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Pseudohyphal growth in Saccharomyces cerevisiae involves protein kinase-regulated lipid flippases

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ABSTRACT

Lipid flippases of the P4 ATPase family establish phospholipid asymmetry in eukaryotic cell membranes and are involved in many essential cellular processes. The yeast Saccharomyces cerevisiae contains five P4 ATPases, among which Dnf3p is poorly characterized. Here, we demonstrate that Dnf3p is a flippase that catalyzes translocation of major glycerophospholipids, including phosphatidylsersine, towards the cytosolic membrane leaflet. Deletion of the genes encoding Dnf3p and the distantly related P4 ATPases Dnf1p and Dnf2p results in yeast mutants with aberrant formation of pseudohyphae, suggesting that the Dnf1p-Dnf3p proteins have partly redundant functions in the control of this specialized form of polarized growth. Furthermore, as previously demonstrated for Dnf1 and Dnf2p, the phospholipid flipping activity of Dnf3p is positively regulated by flippase kinase 1 (Fpk1p) and Fpk2p. Phylogenetic analyses demonstrate that Dnf3p belongs to a subfamily of P4 ATPases specific for fungi and are likely to represent a hallmark of fungal evolution.

KEY WORDS: Polarized growth, Cell budding, Pseudohyphal growth, Lipid flippase, Flippase kinase

INTRODUCTION

Cellular membranes separate cells from the environment and allow for compartmentalization of different processes inside cells. Many membranes present a characteristic asymmetric phospholipid distribution between the two leaflets necessary for their physiological function (Sebastian et al., 2012; Zachowski, 2012). To achieve such transbilayer lipid asymmetry, phospholipids are actively transported across the bilayer at the expense of ATP (van Meer, 2011). P4 ATPases or flippases are integral membrane proteins involved in the control of this asymmetry in eukaryotic cell membranes and are involved in many essential cellular processes. The yeast Saccharomyces cerevisiae contains five P4 ATPases, among which Dnf3p is poorly characterized.

In Saccharomyces cerevisiae, the family of P4 ATPases comprises five members: Drs2p, Neo1p, Dnf1p, Dnf2p and Dnf3p. Drs2p is a well-characterized lipid flippase that functions in the trans-Golgi network (TGN) and is specific for phosphatidylserine (PS) and phosphatidylethanolamine (PE) (Chen et al., 1999; Saito et al., 2004). Drs2p-catalyzed flipping of PS and PE creates membrane curvature and serves as a signal to attract proteins involved in vesicle transport, and both processes contribute to secretory vesicle formation (Xu et al., 2013). Neo1p performs an essential role in vesicle transport between the endoplasmic reticulum (ER) and Golgi, and has been shown to affect the asymmetric distribution of mainly PE, but also PS, at the plasma membrane through an as yet unclear mechanism (Hua and Graham, 2003; Takar et al., 2016; Wicky et al., 2004; Wu et al., 2016). Dnf1p and Dnf2p localize the plasma membrane, where they primarily transport phosphatidycholine (PC) and PE, as well as the corresponding lysophospholipids and glycosphingolipids (Baldridge et al., 2013; Furuta et al., 2007; Hachiro et al., 2013; Pomorski et al., 2003; Rickhof and Voelker, 2006, 2009; Saito et al., 2004; Roland et al., 2019). Like Drs2p and Neo1p, Dnf1p and Dnf2p are required for vesicle transport in the late secretory and endocytic pathways (Hachiro et al., 2013; Hua et al., 2002; Pomorski et al., 2003).

Dnf3p is distantly related to other P4 ATPases in yeast, and is the least characterized P4 ATPase in this model organism. Dnf3p has been identified in intracellular membrane structures that most likely represent the TGN and early endosomes, and was proposed to colocalize with Drs2p (Pomorski et al., 2003). In contrast to Drs2p, Dnf3p has been reported to translocate primarily PC and PE (Alder-Baerens et al., 2006). Dnf3p activity contributes to vesicle formation (Hua et al., 2002) and vascular pH maintenance (Brett et al., 2011) but, due to its low level of expression, it has been suggested that Dnf3p only plays a minor role in the basal cell functions of S. cerevisiae (Furuta et al., 2007; Hua et al., 2002; Saito et al., 2004). However, during shmoof formation in the early phases of yeast mating, Dnf3p has been observed accumulating at the plasma membrane in the emerging tip (Sartorel et al., 2015). Interestingly, in the emerging tip, PS is being concentrated in the inner leaflet of the plasma membrane (Fairn et al., 2011). Members of the Dnf3 protein family in pathogenic filamentous fungi are strictly required for filamentous growth and penetration into the host cell (Balhädere and Talbot, 2001; Gilber et al., 2006; Schultzhaus et al., 2019). This suggests that the Dnf3 protein family in fungi serves a general function in polarized growth.
A previous study demonstrated that Dnf3p can be phosphorylated in vivo by two kinases, flippase kinase 1 and 2 (Fpk1p and Fpk2p) (Nakano et al., 2008), but the physiological relevance of this process was not studied. Notably, Dnf1p and Dnf2p are activated through direct phosphorylation by Fpk1p and Fpk2p (Nakano et al., 2008). Unexpectedly, deletion of the β-subunit for Dnf1p and Dnf2p, Lem3p, does not exactly mimic the fpk1Δfpk2Δ mutant phenotype (Nakano et al., 2008), although both genetic modifications should specifically result in lack of Dnf1p and Dnf2p flippase activity at the plasma membrane. Transport of PS across the yeast plasma membrane is only minimally affected in a dnf1Δdnf2Δ double deletion mutant (Hachiro et al., 2013; Pomorski et al., 2003; Stevens et al., 2008) and in a lem3 deletion mutant, which lacks the β-subunit of Dnf1p and Dnf2p (Kato et al., 2002), suggesting that an alternative PS transport system exists at the plasma membrane.

In this work, we tested the involvement of Dnf3p in flippase-kinase-mediated PS uptake at the plasma membrane in *S. cerevisiae* and examined the putative role for this protein in polarized pseudohyphal growth.

**RESULTS**

**Dnf3p transports endogenous PS**

Several lines of evidence suggest that a PS transport system distinct from Dnf1p and Dnf2p exists at the plasma membrane of *S. cerevisiae* (Hachiro et al., 2013; Nakano et al., 2008; Stevens et al., 2008). It was suggested that this system might be Drs2p, since this protein is known to recycle through the endocytic recycling pathway (Alder-Baerens et al., 2006; Saito et al., 2004). However, confirmation of this hypothesis is lacking. To investigate this, we analyzed the uptake of fluorescent nitrobenzoxadiazole (NBD) acyl-labeled lipid reporters by yeast cells lacking DNF3 alone or DNF1 and DNF2, alone or in combination with DNF3 or DRS2 (Fig. 1A). As expected, deletion of DNF1 and DNF2 resulted in a decrease of lipid uptake for fluorescently labeled PS, PE and PC. Further removal of Drs2p or Dnf3p did not significantly affect transport of NBD–PS, –PE, or –PC, confirming that Dnf1p and Dnf2p are the main contributors to lipid uptake at the plasma membrane. However, deletion of DNF3 alone resulted in a reduction of lipid transport to ∼50–60% of

