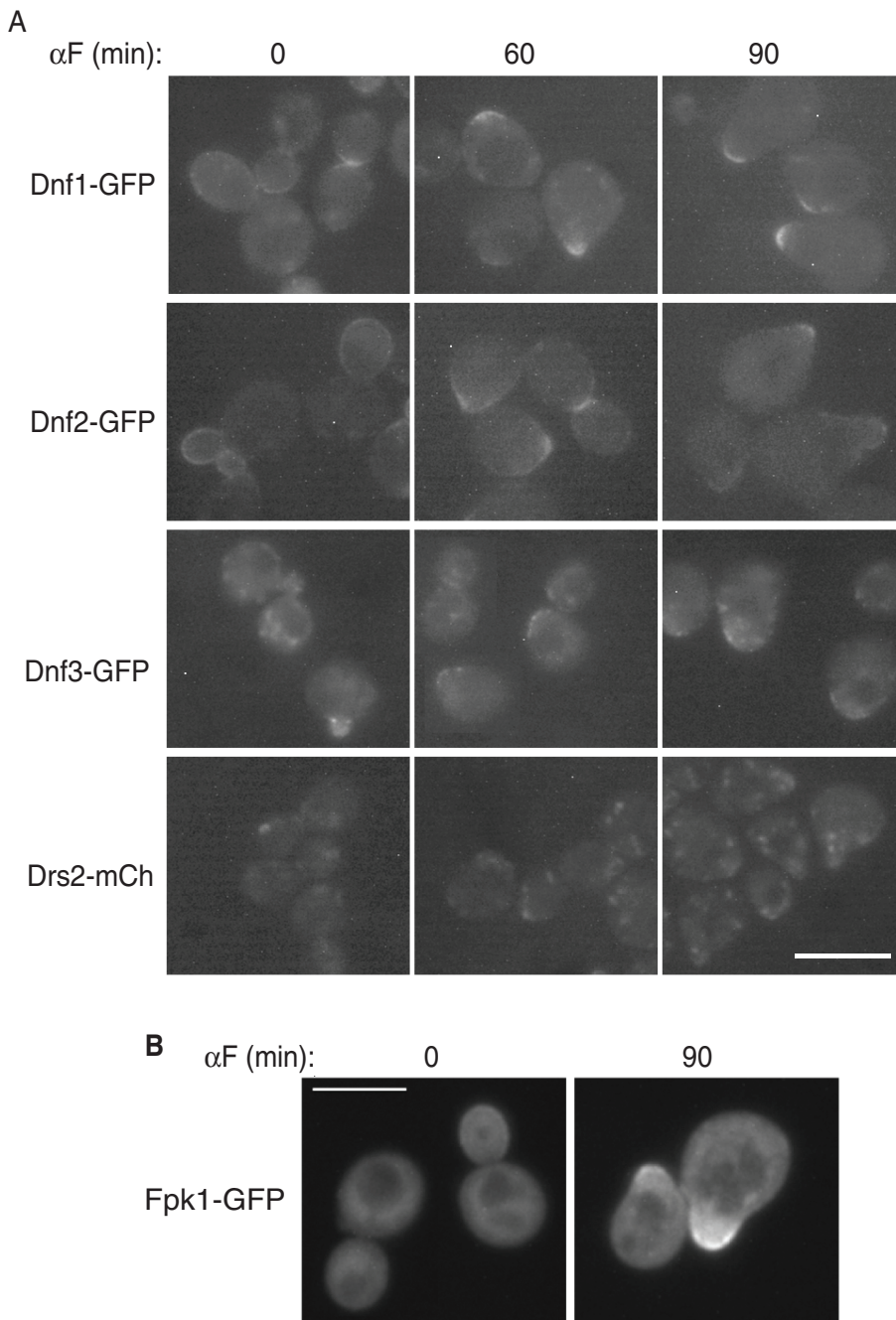


FIGURE 1: Flippase and flippase kinase localization in pheromone-treated cells. (A) MATa cells expressing the indicated flippase from its native promoter at its normal chromosomal locus as the sole source of each protein, Dnf1-GFP (NRY921), Dnf2-GFP (NRY923), Dnf3-GFP (YEB1), and Drs2-mCherry (YEB2), were grown to mid-exponential phase, exposed to α -factor (10 μ M 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 μ m.

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 ap-

proach to monitor localization of Fpk1 and found that it too becomes markedly concentrated at the shmoo tip in pheromone-treated cells (Figure 1B).

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 Δ dnf2 Δ dnf3 Δ drs2 Δ* 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 Δ* , *dnf2 Δ* , *dnf3 Δ* , and *drs2 Δ* 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 *dnf3 Δ drs2 Δ* cells (Figure 2A). In contrast, and in agreement with a largely shared function, we found that two triple mutants, *dnf1 Δ dnf3 Δ drs2 Δ* and especially *dnf1 Δ dnf2 Δ dnf3 Δ* , 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 Δ dnf3 Δ drs2 Δ* cells and especially the *dnf1 Δ dnf2 Δ dnf3 Δ* 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.

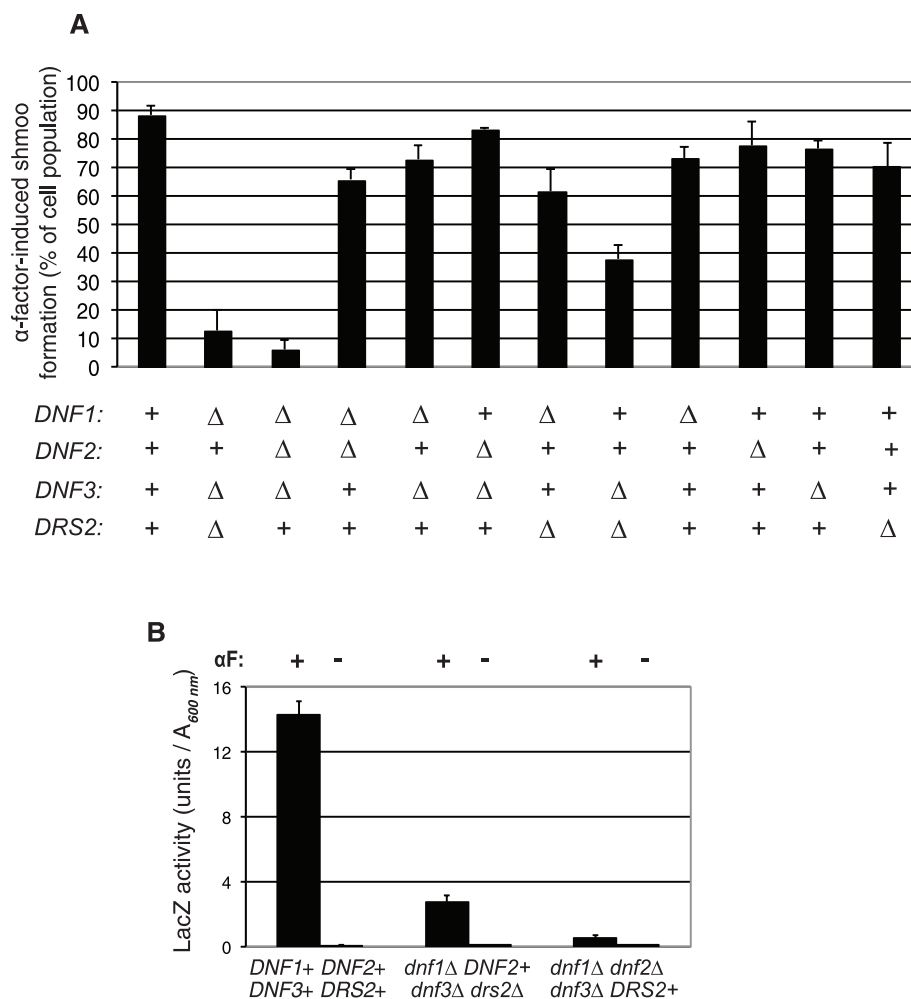


FIGURE 2: Deletion of multiple flippases with overlapping function impairs pheromone response. (A) Exponentially growing cultures of cells of the indicated genotype (see Table 1) were exposed to 10 μ M α -factor for 1.5 h and examined by microscopy. Values are the mean $\pm$ SD of three independent experiments. (B) WT (YLG32), *dnf1Δ dnf3Δ drs2Δ* (YELO4), and *dnf1Δ dnf2Δ dnf3Δ* (YELO3) cells expressing a copy of a *FUS1_{prom}-lacZ::URA3* reporter (pSB286) integrated at the *FUS1* locus were grown to mid-exponential phase, collected, resuspended in either YPD or YPD plus 10 μ M α -factor, and, after 60 min, assayed for galactosidase activity. Values are the mean $\pm$ SD from three independent experiments.

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 neither 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 action 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 *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., 2009; 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* (Figure 4A) or *dnf1Δ dnf3Δ drs2Δ* 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 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 G $\beta\gamma$ 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 G $\beta\gamma$ 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 G $\beta\gamma$ was overexpressed. Therefore we concentrated our attention on the factors that act at the nexus between receptor activation and the function of G $\beta\gamma$.