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**Fig. 1. Dnf3p is involved in phospholipid uptake and maintenance of plasma membrane PS asymmetry.** (A) Yeast strains bearing deletions in the indicated genes were tested for uptake of NBD-lipids. Results are mean±s.e.m. from at least three biological replicates. Numbers above the bars represent geometric mean±s.e.m. of fluorescence values in arbitrary units. a, statistically significant (P<0.01) with respect to the wild type; b, statistically significant (P<0.01) with respect to dnf3Δ; *, statistically different (P<0.01) from all other strains (two-way ANOVA followed by Tukey confidence test). (B) Yeast strains bearing deletions in the indicated genes were grown in SD medium supplemented with papuamide A (PapA) at the indicated concentrations. Values are mean±s.e.m. of three independent experiments. (C) Yeast dnf1,2,3Δdrs2Δ cells transformed with either empty vector (EV) or expression plasmids carrying wild-type DNF1, DNF2, DNF3 or DRS2 were spotted in two serial dilutions onto control plates with glucose (SD) or on galactose-containing plates (SG, induced expression), supplemented with PapA or the cytotoxic PE-binding peptide duramycin (Dura) as indicated. (D) Yeast dnf1,2,3Δdrs2Δ cells transformed with either empty vector (EV) or expression plasmids carrying wild-type DNF3, inactive versions of DNF3 (dnf3E342Q, dnf3D566N), or wild-type DRS2 were grown on control plates with glucose (SD) or on galactose/fructose-containing plates (SGF, induced expression), supplemented with PapA as indicated. Numbers in C and D indicate cell dilutions.
wild-type levels for all lipids tested, with the strongest effect for NBD–PS.

Transport of PC and PE by Dnf3p has previously been reported (Alder-Baerens et al., 2006). To test whether the observed PS translocation is directly caused by Dnf3p activity, we evaluated PS asymmetry at the plasma membrane in yeast mutants lacking different flippase combinations and grown in the presence of small concentrations of the PS-binding peptide papuamide A (Andjelic et al., 2008), which causes cytolysis and cell death by binding to extracellularly exposed PS (Parsons et al., 2006). PS sequestration in the cytosolic leaflet of the plasma membrane due to P4 ATPase-mediated internalization provides wild-type yeast cells with a natural resistance to this peptide. At lower peptide concentrations, growth of all yeast strains was similar to that of the wild type (Fig. 1B). By contrast, at a 1 µg/ml concentration of the peptide, the triple dnf1,2,3Δ and dnf1,2Δrs2Δ mutant strains were more sensitive than dnf1,2Δ or dnf3Δ, which showed a similar growth to the wild type, suggesting that both Drs2p and Dnf3p are contributing to the plasma membrane PS asymmetry. In addition, the triple dnf1,2,3Δ mutant shows sensitivity to the drug at earlier times (24–27 h) than the dnf1,2Δrs2Δ mutant, suggesting that the contribution of Dnf3p to PS asymmetry is stronger than that of Drs2p.

We have previously used a triple dnf1,2Δrs2Δ mutant strain to characterize the lipid specificity of heterologously expressed flippases, due to its low background for lipid translocation (Jensen et al., 2016a,b). Overexpression of DNF1, DNF3 or DRS2 in this strain under the control of a strong galactose-inducible promoter resulted in yeast growth on plates containing papuamide A (Fig. 1C), confirming that all proteins can transport PS. Overexpression of DNF2 seemed to be toxic for the yeast cells. We next employed a yeast strain lacking DNF1, DNF2, DNF3, and DRS2, which is devoid of all P4 ATPases shown so far to be directly involved in lipid translocation. This strain has very limited growth, but it is still viable in our yeast background. Growth of the dnf1,2,3Δ mutant was highly sensitive to papuamide A and this phenotype could be complemented by overexpression of DRS2 (Fig. 1D). Confirming our previous results, expression of DNF3 also resulted in complementation of the peptide-sensitive phenotype (Fig. 1D). To confirm that functional complementation requires an active Dnf3p, we generated two catalytically inactive variants, dnf3E342Q and dnf3D366N. While the dnf3D366N mutant lacks the conserved aspartic acid residue phosphorylated during the P-type ATPase catalytic cycle, the dnf3E342Q mutant lacks the glutamic acid residue in the conserved DGET-motif in P4 ATPases (TGES in other P-type ATPases) required for dephosphorylation (Anthonisen et al., 2006; Lenoir et al., 2009). Both catalytically inactive mutants were unable to support growth of the dnf1,2,3Δ mutant strain on papuamide A (Fig. 1B). Taken together, our results demonstrate that active Dnf3p is indeed capable of promoting endogenous PS transport, in addition to that of PC and PE.

To test whether Dnf3p can reach the plasma membrane under our experimental conditions, we investigated its intracellular localization. We chromosomally tagged the endogenous copy of DNF3 with a SNAP tag at the C-terminal end of the protein in a wild-type yeast strain. The SNAP tag reacts specifically and rapidly with benzylguanine derivatives, allowing for the attachment of virtually any fluorophore after protein expression. Microscopy analysis of fluorescently labeled cells expressing Dnf3p–SNAP revealed numerous cells with a punctate staining pattern of endomembrane structures, in line with previous reports (Fig. S1A, panel I,II) (Hua et al., 2002; Pomorski et al., 2003). However, a large proportion of the cell culture displayed a ring-like peripheral staining pattern characteristic of plasma membrane localization (Fig. S1A, panel I,II). This localization was not an artifact of our labeling procedure, as we could not detect any plasma membrane signal for the actin-binding protein Abp1p, which under the conditions of our experiments resides solely in endomembranes (Fig. S1A, panel III) (Stagge et al., 2013).

To further characterize the plasma membrane localization of Dnf3p–SNAP, we incubated fluorescently labeled yeast cells with Trypan Blue to stain the cell wall (Liesche et al., 2015). This allowed for classification of cells into four different states related to their progression through the cell cycle, with State 1 corresponding to non-budding cells and State 4 corresponding to cells approaching cytokinesis (Fig. S1B). In non-budding cells (State 1), a Dnf3p–SNAP signal in endomembranes was evident in half of the population, whereas the other half showed a signal in the plasma membrane (Fig. S1B). During the initial stages of bud emergence (State 2), Dnf3p was confined to endomembranes, but located primarily to the plasma membrane during the isotropic bud growth phase and in cells approaching cytokinesis (States 3 and 4) (Fig. S1B). While we cannot rule out that vesicles containing Dnf3p are redirected to the plasma membrane in response to electroporation damage during fluorescent dye delivery, the capacity of Dnf3p to reach the plasma membrane in yeast has already been demonstrated during yeast mating (Sartorel et al., 2015), which is a cell-cycle-controlled process. Taken together, our results suggest that Dnf3p might reach the plasma membrane at certain time points during the cell cycle, and is therefore likely to be responsible for the residual PS-translocating activity at the plasma membrane detected in the dnf1,2Δ and lem3Δ mutants.

**Overexpression of FPK1 or FPK2 induces Dnf3p-dependent PS transport**

Given that flippase kinases Fpk1p and Fpk2p activate plasma membrane phospholipid flipping by Dnf1p and Dnf2p (Nakano et al., 2008), we wondered whether they also regulated Dnf3p activity. To study this, we used a fpk1,2Δ yeast strain to generate an fpk1,2Δdnf1,2Δ quadruple mutant lacking both the flippase kinases Dnf1p and Dnf2p. Overexpression of either Fpk1p or Fpk2p in the fpk1,2Δ strain induced translocation of fluorescent NBD–PC, –PE and –PS to the inner leaflet of the plasma membrane (Fig. 2A). Overexpression of the kinases in the fpk1,2Δdnf1,2Δ quadruple mutant increased uptake of labeled PC and especially PS (Fig. 2B). These findings indicate the presence of an Fpk1p- and Fpk2p-regulated system for PS transport at the yeast plasma membrane that is independent of Dnf1p and Dnf2p.

To test whether the observed Dnf1p- and Dnf2p-independent transport of NBD–PS is a result of Drs2p or Dnf3p activity, we attempted to create knockouts of DRS2 or DNF3 in the quadruple fpk1,2Δdnf1,2Δ mutant background. A deletion of CDC50, encoding the β-subunit of Drs2p, is synthetically lethal when combined with deletion of FPK1 (Nakano et al., 2008), which might explain why we were not able to create a quintuple fpk1,2Δdnf1,2Δrs2Δ mutant. However, we succeeded in deleting DNF3 in the quadruple mutant background and in this way obtained a quintuple fpk1,2Δdnf1,2Δrs2Δ mutant strain (henceforth termed *quint*).

We next compared phospholipid transport across the plasma membrane of the *quint* mutant with that of wild-type yeast and its parental strains fpk1,2Δ and fpk1,2Δdnf1,2Δ. In fpk1,2Δ cells, lipid transport was drastically reduced (by ~80%) for all three NBD-labeled lipids (NBD–PC, –PE and –PS) (Fig. 2C). For
Dnf3p is expressed at similar levels in protein expression or its inability to reach the plasma membrane, we control (Fig. 3A). papuamide A-sensitive phenotype (Fig. 3E). By contrast, expression of medium containing papuamide A. Indeed, (cf. Fig. 3C,D).

To verify that catalytically active Dnf3p and Fpk1p are required for PS transport To test for transport of endogenous PS, we examined cell growth on medium containing papuamide A. Indeed, mutant, we employed the quint strain still contains the DR52 gene, which encodes a PS transporter of the TGN (Zhou et al., 2013) that, at least in vitro, is a substrate of Fpk1p (Nakano et al., 2008). To test the possible involvement of Drs2p in Fpk1p- and Fpk2p-regulated PS transport into quint, we overexpressed RGSH10-tagged DR52 and GST–FPK1 in the quint strain and measured transport of NBD–PC, –PE, and –PS. Overexpression of Drs2p alone or in combination with Fpk1p or Fpk2p in quint cells did not significantly alter the transport of NBD–PS or any other labeled phospholipid tested (Fig. 3A; Fig. S2). Similarly, yeast growth was not affected when cultured on papuamide A-containing plates (Fig. 3E), even when our DR52-expressing construct was capable of complementing the papuamide A-sensitive phenotype of dnf1,2Δdrs2Δ and dnf1,2,3Δdrs2Δ strains (Fig. 1C,D), indicating that transport of natural PS was also independent of Drs2p overexpression. The N-terminally decahistidine-tagged Drs2p was expressed at the same level independently of Fpk1p expression (Fig. 3B), and was mainly retained in endomembranes in the absence of Fpk1p (Fig. 3C). Notably, while Fpk1p overexpression did not affect the subcellular localization of Dnf3p, the presence of this kinase increased the amount of Drs2p at the plasma membrane, as indicated by sucrose gradient fractionation (Fig. 3C,D). Taken together, these results suggest that Fpk1p is not capable of activating Drs2p in vivo, at least not in the absence of Dnf3p.

Fig. 2. A phospholipid flipase distinct from Dnf1p and Dnf2p is required for FPK-induced NBD-lipid transport. NBD-lipid uptake by fpk1,2Δ cells (A), fpk1,2Δdnf1,2Δ (B), and fpk1,2Δdnf1,2Δ Δ (D) transformed with either an empty vector (EV) or expression plasmids carrying FPK1 or FPK2. (C) Comparison of NBD-lipid uptake between the deletion strains and wild-type KKT61 cells (WT). Accumulation of NBD-lipids is shown as percentage of fluorescence intensity relative to the indicated control cells. Numbers above the bars represent geometric mean±s.e.m fluorescence values from at least three independent experiments in arbitrary units. *, statistically significant (P<0.01) with respect to all other strains; a, statistically significant (P<0.01) with respect to the empty vector control (A,B,D) or the wild-type strain (C); b, statistically significant (P<0.01) with respect to fpk1,2Δ (two-way ANOVA followed by Tukey confidence test).

Co-expression of Dnf3p and Fpk1p, but not Drs2p and Fpk1p, results in PS transport across the plasma membrane

To confirm that lack of NBD–PS uptake in quint was due to lack of Fpk1p- and Fpk2p-dependent Dnf3p activity, we expressed FPK1 or FPK2 and DNF3 either alone or in combination in the quint background. Introduction of DNF3 alone did not result in increased NBD–PS transport, and the same was true for quint cells expressing only FPK1 (Fig. 3A) or FPK2 (Fig. S2). However, simultaneous expression of DNF3 and FPK1 resulted in an ~3-fold increase in NBD–PS transport compared to that seen with an empty vector control (Fig. 3A).

dpk1,2Δdnf1,2Δ cells, internalization of all three lipids, especially NBD–PE and –PC, was further reduced as compared to fpk1,2Δ cells. Further removal of DNF3 did not have a significant effect, suggesting that Dnf3p activity might be controlled by the Fpk kinases. In agreement to this, we did not observe FPK-induced changes in the uptake of any NBD-phospholipid in the quint strain, in contrast to the results obtained for the quadruple fpk1,2Δdnf1,2Δ mutant (cf. Fig. 2B,D). This finding supports the notion that Dnf3p is responsible for the observed PS transport in the fpk1,2Δdnf1,2Δ background and that, when present, Fpk1p and/or Fpk2p activate this transport.

To confirm that lack of lipid translocation was not due to lack of protein expression or its inability to reach the plasma membrane, we N-terminally tagged Dnf3p and Fpk1p with decahistidine (RGSH10) and glutathione-S-transferase (GST) tags, respectively. Immunoblot analysis of total membrane fractions demonstrated that Dnf3p is expressed at similar levels in quint cells regardless of Fpk1p expression (Fig. 3B). Subcellular localization analysis using sucrose density fractionation demonstrated that Dnf3p is capable of reaching the plasma membrane in an Fpk1p-independent manner (cf. Fig. 3C,D).

To test for transport of endogenous PS, we examined cell growth on medium containing papuamide A. Indeed, quint mutant cells were unable to survive in the presence of this toxin (Fig. 3E). Co-expression of DNF3 and FPK1 resulted in functional complementation of the papuamide A-sensitive phenotype (Fig. 3E). By contrast, expression of each gene individually did not affect yeast growth. This confirms the notion that co-expression of FPK1 and DNF3 generates an activity that results in PS transport at the plasma membrane.

The quint strain still contains the DR52 gene, which encodes a PS transporter of the TGN (Zhou et al., 2013) that, at least in vitro, is a substrate of Fpk1p (Nakano et al., 2008). To test the possible involvement of Drs2p in Fpk1p- and Fpk2p-regulated PS transport into quint, we overexpressed RGSH10-tagged DR52 and GST–FPK1 in the quint strain and measured transport of NBD–PC, –PE, and –PS. Overexpression of Drs2p alone or in combination with Fpk1p or Fpk2p in quint cells did not significantly alter the transport of NBD–PS or any other labeled phospholipid tested (Fig. 3A; Fig. S2). Similarly, yeast growth was not affected when cultured on papuamide A-containing plates (Fig. 3E), even when our DR52-expressing construct was capable of complementing the papuamide A-sensitive phenotype of dnf1,2Δdrs2Δ and dnf1,2,3Δdrs2Δ strains (Fig. 1C,D), indicating that transport of natural PS was also independent of Drs2p overexpression. The N-terminally decahistidine-tagged Drs2p was expressed at the same level independently of Fpk1p expression (Fig. 3B), and was mainly retained in endomembranes in the absence of Fpk1p (Fig. 3C). Notably, while Fpk1p overexpression did not affect the subcellular localization of Dnf3p, the presence of this kinase increased the amount of Drs2p at the plasma membrane, as indicated by sucrose gradient fractionation (Fig. 3C,D). Taken together, these results suggest that Fpk1p is not capable of activating Drs2p in vivo, at least not in the absence of Dnf3p.