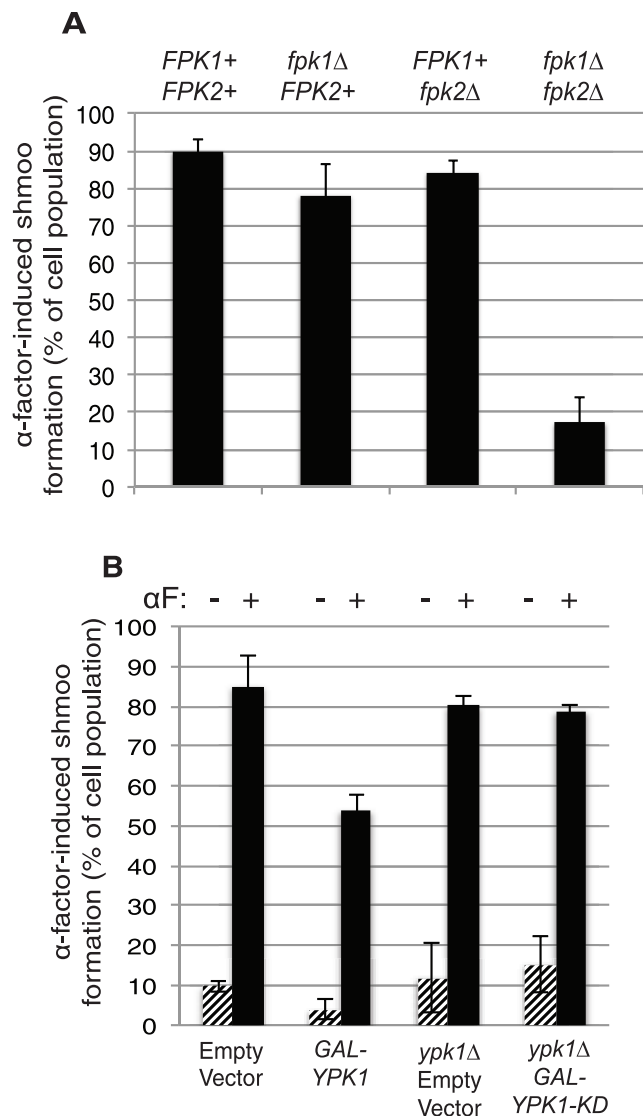


FIGURE 3: Fpk1 and Fpk2 function are required for optimal pheromone response. (A) Cultures of WT (BY4741), *fpk1Δ* (YFR191), *fpk2Δ* (YFR222), and *fpk1Δ fpk2Δ* (YFR205) cells were grown to mid-exponential phase in YPD medium, treated with 10 μ M α -factor for 1.5 h, and examined by microscopy. (B) WT cells (BY4741) carrying empty vector (YE352GAL) or the same vector overexpressing Ypk1 (pAM76), or *ypk1Δ* cells (JTY6142) carrying YE352GAL 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 μ M α -factor for 1.5 h, and examined by microscopy. Values are the mean $\pm$ SD from three independent experiments.

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 G β γ (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

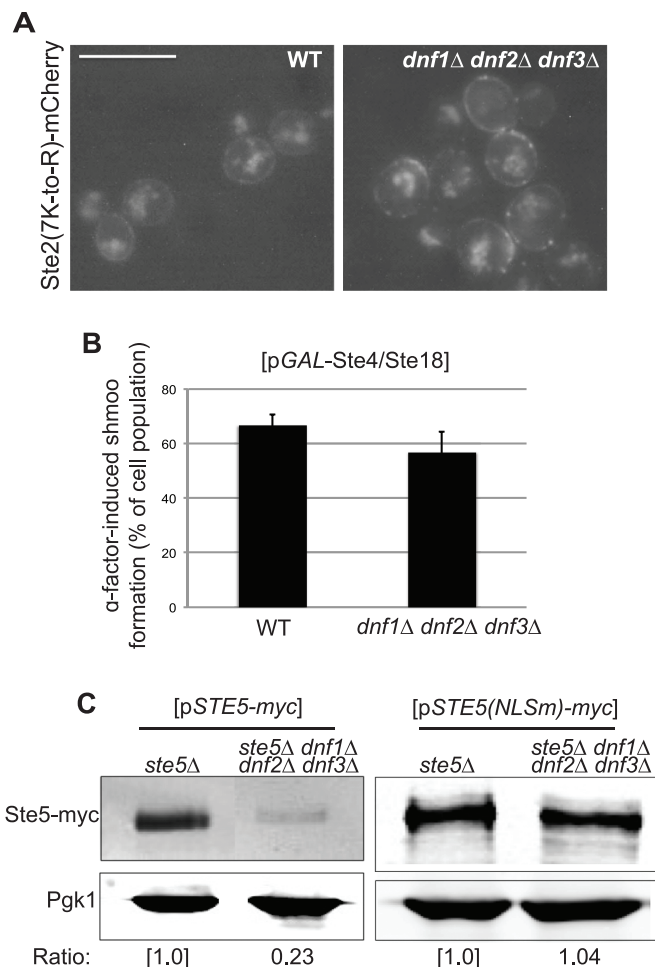


FIGURE 4: Steady-level of Ste5 is markedly decreased in *dnf1Δ dnf2Δ dnf3Δ* cells. (A) WT (YELO17) and *dnf1Δ dnf2Δ dnf3Δ* (YELO18) cells expressing Ste2(7K-to-R)-mCherry from the STE2 promoter at the STE2 locus were grown to mid-exponential phase in YPD and examined by fluorescence microscopy. Scale bar, 10 μ m. (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 $\pm$ SD from three independent trials. (C) Cultures of *ste5Δ* (YELO23) and *ste5Δ dnf1Δ dnf2Δ dnf3Δ* (YELO24) expressing either Ste5-myc from plasmid pSTE5_{prom}-STE5-myc₁₃ or Ste5(NLSm)-myc from plasmid pSTE5_{prom}-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.

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.

Ste5, which undergoes robust nucleocytoplasmic shuttling (Pryciak 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^{Cdc4} (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 *dnf1Δ dnf2Δ 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 mutationally crippled, the steady-state level of Ste5 in *dnf1Δ dnf2Δ 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 Clb-bound Cdk1 might explain the higher level of Cln2 we observed in *dnf1Δ dnf2Δ dnf3Δ* cells. Consistent with this view, we found a modest but reproducible reduction in the cellular content of Clb2 in the *dnf1Δ dnf2Δ 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 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 *dnf1Δ dnf2Δ*

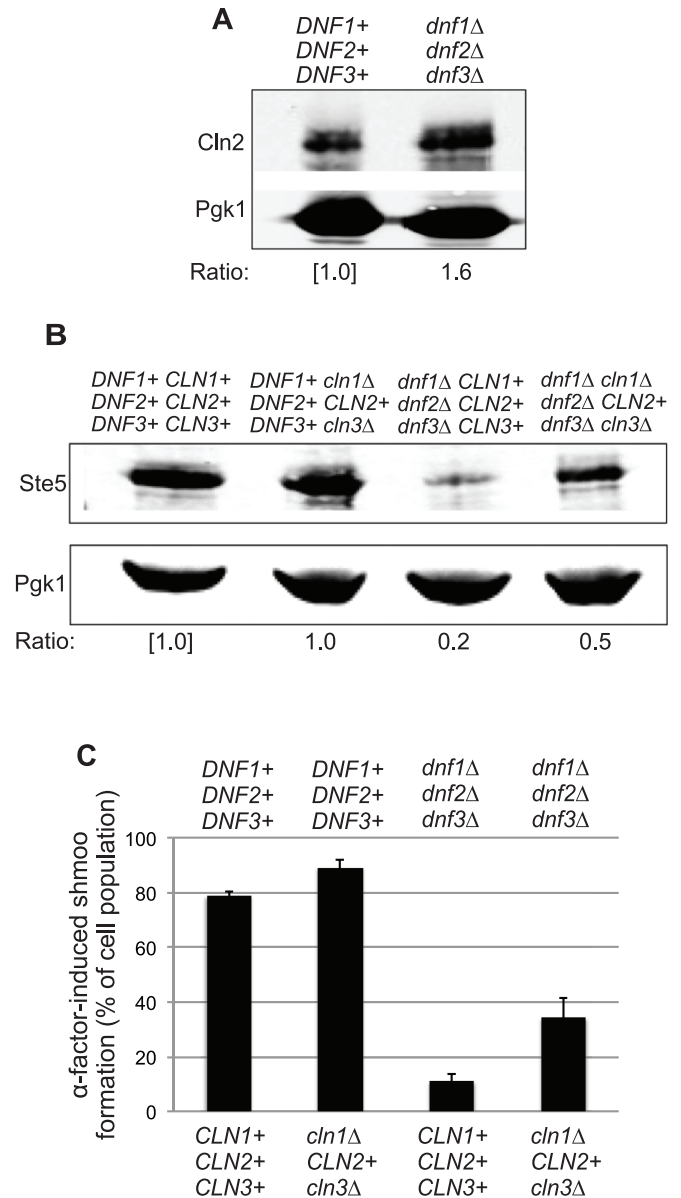


FIGURE 5: Reduction in G1 cyclin activity partially rescues Ste5 protein level and pheromone responsiveness in *dnf1Δ dnf2Δ dnf3Δ* cells. (A) WT (BY4741) and derived *dnf1Δ dnf2Δ dnf3Δ* cells (PFY3272C) were grown to mid-exponential phase in YPD, collected, and lysed, and equal amounts of protein from 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 dnf2Δ dnf3Δ* (PFY3272C), and *cln1Δ cln3Δ dnf1Δ dnf2Δ dnf3Δ* (YELO39) cells. (C) WT (BY4741) and *cln1Δ cln3Δ* (YELO38), *dnf1Δ dnf2Δ dnf3Δ* (PFY3272C), and *cln1Δ cln3Δ dnf1Δ dnf2Δ 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.

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/pss1Δ*) mutant has impaired polarization of Cdc42, causing a delay in bud emergence and defective mating (Fairn *et al.*, 2011).

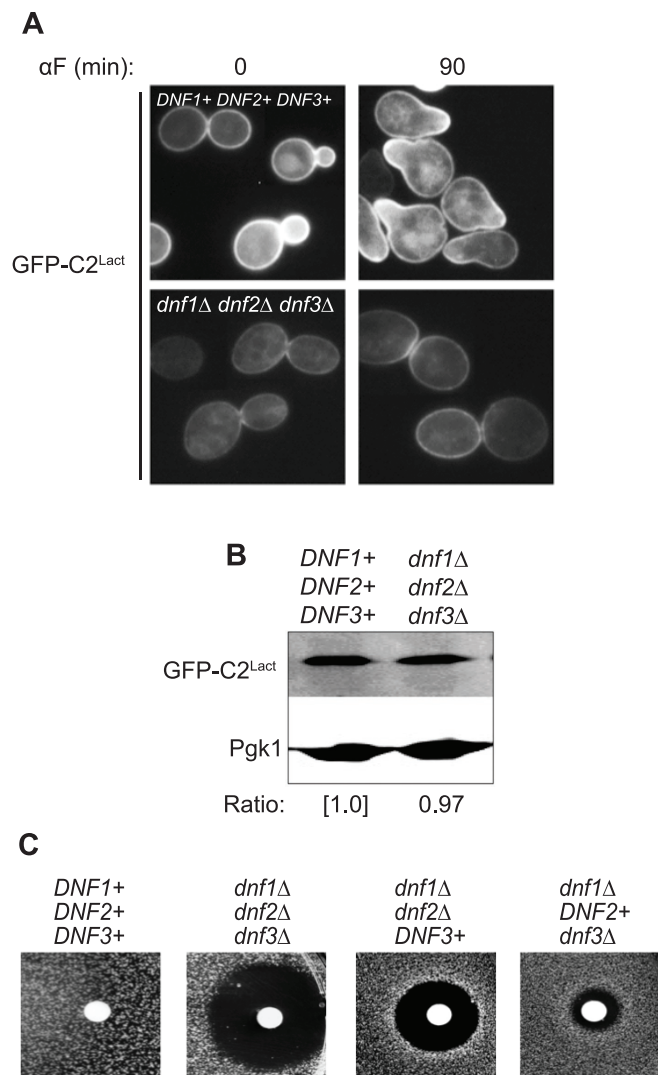


FIGURE 6: Flippase function increases inner-leaflet PtdSer and lowers outer-leaflet PtdEth. (A) WT (BY4741) and isogenic *dnf1Δ dnf2Δ dnf3Δ* (PFY3272C) cells carrying a *URA3*-marked *CEN* plasmid expressing GFP-C2^{Lact} from the *TDH3* (GAPDH/GPD) promoter were grown to mid-exponential phase in SCGlc-Ura medium, treated with 10 μ M 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-C2^{Lact} fusion protein and anti-Pgk1 antibodies as the loading control. (C) Equivalent numbers of WT (BY4741), isogenic *dnf1Δ dnf2Δ dnf3Δ* (PFY3272C), *dnf1Δ dnf2Δ* (YELO9), *dnf1Δ dnf3Δ* (YELO10), and *dnf2Δ dnf3Δ* (YELO13) cells were each plated as a lawn in top agar on YPD plates, and sterile filter paper disks onto which 10 μ l of a stock solution (8 mM) of duramycin had been spotted were immediately placed onto the lawn. After incubation for 2 d at 30°C, the plates were photographed. Response of *dnf2Δ dnf3Δ* cells (not shown) closely resembled that of *dnf1Δ dnf3Δ* cells.

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

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-C2^{Lact} 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 Clb2 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 shmoo 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

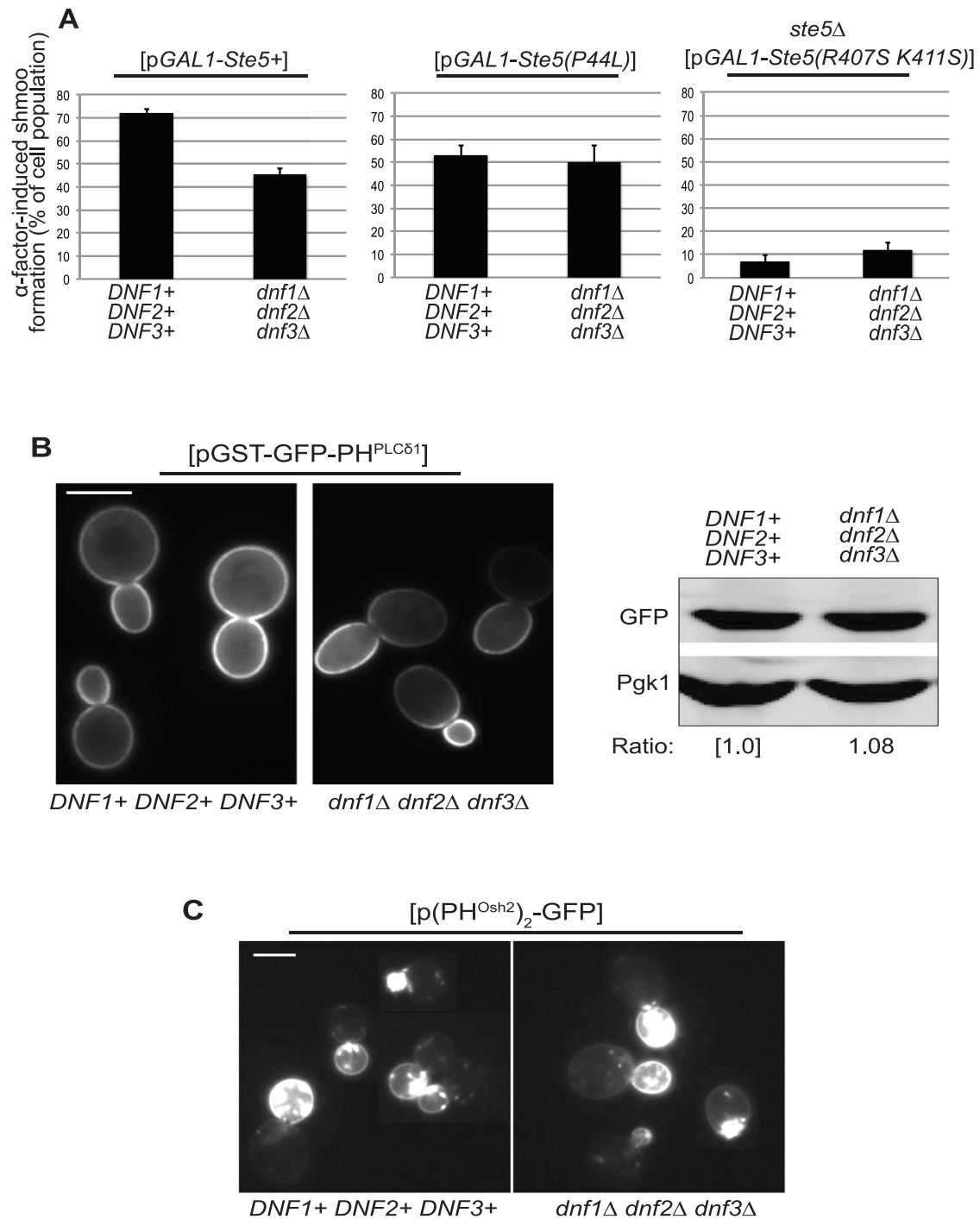


FIGURE 7: Ste5 tethering at the PM is disturbed in *dnf1Δ dnf2Δ dnf3Δ*. (A) Either WT (BY4741) or *dnf1Δ dnf2Δ dnf3Δ* cells (PFY3272C) overexpressing from the GAL1 promoter either Ste5 (pCJ6) or Ste5(P44L) (pCS7), as indicated, from URA3-marked, YEp352-derived plasmids, and *ste5Δ* derivatives of the 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 μ M α -factor for 1.5 h, and examined in the microscope. Values are the mean $\pm$ SD from three independent trials. (B) Left, a URA3-marked *CEN* plasmid (pPP1872) expressing the PtdIns(4,5)P₂-specific probe GST-GFP-PH^{PLCδ1} was introduced into the control and the *dnf1Δ dnf2Δ dnf3Δ* mutant, which were then treated as in A and viewed by fluorescence microscopy. Scale bar, 5 μ m. 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^{Osh2}-GFP.

wild-type Ste5 and the constitutively active Ste5(P44L) allele were recruited to the shmoo tip in the pheromone-treated cells (Supplemental Figure S7).