Catalytically active Dnf3p and Fpk1p are required for PS transport

To verify that catalytically active Dnf3p and Fpk1p are required for PS transport in the quint mutant, we employed the dnf3E342Q mutant described above and generated an inactive version of the kinase, fpk1K525R, which lacks a conserved lysine residue in the phosphotransfer site (Hanks et al., 1988; Nakano et al., 2008). We expressed combinations of active and inactive versions of Dnf3p and Fpk1p in the quint strain and assayed the effect on phospholipid transport and papuamide A sensitivity. NBD–PS transport was only
detected in cells expressing active versions of both proteins (Fig. 4A). Similarly, inactive mutants did not complement the papuamide A-sensitive growth defect of the quint strain (Fig. 4B). All protein variants were expressed at similar levels as detected by immunoblotting (Fig. 4C). We noted that the fpk1K525R mutant protein migrated faster in SDS-PAGE gels than did active Fpk1p, probably due to lack of autophosphorylation (Roelants et al., 2010).

Fpk1p has previously been shown to phosphorylate Dnf3p in vitro (Nakano et al., 2008). To verify that Fpk1p is an in vivo activator of Dnf3p, we employed a previously devised strategy, in which Dnf3p and its β-subunit, Crf1p, are both overexpressed (Nakano et al., 2008). The rationale behind this approach is that overexpression of Dnf3p and Fpk1p, but not Crf1p, results in a relatively small number of catalytically competent Dnf3p–Crf1p complexes that can become highly activated by Fpk1p, whereas overexpression of Dnf3p together with Crf1p produces a large number of catalytically competent complexes with a lower activity that impact phospholipid transport even in the absence of Fpk1p. Therefore, we expressed Dnf3p and Crf1p in the quint strain under the control of a strong inducible galactose promoter (Fig. S3). Overexpression of Crf1p and Dnf3p in the absence of Fpk1p complemented the papuamide A-sensitive phenotype of quint, confirming that Fpk1p acts upstream of Dnf3p.

Dnf1p, Dnf2p and Dnf3p are involved in morphogenesis during pseudohyphal growth

Under conditions of nitrogen or glucose starvation, diploid S. cerevisiae cells switch from the so-called yeast form, characterized by independent budding cells, to a pseudohyphal form, in which cells present an elongated shape and cell division is incomplete, generating short multicellular filamentous structures (Ceccato-antonini and Sudbery, 2004; Karunanithi and Cullen, 2012; Song and Kumar, 2012). These pseudohyphae actively penetrate the growth medium, allowing the cells to scavenge for nutrients that are then delivered to all cells along the filament. In some filamentous fungi, the equivalent process of formation of hyphal structures requires the action of lipid flippases related to yeast Dnf1p and Dnf2p, which are suggested to be involved in lipid redistribution at the point of polarized growth (Schultzhaus et al., 2015). In addition, a relative of Dnf3p in the pathogenic fungi Magnaporthe grisea has been shown to be necessary for formation of specialized polarized structures at the tip of penetration hyphae (Balhadère and Talbot, 2001). Based on their common regulation by FPKs, we wondered whether Dnf3p served a role in formation and development of the pseudohyphae in S. cerevisiae, in a manner coordinated with Dnf1p and Dnf2p. To investigate this, we
generated single and double mutants of DNF genes in a diploid yeast strain, and grew them on solid media lacking nitrogen or glucose to trigger pseudohyphal growth. For quantitation, and considering that pseudohyphal formation is a balance between cell elongation and cell division, we defined several parameters that characterize the process: (1) the average pseudohyphal colony size (based on the number of cells forming the colony; Fig. 5A); (2) the proportion of pseudohyphal colonies containing a certain number of cells (e.g. between 20 and 30 cells); (3) the average number of cells per pseudohyphal filament in the colony; and (4) the presence of cells with an elongated shape.

While diploid yeast cells showed a small tendency to form pseudohyphal colonies even when grown under control conditions, strains lacking DNF flippases did not show any significant differences with respect to the wild type for any of the tested parameters (Fig. S4). After 12 h of starvation, strains lacking DNF2 showed an increased colony size in comparison to the wild type or a dnf1Δ mutant (Fig. 5B). Interestingly, while deletion of DNF1 or DNF3 in the dnf2Δ background did not generate any additional effect, an increased colony size was also observed for the dnf1,3Δ strain, suggesting that Dnf1p and Dnf3p might have overlapping functions that are similar to those of Dnf2p. In line with this notion, the effect on colony size was exacerbated in the triple dnf1,2,3Δ mutant. Analysis of the colony size distribution revealed that a dnf2,3Δ mutant accumulated large colonies (>30 cells), while dnf1,3Δ accumulated colonies containing 20 to 30 cells. Simultaneous deletion of DNF1 and DNF2 also resulted in a higher proportion of large colonies, especially those containing more than 40 cells. This effect was exacerbated in a triple mutant lacking all DNF genes, suggesting that coordinated action of all three flippases is required for control of the cell division rate during pseudohyphal growth. Despite the enlarged colony size, the average number of cells per pseudohyphal filament in a colony did not change with respect to the wild type for any of the tested mutants (Fig. 5D), indicating that cell budding was not compromised. After 12 h of starvation, some wild-type cells showed the presence of elongated buds (Fig. 5E). This effect was even more pronounced for dnf2Δ cells and exacerbated by deletion of DNF1. Further deletion of DNF3 did not result in any additional effect. These results suggest that Dnf1p and Dnf2p, but not Dnf3p, are required for the control of cell elongation during pseudohyphal development. The involvement of Dnf1p and Dnf2p in cell elongation has been previously demonstrated in haploid cells (Saito et al., 2007). Interestingly, diploid cells grown in control medium containing all nutrients showed a very small proportion of elongated cells that was not significantly affected by deletion of DNF genes (Fig. S4).

To further characterize the observed phenotypes, we spotted wild-type and dnf diploid mutant cells on plates lacking either nitrogen or glucose and observed the growth after a 12-day incubation (Fig. 6). On these plates, lack of nutrients forces the yeast to penetrate the medium through the formation of pseudohyphae. In order to extend the penetration area, cells divide and expand laterally, generating an outer ring of cells, whose size can be easily measured after microscopic visualization. Deletion of different DNF combinations did not cause any significant effects on outer ring expansion under nitrogen-starvation conditions (Fig. 6A,C, unwashed). On glucose-depleted medium, where cells are subjected to an additional metabolic stress, deletion of DNF1 caused an increase in the size of the outer ring, while deletion of DNF2 resulted in a reduced size (Fig. 6B,D, unwashed). A double dnf1,2Δ mutant showed a reduction of the outer ring size in addition to that for dnf2Δ, suggesting that DNF1 and DNF2 have complementary functions in outer ring expansion and that the increased ring size observed for dnf1Δ might be due to a compensatory effect of Dnf2p or Dnf3p activity in the absence of DNF1. Interestingly, a double dnf2,3Δ mutant did not show any significant differences with respect to the wild type, suggesting...
that either Dnf2p and Dnf3p have opposite functions in outer ring expansion, or that Dnf1p activity is increased to compensate for absence of DNF3 in the double dnf2,3Δ mutant. The changes in outer ring size seemed to be concomitant with a change in the penetrating capacity of the pseudohyphae, which can be observed after removal of surface cells by washing (Fig. 6, washed). In this case, a statistically significant reduction of the outer ring size was observed for dnf1,2,3Δ with respect to a double dnf1,2Δ mutant under all nutritional conditions tested, suggesting DNF3 might be partly compensating for loss of DNF1 function in dnf1,2Δ.