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

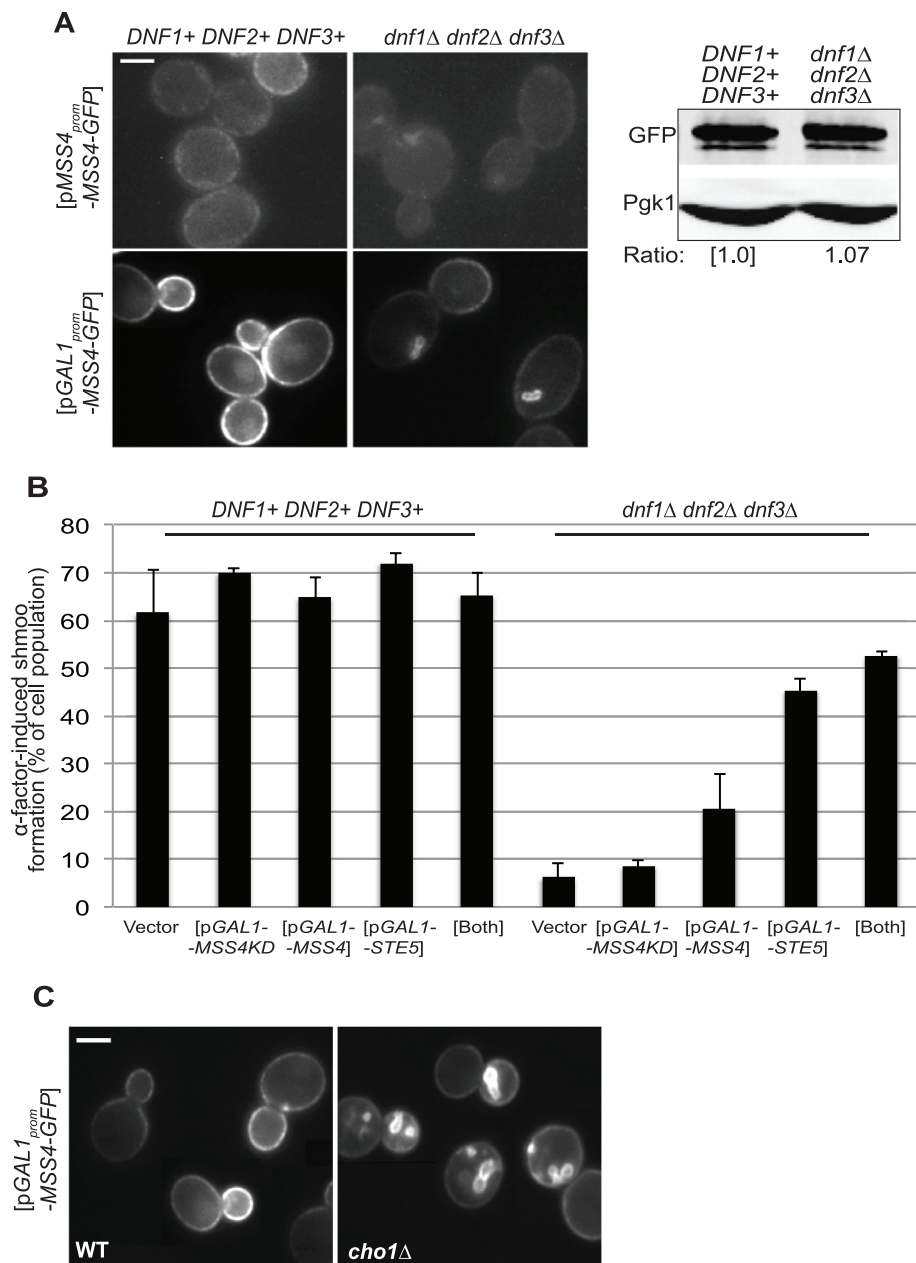


FIGURE 8: Mislocalization of Mss4 in *dnf1Δ dnf2Δ dnf3Δ* cells. (A) Left, *URA3*-marked *CEN* plasmids expressing Mss4-GFP from the *MSS4* promoter (pCS321; top) or from the *GAL1* promoter (pRB2; bottom) were introduced into WT (BY4741) and *dnf1Δ dnf2Δ dnf3Δ* (PFY3272C) cells. The resulting transformants were grown to mid-exponential phase in SCGlc-Ura (top) or shifted to SCGal-Ura for 3 h (bottom) and then examined by fluorescence microscopy. Scale bar, 5 μ m. 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 (YE351GAL) or the same vector expressing from the *GAL1* promoter *MSS4* (pES4), a catalytically inactive (KD) allele Mss4(D636A) (pES20), a *URA3*-marked multicopy vector expressing *STE5* from the *GAL1* promoter (pCJ6), or both the *MSS4*- and *STE5*-expressing plasmids together. The resulting transformants were grown to mid-exponential phase in SCGlc-Leu, SCGlc-Ura, or SCGlc-Leu-Ura, as appropriate, collected, resuspended in SCGal-Leu, SCGal-Ura, or SCGal-Leu-Ura for 3 h, incubated with 10 μ M α -factor for 1.5 h, and examined by microscopy. Values are mean $\pm$ SD from three independent experiments. (C) WT (BY4741) and otherwise isogenic *cho1Δ* (JTY6525) carrying a plasmid (pRB2) expressing *MSS4* under control of the *GAL1* promoter were grown to mid-exponential phase in SCGal-Ura and examined by fluorescence microscopy. Scale bar, 5 μ m.

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^{PLCδ1}) 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 PtdIns4P 5-kinase in yeast Mss4 (Audhya and Emr, 2003; Strahl and Thorner, 2007), and an *mss4^{ts}* 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 cells]: WT, 2.6 $\pm$ 0.5; *dnf1Δ dnf2Δ dnf3Δ*, 0.7 $\pm$ 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

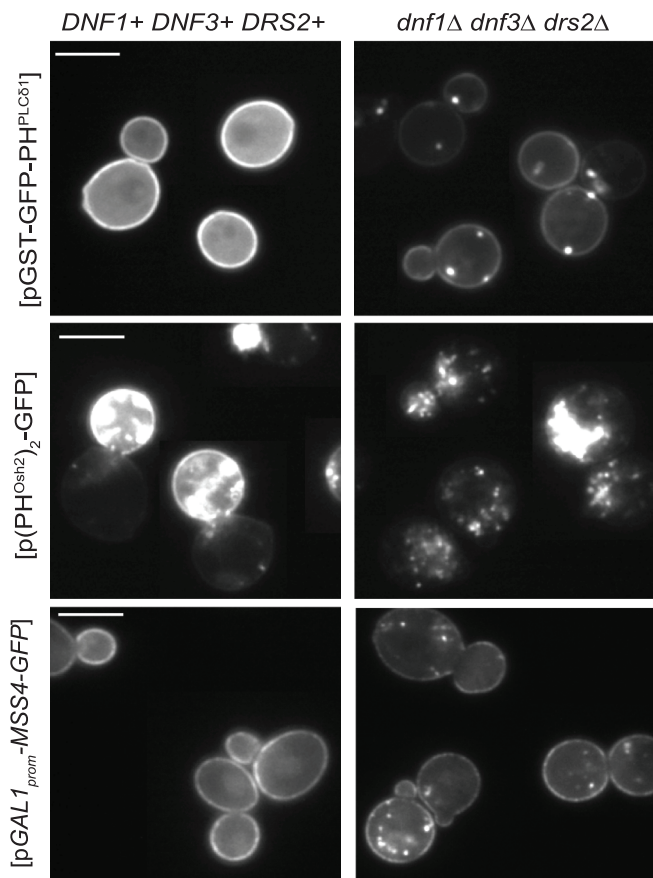


FIGURE 9: Phosphoinositide distribution is markedly perturbed in *dnf1Δ dnf3Δ drs2Δ* cells. *URA3*-marked plasmids expressing under the *GAL1* promoter either GST-GFP-PH^{PLCδ1} (top), (PH^{Osh2})₂-GFP (middle), or Mss4-GFP (bottom) were introduced into wild-type cells (BY4741) or an otherwise isogenic *dnf1Δ dnf3Δ drs2Δ* (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.

(Figure 8A, right). When Mss4-GFP was overexpressed, the PM decoration was much brighter, and some nuclear localization was observed in wild-type cells, as also observed previously (Audhya and Emr, 2003); however, in the *dnf1Δ dnf2Δ dnf3Δ* mutant, the PM decoration was increased only slightly, and a unique, internal, apparently vesicular compartment was prominent (Figure 8A, left, bottom).

If mislocalization of Mss4 and a resulting diminution of PM PtdIns(4,5)P₂ in *dnf1Δ 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 *dnf1Δ 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 shmoo 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 *dnf1Δ dnf2Δ dnf3Δ* cells have lower levels of inner-leaflet PtdSer and PtdEth (Figure 6). 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 phosphatidylserine 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 *dnf1Δ 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 *dnf1Δ dnf2Δ dnf3Δ* mutant (Figure 8C).

Through its generation of PtdIns(4,5)P₂, 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 *dnf1Δ 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 *dnf1Δ dnf2Δ dnf3Δ* cells appeared quite comparable to those in control cells (Supplemental Figure S8). Hence the failure of shmoo formation in *dnf1Δ dnf2Δ dnf3Δ* cells is not likely an indirect consequence of defects in the actin cytoskeleton.

Distribution of phosphoinositides is grossly aberrant in *dnf1Δ dnf3Δ drs2Δ* cells

We found that another flippase triple mutant, *dnf1Δ dnf3Δ drs2Δ*, also was defective in pheromone response (Figure 2, A and B), although not quite as severe as *dnf1Δ dnf2Δ dnf3Δ* cells.

However, *dnf1Δ dnf3Δ drs2Δ* 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)P₂ level and Ste5 stability and function that we uncovered in the course of analyzing the signaling defect in the *dnf1Δ dnf2Δ dnf3Δ* cells, we used the same probes to examine the distribution of PtdIns(4,5)P₂, PtdIns4P, and Mss4 in the *dnf1Δ dnf3Δ drs2Δ* cells.

The PtdIns4P that is converted to PtdIns(4,5)P₂ 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 Pik1 (Strahl and Thorner, 2007). We found that, compared with control cells, the distribution of the PtdIns(4,5)P₂-specific probe was strikingly different in the *dnf1Δ dnf3Δ drs2Δ* mutant. Although there was faint decoration of the PM, the most prominent fluorescent feature was several bright internal puncta (Figure 9, top). In the *dnf1Δ dnf3Δ drs2Δ* 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 *dnf1Δ dnf3Δ drs2Δ* 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)P₂ could be sufficient, by themselves, to compromise the efficiency of Ste5 recruitment to the

PM and hence readily explain the signaling defect observed in *dnf1Δ dnf3Δ drs2Δ* cells.

Similarly, localization of the PtdIns4P-specific probe in *dnf1Δ dnf3Δ drs2Δ* 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 drs2Δ* 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 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 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 *dnf1Δ dnf2Δ dnf3Δ* cells, we traced the primary problem to inefficient PM recruitment of Mss4 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 explains 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 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-associated 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 *dnf1Δ dnf3Δ drs2Δ* 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-derived 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 drs2Δ* 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 preference 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 drs2Δ* 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 (MAPKKK Ste11) in the MAPK cascade. These considerations likely explain the signaling deficiency of *dnf1Δ dnf3Δ drs2Δ* 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 drs2Δ* 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 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 *dnf1Δ dnf2Δ dnf3Δ* cells, as judged by the GFP-C2^{lact} 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 PtdEth 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, Dnf2 was identified among likely targets of Cdk1-Clb2 in an unbiased in vitro screen for novel substrates (Ubersax et al., 2003). In addition, the flippase-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 flippases 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 leaflet 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 for the negatively charged phosphate groups in the head groups of neighboring phosphoinositides (Slochow et al., 2014). PtdIns4P and PtdIns(4,5)P₂ have been implicated in PM recruitment of proteins involved in the cell wall integrity pathway, such as Rho1 and some of its effectors (Levin, 2011). Hence, in addition to mating pheromone response, other specific PM-associated signaling processes may be similarly defective in flippase-deficient cells.

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 SC_{Raf-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-*NdeI* 5'-GGGTAAGTACATGATGAAACACA-TATGAA-GAAA-3', and Ste2-*EcoRI*, TTGTAGAGCATTCATCACCATCT-TAAGCGC-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-1_{prom}-lacZ* reporter gene was accomplished by linearizing pSB286 (Trueheart et al., 1987) with *SphI*, 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'-TACCTCTATACTTTAACGTC AAGGAGAAAA-AACCCCATGTCAGTCTTGCATCACCACCTCT-3', and Mss4-2, 5'-ATGGGTACCCTACCTACTTGATATGTTACTGGTGGCGCCAC-CGCCGCGAGATCTTGATC-3'). The resulting product and *URA3*-marked plasmid pRS316-*GAL1*,10, which had been digested with *Bam*HI and *Spe*I restriction enzymes, were introduced by DNA-mediated transformation (Pham et al., 2011) into BY4741, and Ura⁺ transformants were selected on SC_{Glc}-Ura medium. Candidate clones were extracted, plasmid DNA was recovered in *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" [KD]) 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'-TTATTCCTGTGTAATTTGCATT-CATGACAT-3' and 5'-GCCATTGTATTCAA-TTTAGCAAGCAATTC-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'-TAAGTTTTCCGTATGTTG-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 A_{600nm} 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.