In addition to the changes in outer ring size, pseudohyphal colonies lacking DNF proteins showed several morphological defects (Fig. 6A,B; Fig. S5). While single dnf1Δ and double dnf1,3Δ mutants showed more or less circular colonies with a homogenous perimeter, removal of DNF2 resulted in the formation of pseudohyphae that project out of the colony generating an uneven perimeter. This effect was exacerbated in dnf2,3Δ, but not in a dnf1,2Δ mutant, while it seemed to be reduced in dnf1,2,3Δ. However, the morphological defects in dnf1,2,3Δ might be masked by the heavily reduced size of the outer ring. Interestingly, dnf3Δ showed a similar, albeit less pronounced, phenotype to dnf2Δ with respect to the circularity of the colony (Fig. 6; Fig. S5). In addition, single deletion of DNF3 resulted in the formation of drop-like structures on the surface of the colony. Taken together, these results suggest that DNF2 and DNF3 are important for uniform pseudohyphal expansion.

Dnf3p appeared at the origin of fungal evolution

Previous phylogenomic analysis of P4 ATPase sequences from fungi, animals and plants revealed that Dnf3p is related to a group of fungal sequences with no obvious relationship to any other P4 ATPases (Palmgren et al., 2019; Poulsen et al., 2015). Thus, having identified Dnf3p as a novel system for flipping PS at the plasma membrane in S. cerevisiae, we wondered how this system had evolved in fungi. For this purpose, we first selected 37 fungi from all major fungal lineages, identified all P4 ATPases in their genomes and subjected them to a phylogenetic analysis (Fig. 7). Two subfamilies of P4 ATPases, P4A and P4B could be identified, as previously described (Palmgren et al., 2019). Fungal P4B ATPases
comprised a single monophyletic clade represented in *S. cerevisiae* by Neo1p, and all fungal genomes analyzed included at least one gene belonging to this clade. Fungal P4A ATPases could further be divided into four clades. A minor basal clade only had members from early diverging lineages of fungi, whereas three remaining clades each had a *S. cerevisiae* representative (designated the Drs2p, Dnf1p/2p, and Dnf3p clades, respectively). The Dnf1p/2p clade showed signs of an early gene duplication and subsequent gene loss of one of the copies in Ascomycota (Fig. 7). A Dnf3p homolog could be found in all fungal genomes except in those from Basidiomycota (Fig. 7A). Taken together, Dnf3p-like sequences are present in the earliest fungi but have been subsequently lost in

**Fig. 6.** Yeast strains lacking DNF proteins show morphological defects in pseudohyphal expansion that are concomitant with changes in the invasive growth profile. Yeast wild-type and *DNF* knockout cells were spotted onto plates (A) without nitrogen or (B) without glucose. Plates were observed at the microscope after a 12-day incubation and images were taken before and after wash with water to study colony morphology and pseudophyphae invasiveness, respectively. (C,D) The average size of the outer ring was measured for each colony as described in the Materials and Methods and normalized to wild-type values. Results are means ± s.e.m. from at least three independent experiments, in which at least 900 cells per strain were counted. Values not sharing a common superscript letter differ significantly at *P*<0.01 according to a two-way ANOVA followed by Tukey confidence test.
Basidiomycota, which, on the other hand, have retained both copies of an early Dnf1p/2p ATPase duplication, among which one was lost in Ascomycota.

To determine whether Dnf3p-like sequences are specific for the fungal kingdom, we searched for homologs among all P4 ATPase sequences extracted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) collection of organisms with complete genomes. We then expanded the phylogenetic analysis to include the fungal sequences identified above and, in addition, identified the 100 sequences with highest similarity to Dnf3p that resulted from a BLAST search in the National Center for Biotechnology Information (NCBI) protein database, both when ‘Fungi’ were excluded from the search and when ‘Fungi’ were searched excluding Basidiomycota. The resulting tree (Fig. 8) included the Dnf3p clade within P4A ATPases (Palmgren et al., 2019), but this clade did not group with any other clade in the tree. The only exception to fungal sequences in the Dnf3p clade was a sequence (XP_009493813) from Fonticula alba, which appeared at the base of the clade. Fonticula is an amoeboid genus that has been characterized as the sister group to fungi (Brown et al., 2009; Kiss et al., 2019). A F. alba sequence was also found at the base of the fungal Dnf1p/2p clade. Taken together, these results show that the Dnf3p clade is unique for fungi and evolved at the very onset of fungal evolution.

**DISCUSSION**

In this work, we demonstrate that Dnf3p is a transporter of endogenous PS in both endomembranes and the plasma membrane. Previous work presented evidence that Dnf3p primarily transports PC and PE (Palmgren et al., 2006). This conclusion was based on studies of late post-Golgi vesicles purified from a yeast strain lacking DRS2 and carrying the sec6-4 mutation (Alder-Baerens et al., 2006; Lamping et al., 2005). As this strain still expresses Dnf1p and Dnf2p, which are known to primarily transport PC and PE (Pomorski et al., 2003), it is possible that the measured fluorescent lipid uptake might partially have been caused by the activity of Dnf1p and Dnf2p, which may explain the discrepancy with the results reported here. In support of this, the amount of Dnf3p in metabolically active yeast cells is ~40 times lower than that of Dnf1p and 15 times lower than that of Dnf2p (Hua et al., 2002). Moreover, Dnf1p and Dnf2p are required for proper sorting of tryptophan permease Tal2p at the TGN, which suggests these flippases are active in endomembranes (Hachiro et al., 2013).

One prominent example of polarized growth in yeast cells is pseudohyphal growth. Our phenotypic analysis of yeast mutants lacking different combinations of the Dnf proteins indicates that Dnf3p is involved in control of lateral expansion during pseudohyphal growth, and that its physiological function is coordinated with that of Dnf1p and Dnf2p during this process. In addition, Dnf3p is also involved, together with Dnf2p, in controlling homogenous pseudohyphal expansion. The Dnf3p homolog PDE1 from the pathogenic fungi Magnaporthe grisea plays an essential function in host-cell penetration, a process that, similar to mating tip homogenous pseudohyphal expansion. The Dnf3p homolog PDE1 is involved in control of lateral expansion during pseudohyphal growth. Our phenotypic analysis of yeast mutants flippases are active in endomembranes (Hachiro et al., 2013).

The emergence of a common regulatory mechanism for all Dnf proteins also has consequences for our understanding of the formation of the shmoo, another example of cell polarity in S. cerevisiae. Shortly after exposure to α-mating factor, Dnf1p, Dnf2p, Dnf3p and Fpk1 are all localized to the mating tip, and a dnf1Δdnf2Δdnf3Δ mutant has a defect in mating (Sartorel et al., 2015). Cdc42p is a small GTPase required for the recruitment and function of effectors that promote bud emergence (Atkins et al., 2013; Lo et al., 2013; Saito et al., 2007; Tong et al., 2007). As recruitment of Cdc42p to the plasma membrane depends on PS internalization (Fairn et al., 2011), our results suggest that Gin4p might exert at least parts of its effect indirectly via inhibition of Dnf3p.

Our findings that Fpk1p activates Dnf3p and that Dnf3p reaches the plasma membrane in a cell cycle-dependent manner have further implications for our understanding of the role of flippase kinases in the control of the yeast cell cycle. During progression through the cell cycle, the protein kinase Gin4p localizes at the incipient bud site, and phosphorylates and inhibits Fpk1p at the same location, which in turn increases the activity of Cdc42p (Roelants et al., 2015). Cdc42p is a small GTPase required for the recruitment and function of effectors that promote bud emergence (Atkins et al., 2013; Lo et al., 2013; Saito et al., 2007; Tong et al., 2007). As recruitment of Cdc42p to the plasma membrane depends on PS internalization (Fairn et al., 2011), our results suggest that Gin4p might exert at least parts of its effect indirectly via inhibition of Dnf3p.

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Our results indicate that Dnf3p is a PS transporter that evolved to complement a flippase kinase-controlled system at the plasma membrane that also includes Dnf1p and Dnf2p. The emergence of a system that created PS asymmetry in the plasma membrane may have been a crucial early event in fungal evolution that coincided with the appearance of polarized growth processes such as substrate penetration and the formation of hyphae. Notably, Dnf3p-like sequences are absent from Basidiomycota, which instead, with no exceptions, harbored representatives of a subclade in the Dnf1p/2p clade not present in Ascomycota, but shared with primitive fungal groups. This raises the question of whether sequences in this subclade replaced Dnf3p to carry out a similar function in polar growth in Basidiomycota.

Our phylogenetic analyses of P4 ATPase sequences demonstrate that the ancestor of the Dnf3p subclass of P4 ATPases emerged at the very base of the fungal tree. Their presence in Fonticula, a sister group...
Fig. 7. Five clades of P4 pumps are present in fungi. (A) Schematic of the relationship between fungal phyla represented in B. (B) Phylogenetic tree of 202 sequences representing all P4 ATPases from 37 fungal species and a rabbit (Oryctolagus cuniculus) SERCA pump protein sequence as outgroup. The fungal species represent the major phyla of the fungal kingdom. The phylogenetic tree was inferred from maximum likelihood analysis with 1000 bootstrap iterations, performed using the RAxML program version 8.2.1. Node values represent maximum likelihood statistical values with a maximum of 100%. All nodes with statistical values less than 60% were collapsed into multifurcations and only values of 75% or above are shown. In the phylogenetic tree, proteins belonging to the P4A clade are outlined in light gray, with individual groups in light blue. The P4B clade is outlined in blue. Proteins are preceded by a colored circle representing the corresponding phylum of the species. The species are as follows. Light blue: Basidiomycota: Aursu, Auricularia subglabra; Fompi, Fomitopsis pinicola; Fibra, Fibroporia radiculosa; Pleos, Pleurotus ostreatus; Copci, Coprinopsis cinerea; Cryga, Cryptococcus gattii; Treme, Tremella mesenterica; Pucgr, Puccinia graminis; Spore, Sporisorium reilianum; Ustim, Ustilago maydis; Walme, Wallisia mellicola. Dark blue: Ascomycota: Pyrom, Pyronema omphalodes; Macph, Macrophomina phaseolina; Baupa, Baudoinia panamericana; Exstu, Exserohilum turcicum; Aspni, Aspergillus niger; Penru, Penicillium rubens; Glalo, Glarea lozoyensis; Neuro, Neurospora crassa; Canai, Candida albicans; Sacce, Saccharomyces cerevisiae; Pnemu, Pneumocystis murina; Schpo, Schizosaccharomyces pombe; Tapde, Taphrina deformans. Green: Mucormycota: Gloce, Glomus cerebriforme; Rhii, Rhizofaghus irregularis; Morve, Mortierella verticillata; Mucci, Mucor circinelloides; Rhide, Rhizopus delemar. Yellow: Blastocladiomycota: Allomyces macrogynus; Catan, Catenaria anguillulae. Chytridiomycota: Rhig, Rhizoclozymatium globosum; Balde, Batrachochytrium dendrobatidis; Anaro, Anaeromyces robustus; Pirsp, Piramyces sp. Cryptomycota: Rozal, Rozella allomycis.
to true Fungi, is noteworthy. *Fonticula* morphologically resembles slime molds and do not develop hyphae. It may be that *Fonticula* has evolved an as yet uncharacterized mechanism for polarized growth that, in true fungi, became a basis for hyphal growth. This would suggest that the emergence of Dnf3p to complement the work of Dnf1p and Dnf2p represents a hallmark of fungal evolution.

To conclude, we present evidence that Dnf3p is able to transport PS, in addition to PC and PE, and is subject to regulation by Fpk kinases that control Dnf1/2p activity. Through phenotypic analysis we demonstrate that the action of Dnf1p, Dnf2p and Dnf3p is required for pseudohyphal growth. This, together with the results of our phylogenetic analyses and the physiological evidence gathered from filamentous fungi, suggests that Dnf1p, Dnf2p and Dnf3p play important roles in regulating polarized growth.

**MATERIALS AND METHODS**

All chemicals were purchased from Sigma-Aldrich, St Louis, MO, USA, unless otherwise stated.

**Yeasts strains**

Yeast strains used in this study are listed in Table S1. *DNF1, DNF2* and *DNF3* were sequentially removed in an *fpk1,2A* strain (Nakano et al., 2008).
kindly provided by Kazuma Tanaka, Hokkaido University, Japan (KKT268), by transforming yeasts with linearized gene-deletion constructs consisting of a loxp:HIS3:loxp cassette flanked by 500 base pair (bp)-long recombination sites corresponding to the promoter and terminator regions for the desired gene (Pomorski et al., 2003). HIS3 was subsequently removed by expression of Cre recombinase from a plasmid (Sauer, 1987). All integration and dis-integration events were verified by yeast colony PCR with Phusion polymerase (Fisher Scientific Fermentas, Waltham, MA) according to manufacturer’s instructions and the primers listed in Table S2. For C-terminal tagging of the endogenous copy of DNF3, a DNA construct containing the SNAP tag sequence followed by a HIS3 reporter gene, using primers that generate 30 bp-long homologous regions to the final nucleotides of the DNF3 open reading frame and the DNF3 3′-UTRs, respectively. The PCR product was subsequently transformed into wild-type BY4741 and integration of the fragment was checked by PCR as above. For cell morphology, pseudohyphae formation and invasive growth studies Δdnf1, Δdnf2 and/or Δdnf3 single, double and triple knockout yeast in MAtr-Y825 and MAte- HLY337 backgrounds (Johnson et al., 2014) (a kind gift from Anuj Kumar, University of Michigan) were generated. Gene deletions were carried out by transforming yeast with respective knockout constructs consisting of the loxp::URA3:loxP cassette with ~500 base pair recombination sites for the promoter region and 3′-end region of the DNF genes. DNF gene knockout cassettes were obtained by PCR using oligo3339 and oligo3339 with pMP5029, pMP5030 and pMP5031 as templates to amplify, respectively, DNF1, DNF2 and DNF3 knockout cassettes. A subsequent removal of the URA3 marker was performed by excision recombination using Cre recombinase (Sauer, 1987). The generation of double and triple gene deletions were performed sequentially by repeated use of the loxp::URA3:loxP cassette and subsequent removal of the URA3 marker by excision recombination using Cre recombinase. All integration and dis-integration events were verified by PCR. Diplodil strains were then generated by mating the respective knockout haploid strains.