Strain	Genotype or description	Reference or source	Strain	Genotype or description	Reference or source
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Brachmann et al. (1998)	YEB1	BY4741 <i>DNF3-GFP::URA3</i>	This study
ZHY708	BY4741 <i>drs2Δ::LEU2 dnf1Δ::KanMX4 dnf3Δ::KanMX4</i>	Hua et al. (2002)	YEB2	BY4741 <i>DRS2-mCherry::URA3</i>	This study
PFY3272C	BY4741 <i>dnf1Δ::KanMX4 dnf2Δ::KanMX4 dnf3Δ::KanMX4</i>	Hua et al. (2002)	YEB6	BY4741 <i>NEO1-GFP::URA3</i>	This study
YLG32	BY4741 <i>FUS1_{prom}::FUS1-lacZ::URA</i>	Garrenton et al. (2009)	YFR191	BY4741 <i>fpk1Δ::KanMX4</i>	Roelants et al. (2010)
YELO4	BY4741 <i>drs2Δ::LEU2 dnf1Δ::KanMX4 dnf3Δ::KanMX4 FUS1_{prom}::FUS1-LacZ::URA</i>	This study	YFR222	BY4741 <i>fpk2Δ::KanMX4</i>	Roelants et al. (2010)
YELO3	BY4741 <i>dnf1Δ::KanMX4 dnf2Δ::KanMX4 dnf3Δ::KanMX4 FUS1_{prom}::FUS1-LacZ::URA</i>	This study	YFR205	BY4741 <i>fpk1Δ::KanMX4 fpk2Δ::KanMX4</i>	Roelants et al. (2010)
YELO9	BY4741 <i>dnf1Δ::KanMX4 dnf2Δ::KanMX4</i>	This study	JTY6142	BY4741 <i>ypk1Δ::KanMX4</i>	Research Genetics
YELO10	BY4741 <i>dnf1Δ::KanMX4 dnf3Δ::KanMX4</i>	This study	YELO23	BY4741 <i>ste5Δ::HIS3</i>	This study
YELO13	BY4741 <i>dnf2Δ::KanMX4 dnf3Δ::KanMX4</i>	This study	YELO 24	BY4741 <i>ste5Δ::HIS3 dnf1Δ::KanMX4 dnf2Δ::KanMX4 dnf3Δ::KanMX4</i>	This study
YELO12	BY4741 <i>drs2Δ::KanMX4 dnf1Δ::KanMX4</i>	This study	YELO38	BY4741 <i>cln1::HIS3 cln3::LEU2</i>	This study
YELO11	BY4741 <i>drs2Δ::KanMX4 dnf3Δ::KanMX4</i>	This study	YELO39	BY4741 <i>cln1::HIS3 cln3::LEU2 dnf1Δ::KanMX4 dnf2Δ::KanMX4 dnf3Δ::KanMX4</i>	This study
YELO5	BY4741 <i>dnf1Δ::KanMX4</i>	This study	YEB3	BY4741 <i>DRS2-myc13::HIS3MX6</i>	This study
YELO6	BY4741 <i>dnf2Δ::KanMX4</i>	This study	YEB4	BY4741 <i>DNF1-myc13::HIS3MX6</i>	This study
YELO7	BY4741 <i>dnf3Δ::KanMX4</i>	This study	YEB5	BY4741 <i>DNF3-myc13::HIS3MX6</i>	This study
YELO8	BY4741 <i>drs2Δ::LEU2</i>	This study	YEB7	BY4741 <i>NEO1-myc13::HIS3MX6</i>	This study
NRY921	BY4741 <i>DNF1-GFP::HISMX6</i>	Rockwell et al. (2009)	YDB119	BY4741 <i>SST2-GFP::KanMX6 STE2(7K-to-R)-mCherry::CaURA3</i>	Ballon et al. (2006)
NRY923	BY4741 <i>DNF2-GFP::HISMX6</i>	Rockwell et al. (2009)	YELO17	BY4741 <i>STE2(7K-to-R)-mCherry::CaURA3</i>	This study
			YELO18	BY4741 <i>dnf1Δ::KanMX4 dnf2Δ::KanMX4 dnf3Δ::KanMX4 Ste2(7KtoR)-mCherry::CaURA3</i>	This study
			JTY6525	BY4741 <i>cho1Δ::KanMX4</i>	Open Biosystems

TABLE 1: Yeast strains used in this study.

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 antiserum (Thomson et al., 2011; gift of Kirsten Benjamin, 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-Clb2 (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

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 shmoo was assessed after growing the culture to mid–exponential phase, treating it with α -factor (10 μ 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 α -factor (10 μ M 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).

Plasmid	Description	Reference or source
pSB286	<i>FUS1_{prom}::FUS1-LacZ</i>	Trueheart et al. (1987)
pFR150	pRC181- <i>TPI1_{prom}-FPK1-GFP::LEU2</i>	Roelants et al. (2010)
YEp352GAL	2 μ m DNA ORI, <i>URA3</i> , <i>GAL1_{prom}</i> vector	Benton et al. (1994)
pAM76	YEp352- <i>GAL_{prom}-YPK1-myc</i>	Roelants et al. (2002)
pJT1126	YEp352- <i>GAL_{prom}-ypk1(K376A)-myc</i>	Roelants et al. (2002)
pS5Kmyc	<i>STE5_{prom}-STE5-myc13</i>	Winters et al. (2005)
pCS52	YCpUGAL- <i>STE5-GFP</i>	Sette et al. (2000)
pCS7	YEp352- <i>GAL-STE5(P44L)</i>	Sette et al. (2000)
pCS18	YCplac111- <i>GAL1_{prom}-STE5(P44L)-GFP</i>	Sette et al. (2000)
pLG35	YCplac111- <i>GAL1_{prom}-ste5(R407S K411S)-GFP</i>	Garrenton et al. (2006)
pS5Kmyc/NLSm	<i>Ste5_{prom}-ste5(NLSm)-myc</i>	Winters et al. (2005)
pGFP-C2 ^{Lact}	p416- <i>GPD_{prom}-GFP-C2^{Lact}</i>	Addgene #22853
pG-Ste4/18	pRS316- <i>GAL_{prom}-STE4/STE18</i>	Song et al. (1996)
pCJ6	YEp352- <i>GAL1-(His)₆-myc-STE5</i>	Inouye et al. (1997)
pPP1872	pGAL- <i>GST-GFP-PH^{PLCδ1}</i>	Garrenton et al. (2010)
pGFP-OSH2-PH	<i>GFP-2X-PH^{OSH2}</i>	Roy and Levine (2004)
pCS321	pRS416- <i>MSS4_{prom}-MSS4-GFP</i>	Audhya and Emr (2003)
pRB2	pRS316- <i>GAL1-MSS4-GFP</i>	This study
YEp351GAL	2 μ m DNA ORI, <i>LEU2</i> , <i>GAL1_{prom}</i> vector	Benton et al. (1994)
pES4	YEp351- <i>GAL1-MSS4-GFP</i>	This study
pES20	YEp351- <i>GAL1-mss4(D636A)-GFP</i>	This study
pCdc24	p415- <i>MET25_{prom}-GFP-8A-CDC24</i>	Toenjes et al. (1999)

TABLE 2: Plasmids used in this study.

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 $\times$ 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-X1TM; Yokogawa Electric Corp., Newman, GA), a piezo-controlled motorized XYZ stage, and two charge-coupled device cameras. A PlanSApo 1.4 numerical aperture/100 $\times$ 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 μ l 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 μ l of a solution containing Alexa Fluor 488-phalloidin (Life Technologies, Grand Island, NY; 3.3 μ M 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 μ l 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 *dnf1 Δ dnf2 Δ dnf3 Δ* 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.

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Supplemental Materials

Molecular Biology of the Cell

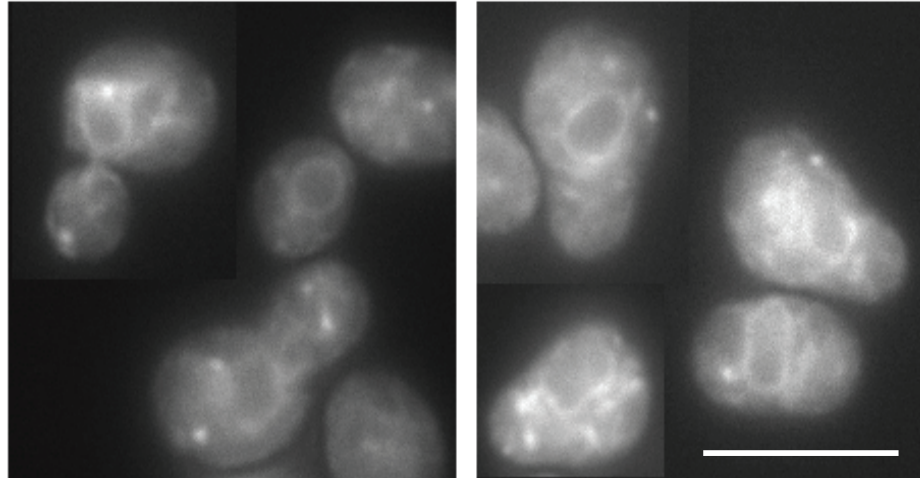
Sartorel et al.