**Yeast plasmids and cloning**

All plasmids (listed in Table S2) were constructed by PCR amplification using Phusion polymerase (Fisher Scientific Fermentas, Waltham, MA, USA) following manufacturer’s instructions. Primers are listed in Table S3. FPK1 and FPK2 were amplified from S. cerevisiae strain BY4741 genomic DNA. PCR-products were cloned into pENTR™/D-TOPO (Invitrogen, Carlsbad, CA, USA) and subsequently transferred to the Gateway™-compatible destination vector pMP2355, created by inserting Gateway® Reading Frame Cassette A in the unique HincII site of pRS425-GAL (Burgers, 1999), using the Gateway® Vector Conversion System (Invitrogen, Carlsbad, CA, USA). This resulted in plasmids pMP3859 (FKP1) and pMP3714 (FKP2). Full-length FPK1 was N-terminally tagged by two-step PCR-cloning. First, two overlapping PCR fragments corresponding to GST and FPK1 were amplified from pGEX-4T-1 (GE Healthcare, Amersham, UK) and pMP3859, respectively. A full-length PCR product was then used to purify the PCR products as templates. The final product was ligated into pENTR™/D-TOPO and transferred to pMP2355 using Gateway® technology (Invitrogen, Carlsbad, CA, USA). DNF3 was amplified from a plasmid (kindly provided by Joost Holthuis, University Osnabrück, Germany) with primers including EcoRI and Sall restriction sites at the 5′- and 3′-ends, respectively. The resulting PCR-product was ligated into pENTR™/D-TOPO and inserted into the Gateway®-compatible vector pMP1965 (Poulussen et al., 2008), rendering plasmid pMP4077. DNF3 was transferred to pMP4062 (López-Maqués et al., 2012) by EcoRI/Sall digestion to obtain N-terminally RGSH10-tagged DNF3 under the control of a GAL1-10 promoter. The same strategy was used to generate N-terminally RGSH10-tagged DR52 by transferring the gene from pMP2340 (López-Maqués et al., 2010) to pMP4062. DNF1 and DNF2 were amplified from plasmids (kindly provided by Joost Holthuis) and cloned by homologous recombination into pMP4062 using the ZHY709 yeast strain (Hua et al., 2002) as a host. Site-directed mutagenesis to obtain dnf3E343Q, dnf3D556N and fpk1K525R was performed with the QuikChange XL Gold Mutagenesis Kit (Stratagene, La Jolla, CA, USA), according to manufacturer’s instructions and the primers listed in Table S3.

DNF1-, DNF2- and DNF3-URA3 knockout cassettes were generated through a homologous recombination strategy in which the URA3 fragment was amplified by PCR (Oli5338 and Oli5339 with loxp sequence overhangs) using pRS423-GAL as template (Burgers, 1999), resulting in PCR fragments containing a loxp sequence overlap in the loxp::HIS3::loxP plasmids pMP4967, pMP4968 and pMP4969 (Pomorski et al., 2003). PCR fragments were transfomred into yeast strain ZHY709 together with linearized plasmids digested with BclI. This resulted in pMP4029, pMP5030 and pMP5031 containing, respectively, DNF1-, DNF2- and DNF3-URA3 knockout cassettes.

For cloning of GFP-tagged DR52 and DNF3, yEGFP was PCR amplified with primers oligo3727 and oligo3775 (Table S3). Homologous recombination in yeast of the PCR fragment into EcoRI linearized pMP423-GAL (Burgers, 1999) resulted in plasmid pMP3129. Constructs in pMP4062 (see above) were then used to transfer DR52 and DNF3 into pMP3129 by EcoRI/Sall digestion.

**Yeast transformation and media**

All yeast media reagents were purchased from BD Bioscience (Alburtland, Denmark), unless otherwise stated. Yeast transformations were performed by the lithium acetate method (Gietz and Woods, 2002). Cells were grown at 30°C in YPD (1% w/v yeast extract, 2% w/v bacto-peptone, 2% w/v glucose) or in rich synthetic medium containing 0.7% w/v Yeast Nitrogen Base, 1.4 g/l of Yeast Synthetic Drop-out Medium Supplements without the desired amino acids (Sigma-Aldrich), and 2% w/v glucose (SD), galactose (SG) or galactose/fructose (SGF). For cell elongation/pseudohyphae formation and invasive growth studies, medium without a nitrogen (2% w/v glucose, 0.7% w/v yeast nitrogen base without nitrogen, 0.05 mM ammonium sulfate, 0.03 mg/ml leucine, 0.02 mg/ml tryptophan and uracil) or glucose (1% w/v yeast extract and 2% w/v peptone) was used. For solid media, 2% agar was added.

**Membrane preparation and protein immunodetection**

Total membrane preparations were performed essentially as described previously (Villalba et al., 1992). Fresh yeast transformants were inoculated in selective SD medium and grown for 24 h at 28°C with 100 rpm shaking, before inoculation into SG medium at a 1:100 dilution. After another 24-h incubation period under the same conditions, cells were harvested by centrifugation (5000 g, 4°C, 5 min), washed twice in ice-cold water and resuspended 1/1 (w/v) in homogenization buffer (45% glycerol, 200 mM Tris-HCl pH 7.5, 40 mM EDTA, 0.5 µg/ml pepstatin A, 50 µM phenylmethylsulfonyl fluoride, 0.03 mM dithiothreitol). Cells were lysed by addition of one volume ice-cold acid-washed glass beads (0.5 mm), followed by four cycles of 1 min vortex and 1 min incubation on ice. Samples were diluted to a half with GTED20 (20% glycerol, 100 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 µg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and centrifuged (3000 g, 4°C, 10 min) to eliminate glass beads and cell debris. Total microsomal membrane fractions were collected by centrifugation at 100,000 g at 4°C for 1 h and homogenized in GTED20. Protein samples were quantified by the method of Bradford using bovine serum albumin (BSA) as a standard. For protein blot analysis of total membrane fractions, 40 µg total protein were precipitated with trichloroacetic acid.

For sucrose-density fractionation, fresh yeast transformants were inoculated in 5 ml selective SG media and grown for 24 h at 28°C under 160 rpm shaking. Cells were harvested (1500 g, 5 min, 24°C) and inoculated into 500 ml fresh SG medium. Cultures were grown to an optical density at 600 nm (OD600) of 1.1-2 at 28°C under 160 rpm shaking. Cells (400 ml) were harvested by centrifugation (1500 g, 4°C, 5 min), and washed in 25 ml ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.6 M sorbitol, 1 µg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). After centrifugation, cells were resuspended in 4 ml of lysis buffer, and 2-ml aliquots were vortexed with glass beads (1.2 g; 200 µm). The cell lysate was clarified by centrifugation (1000 g, 10 min, 4°C), and plasma membrane-enriched membranes were subsequently
collected by centrifugation (9000 g, 30 min, 4°C). Pellets were homogenized in 500 µl lysis buffer and centrifuged (1000 g, 10 min, 4°C) before loading onto two-step sucrose gradients prepared in lysis buffer: 7.6 ml 43% w/w sucrose on top of 3.8 ml 53% w/w sucrose. After centrifugation (120,000 g, 17 h, 4°C, rotor type Ti SW41), 1.2-ml fractions were collected from the top, diluted with 2 ml GTED20 and collected by centrifugation (100,000 g, 1 h, 4°C) before resuspension in 40 µl SDS-loading buffer (0.3 M Tris-HCl pH 6.8, 37.5% v/v SDS 10%, 0.05 M DTT, 13% w/w sucrose, 25% w/v Bromophenol Blue, 0.01 M EDTA). Equal volumes per fraction (20 µl) were used for immunodetection.

Protein samples were loaded onto 8% acrylamide SDS-PAGE gels, transferred to nitrocellulose membranes, and immunodetected using antibodies targeting the yeast plasma membrane marker Pma1p (Monk et al., 1991), the Golgi marker Sed5p (Sappérestein, 1996), and the ER marker Dpm1p (Invitrogen, Carlsbad, CA, USA). A monoclonal mouse anti-RGSHis antibody (cat. no. 34650, Qiagen, Venlo, The Netherlands) was used to detect decahistidine-tagged proteins. GST was detected with a goat anti-GST antibody (cat. no. 27-4577-01, GE Healthcare, Brondby, Denmark). As secondary antibodies, a rabbit anti-mouse-IgG (Dako, Glostrup, DK), goat anti-rabbit-IgG (Dako, Glostrup, Denmark) and a polyclonal rabbit anti-goat-IgG (Sigma, St. Louis, MO, USA) conjugated to alkaline phosphatase were used. Commercially available antibodies were used at the recommended dilutions. All other antibodies were used at a 1:5000 dilution. Bands were visualized with the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) color development substrate (Promega, Madison, WI, USA).