ATime after α -factor
treatment (min):

0

90

Neo1-GFP

**B**Time after α -factor
treatment (min):

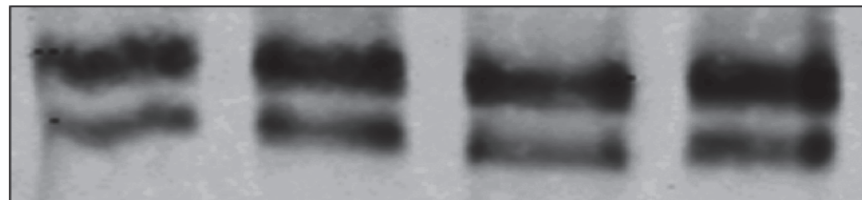
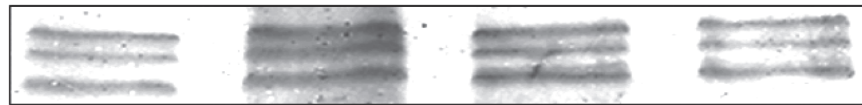
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30

60

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Neo1-myc

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

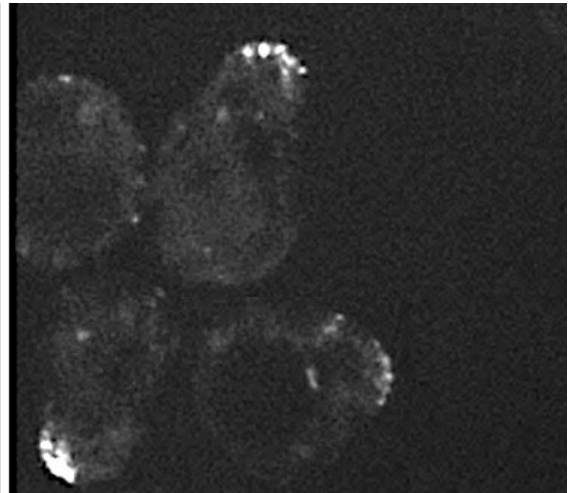
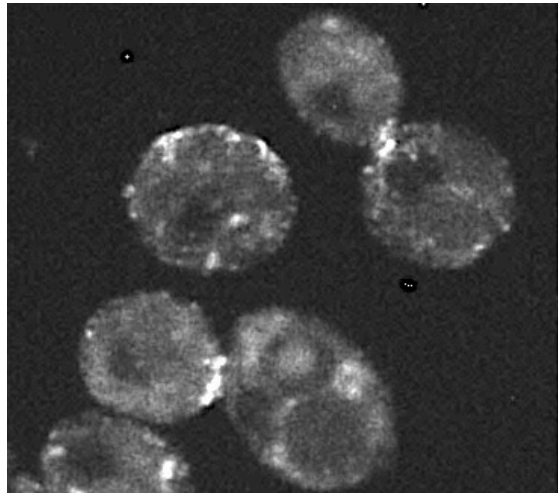
(A) Cells (YEB6) expressing Neo1-GFP from its native promoter at its normal chromosomal locus as the sole source of this protein were grown in YPD medium to mid-exponential phase, exposed to α -factor (10 μ M final concentration) for the indicated time and viewed by fluorescence microscopy. Scale bar, 5 μ m. (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 μ M 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).

Time after α -factor
treatment (min):

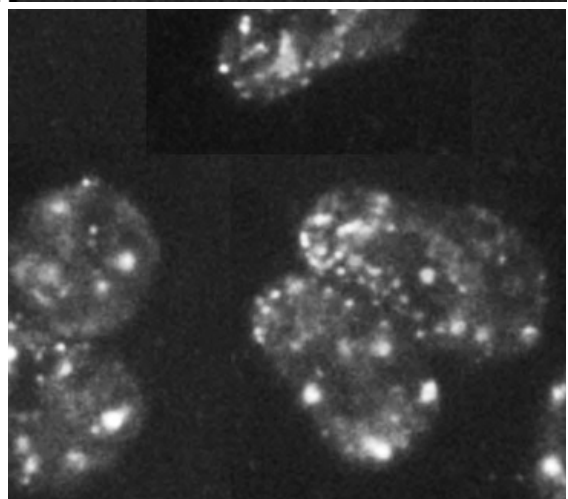
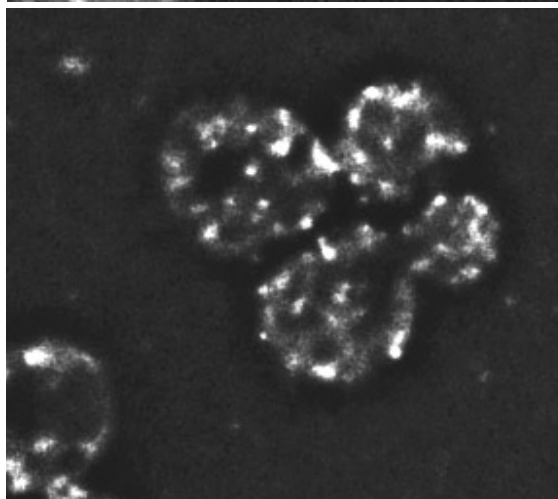
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Dnf1-GFP



Dnf3-GFP



Drs2-mCh

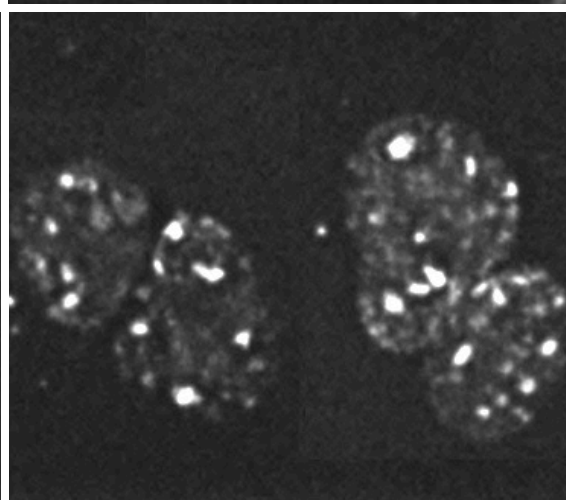
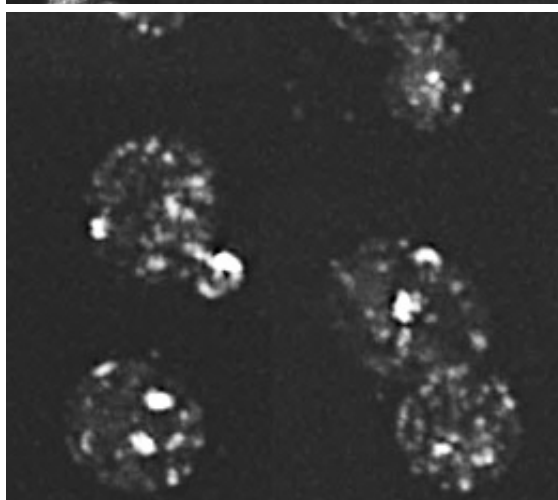


Fig. S2. Flippase redistribution in response to pheromone. To examine flippase localization, cells (BY4741) carrying an integrated copy of Dnf1-GFP (NRY921), Dnf3-GFP (YEB1) or Drs2-mCherry (YEB2) were grown to mid-exponential phase, incubated with 10 μ M α -factor for the indicated time, and examined 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.

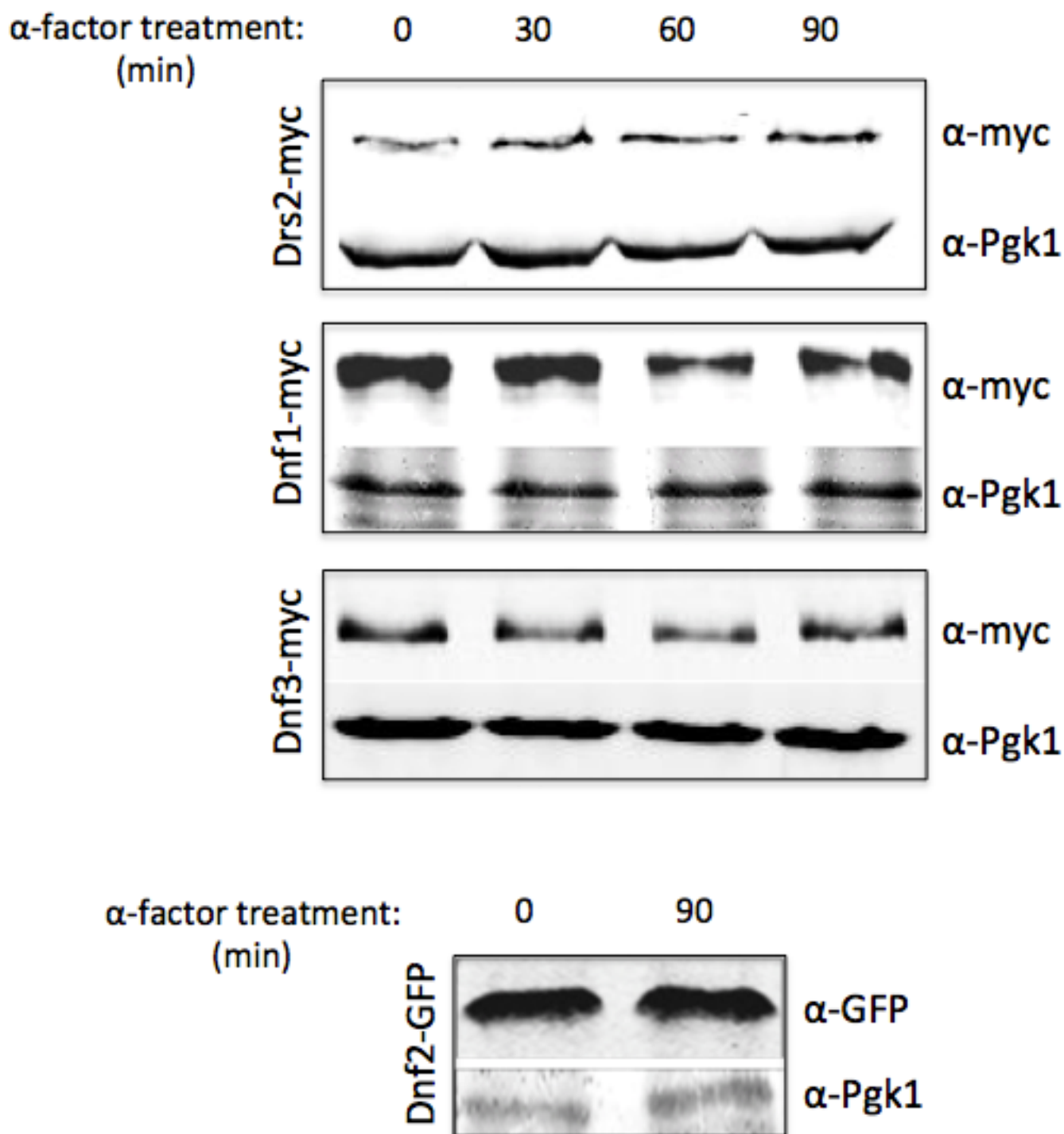


Fig. S3. Flippase protein level does not change in response to pheromone. Cells (BY4741) expressing Dnf1-myc (YEB4), Dnf2-GFP (NRY923), Dnf3-myc (YEB5) or Drs2-myc (YEB3), as indicated, were grown to mid-exponential phase in YPD medium, incubated with 10 μ M α -factor, and harvested at the indicated 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.

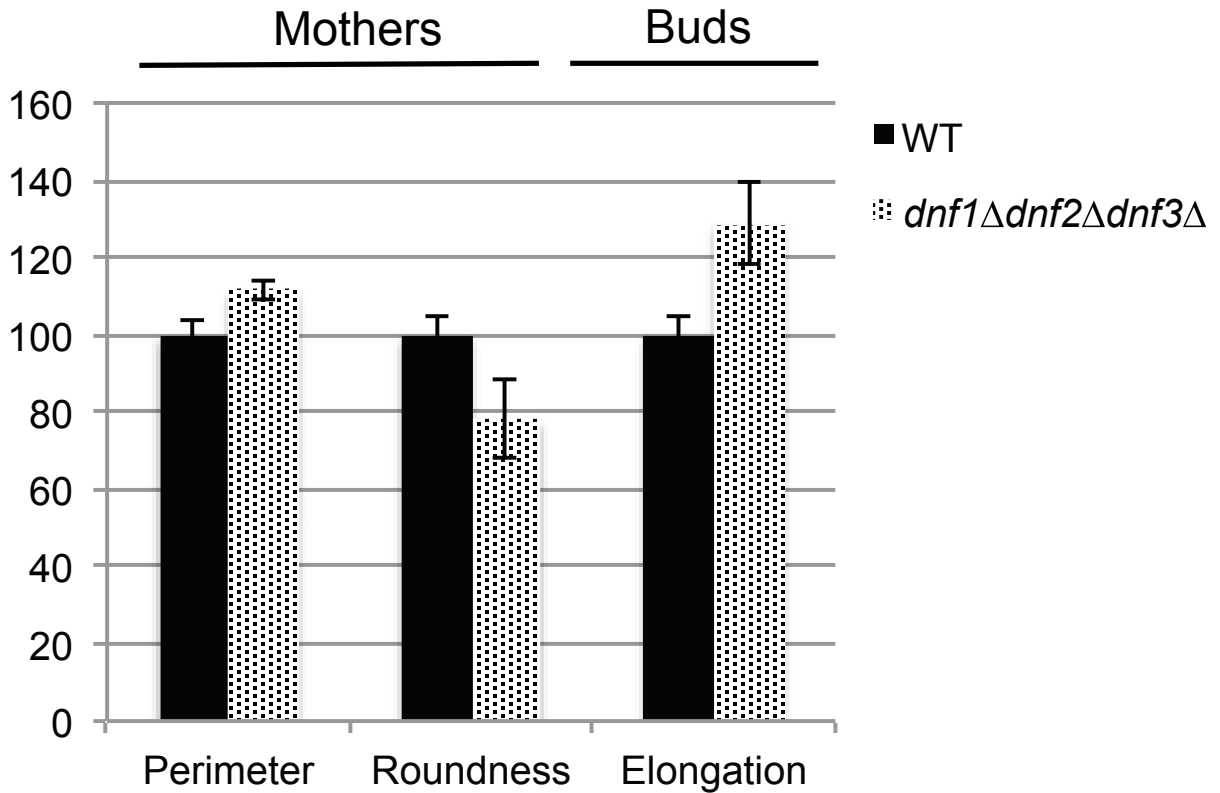


Fig. S4. Comparison of morphological parameters of wild-type and *dnf1*Δ *dnf2*Δ *dnf3*Δ cells. Digital images of fields of WT (n = 100) and *dnf1*Δ *dnf2*Δ *dnf3*Δ (n = 110) cells expressing plasmid pGFP-C2^{Lact} to highlight the plasma membrane were analyzed using ImageJ software (Collins, 2007), 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 ($4 \times [\text{Area}] / \pi [\text{major axis}]^2$) gives an estimate of its approximate roundness, and measurement of ratio of the longest axis to the shortest axis of the bud (aspect ratio: $[\text{major axis}] / [\text{minor axis}]$) 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 *dnf1*Δ *dnf2*Δ *dnf3*Δ cells then normalized accordingly. Error bars represent standard deviation of the mean. Comparing *dnf1*Δ *dnf2*Δ *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.

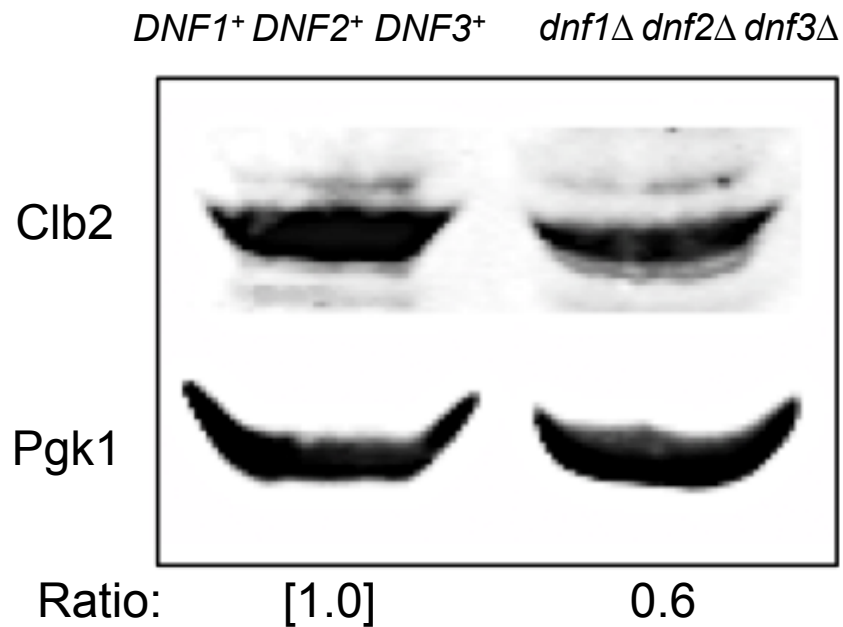


FIG. S5. Flippase-deficient cells have a lower level of Clb2.

WT (BY4741) and otherwise isogenic *dnf1Δ dnf2Δ dnf3Δ* 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.

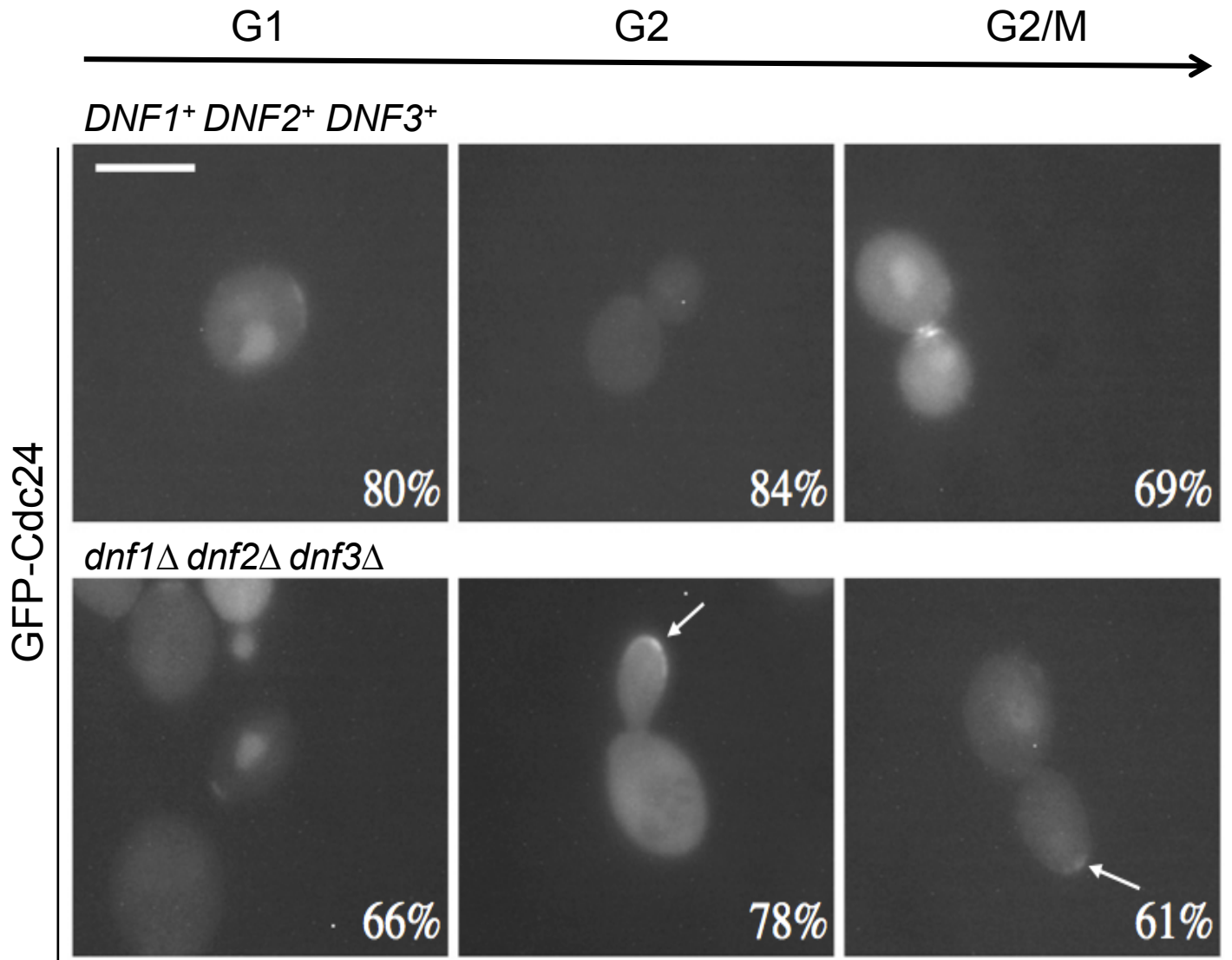


Fig. S6. Cdc24 is sequestered at the bud tip in *dnf1Δ dnf2Δ dnf3Δ* cells.

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).

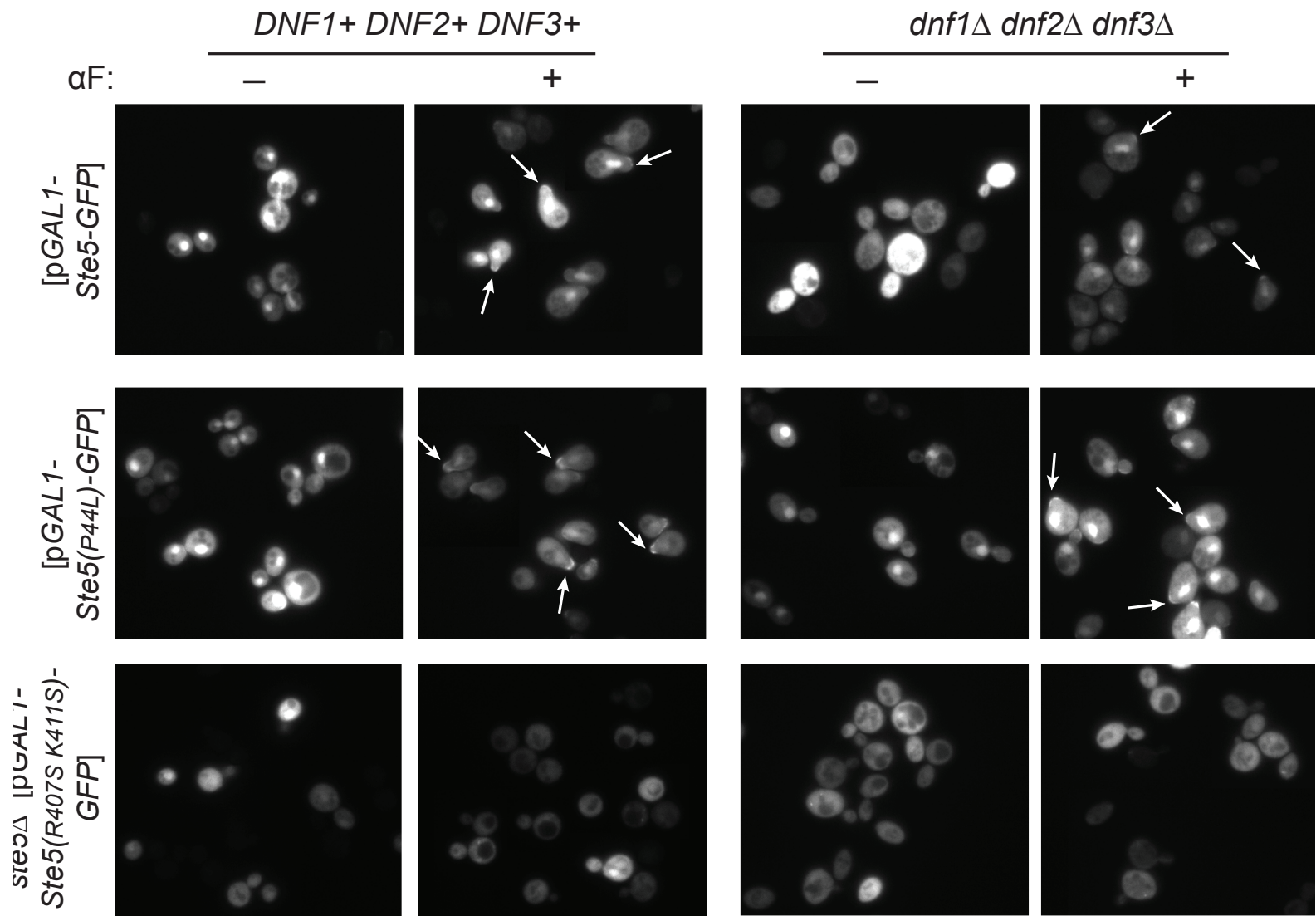
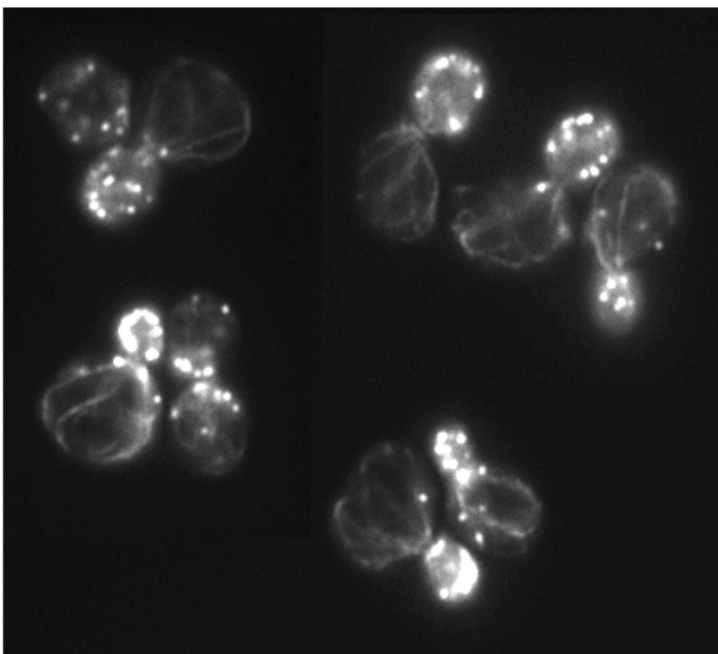


Fig. S7. Recruitment of Ste5 and Ste5 mutants to the plasma membrane at the shmoo tip in pheromone-treated cells.

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 (PFY3272C), 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 μ M 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 is defective in stable plasma membrane association 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 μ M α -factor for 1.5 h, and examined by fluorescence microscopy. Arrows, Ste5 localization at the shmoo tip.

DNF1⁺ DNF2⁺ DNF3⁺



dnf1 Δ dnf2 Δ dnf3 Δ

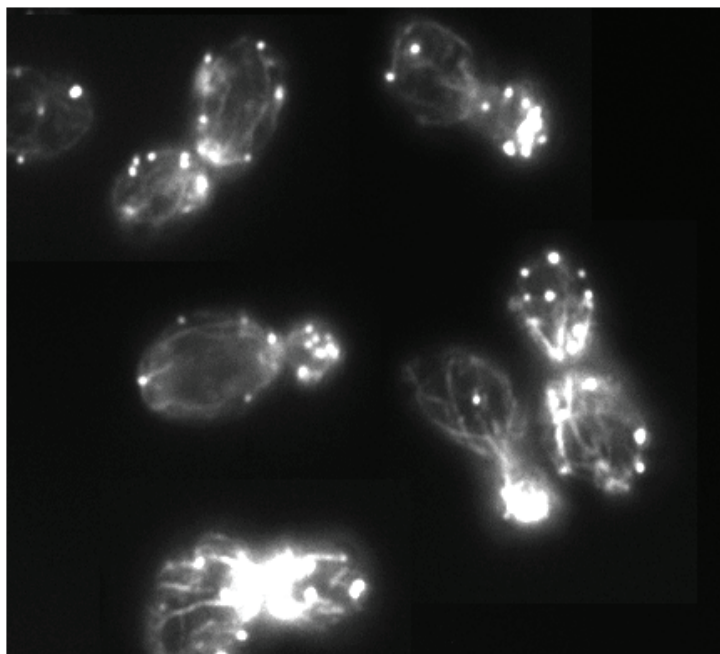


Fig. S8. Organization of the actin cytoskeleton in WT and *dnf1 Δ dnf2 Δ dnf3 Δ* cells.

WT (BY4741) and otherwise isogenic *dnf1 Δ dnf2 Δ dnf3 Δ* 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.

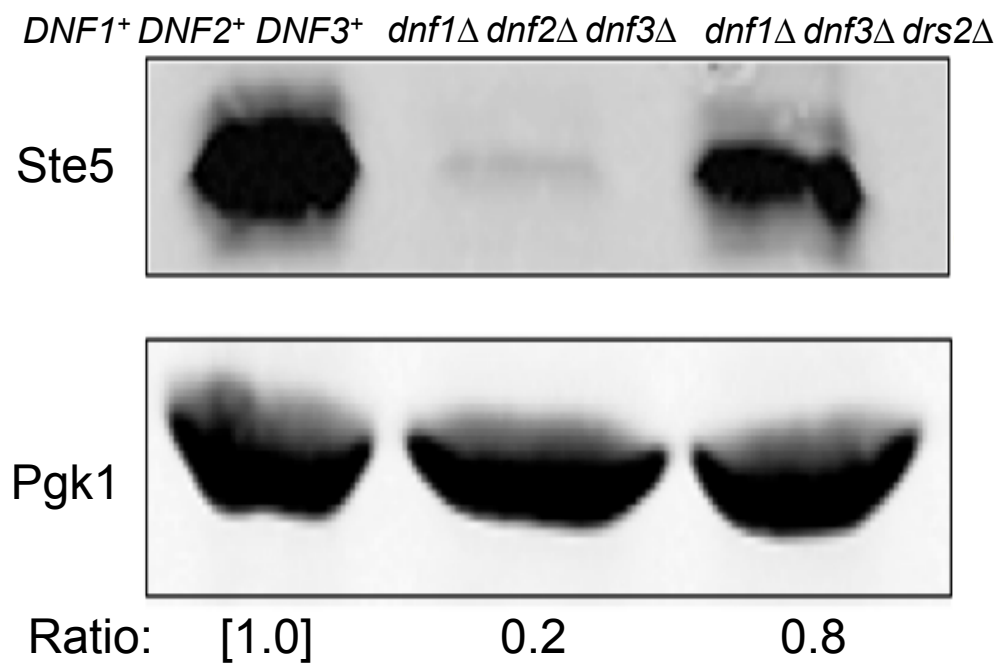


Fig. S9. Ste5 protein is not unstable in *dnf1 Δ dnf3 Δ drs2 Δ* cells.

Cultures of WT (BY4741), *dnf1 Δ dnf2 Δ dnf3 Δ* (PFY3272C) and *dnf1 Δ dnf3 Δ drs2 Δ* (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.