**Drop test assays**

For drop test assays, transformants were incubated in liquid SG medium for 4 h and then diluted with water to 0.1 and 0.01 OD600/ml. Drops (5 µl) were spotted on plates and incubated at 30°C for 4 days. When indicated, papuamide A (Flintbox, LynseyHuxham; www.flintbox.com), was added to the medium at a final concentration of 0.75 µg/ml for the mutant strain Δdnf1,2,3 mutants and 0.2 µg/ml for the triple Δdnf1,2,3 mutants. After 17 h until early logarithmic phase (0.2-0.5 OD600/ml), the growth was transferred to 50 ml SG medium to an initial OD600/ml of 0.009 for wild-type yeast or 0.03 for mutant strains. Cells were grown under the same conditions for another 16 h, 10 min after washing under a stream of water. ImageJ was used for quantification of fluorescent protein visualization and image analysis.

**Fluorescent protein visualization and image analysis**

The protocol for SNAP labeling of yeast cells was adapted from Stagge et al. (2013). Cells were grown in 1 ml YPD medium at 30°C for 16–18 h with 150 rpm shaking to an OD600/ml between 0.5 and 0.8, and harvested by centrifugation at 800 g for 2.5 min. After washing with deionized water, cells were resuspended in 100 µl SNAP-Cell Oregon Green solution (5 µM final concentration; New England Biolabs, Ipswich, MA, USA). The suspension was transferred to a 0.2 cm gap electroporation cuvette and cells were electroporated using an E. coli pulser™ (Bio-Rad, Hercules, USA) with a pulse strength of 800 V. Immediately after electroporation, 400 µl YPD medium were added to the cells inside the cuvette. The suspension was transferred to a round-bottom culture vial and incubated at 30°C for 90 min under 150 rpm shaking. For identification of dead cells, Trypan Blue staining was carried out as previously described (Liesche et al., 2015). For imaging, cells were harvested, resuspended in 100 µl phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) and analyzed within 30 min. A Zeiss Elyra PS1 microscope (Zeiss, Jena, Germany) with a 100×/1.46 numerical aperture objective was used. Wide-field settings and detection with an Andor iXon 860 EMCCD camera (Andor Technology, Belfast, Ireland) provided the high sensitivity necessary to visualize the signal even in cells with very low intensity. Oregon Green was excited at 488 nm with 5 mW output laser power. The signal was detected through a 495–565 nm band pass emission filter. The exposure time was 300 ms and two times frame averaging was used to obtain images with sufficient signal-to-noise ratio.

All image processing and analysis was performed in ImageJ (National Institutes of Health, Bethesda, MD, USA). For determining the percentage of cells with Dnf3p-SNAP endomembrane signal versus those only showing plasma membrane signal, every cell on 10 images (5 to 20 cells per image) was visually analyzed and categorized into one of the four states shown in Fig. S1B. Dead cells, which constituted 20% of all cells, were identified by strong internal Trypan Blue signal and excluded from the analysis. The experiment was repeated three times with at least 50 cells being analyzed in each repetition. For the quantification of the Dnf3p–SNAP signal distribution, the signal background, which was the same on all images, was subtracted.

**Cell morphology analysis, pseudohyphae formation and invasive growth**

Wild-type and Δdnf1 yeast knockout cells were grown for 18–24 h in YPD at 30°C and 150 rpm shaking. Cultures were diluted in water to 0.1 OD600/ml and drops (3 µl) were spotted onto agar plates lacking nitrogen or glucose. Colonies were imaged after incubation for 12 days at 30°C. A Leica MZ FLIII stereomicroscope (Leica microsystems, Wetzlar, Germany) with a transmitted light cone base and 2× magnification was used. Petri plates were placed directly on the microscope stage. Imaging was done before and after washing under a stream of water. ImageJ was used for quantification of the outer ring size. For this, each image of a single pseudohyphal colony was divided into six equal quadrants starting on the vertical axis of the image. A measurement of the outer ring size was taken along each of the six quadrant-delimiting axes and the average of the six measurements was used as the final outer ring size for each colony.

To study cell elongation and pseudohyphal growth parameters, cells were incubated in YPD for 18–24 h and then transferred to medium without nitrogen (starting OD600/ml 0.5) for another 18–24 h. Cultures were adjusted to 0.1 OD600/ml with water before 100 µl culture was spread onto agar plates without nitrogen. Plates were incubated at 30°C for 12 h before imaging. Blocks of agar containing the spread cultures were lifted from the plate with a scalpel, transferred to a slide and cells were observed without a cover slip. For control experiments, yeast strains were grown in YPD for 18 h, washed in water and resuspended to 0.1 OD600/ml in 2 ml complete SD medium. After 16 h, cells were mounted on microscopic slides for visualization. Light microscopy was performed with a Leica DMIR HC microscopy (Leica microsystems, Wetzlar, Germany) using a 100×/1.3 numerical aperture oil-immersion objective. Data were acquired for at least 900 cells per replicate for each yeast strain. Budding cells were treated as single cells. Any cell group containing three or more cells was considered a pseudohyphal colony.
Phylogenetic analysis

The collection of protein sequences for the phylogenetic analyses involved different steps. For the fungus-specific phylogenetic analysis, all P4 ATPases present in 37 fungal genomes, representing the major phyla of the fungal kingdom, were collected and curated as previously described (Palmgren et al., 2019). For the large analysis of eukaryotic P4 ATPases of various origins an extensive collection of P4 ATPases from the KEGG database was collected as previously described (Palmgren et al., 2019). To reduce the dataset, every sixth sequence in the collection was sampled and the rest discarded. As the KEGG database is biased towards dikaryotic fungi (Ascomycota and Basidimycota), the P4 ATPase sequences in the fungus-specific collection above (excluding those from dikaryotic fungi) were added to the collection. Further sequences were collected from the NCBI database by two BLASTp searches performed with standard settings using the S. cerevisiae DnEp sequence (Uniprot Q12764) as a protein query. The first search was against the Non-Redundant Protein(NR) database excluding fungi, and the second against the fungi in the NR database excluding Ascomycota. Following both searches, the top 100 hits were collected and curated as described in Palmgren et al. (2019), and approved sequences were added to the sequence collection. The sequences in the final dataset were aligned using the MUSCLE algorithm (Edgar, 2004) in MEGA6 (Tamura et al., 2013) with standard settings except for a gap extension value of −5. The resulting alignment was analyzed using the GBLOCKS program, with a minimum of 376 positions for a conserved position, a minimum of 376 positions for a flank position, a maximum of 15 contiguous non-conserved positions, a minimum length of 2 for a block and gap positions allowed for half of the sequences. This resulted in an alignment of 600 positions in 55 blocks. The maximum likelihood analyses were performed employing RAxML 8.2.4 (Stamatakis, 2014) using the LG+G+I substitution model and included 1000 rounds of bootstrapping. The analyses were run on the CIPRES Science Gateway (Miller et al., 2010) in the Extreme Science and Engineering Discovery Environment (XSEDE). Accession numbers for the proteins indicated in Fig. 8 are: Arabidopsis thaliana: ALA1, P198204; ALA2, P189205; ALA3, Q9X166; ALA4, Q9NL4Q; ALA5, Q9SGSG; ALA6, Q9LSK6; ALA7, Q9LVK9; ALA8, Q9LKO0; ALA9, Q9XS33; ALA10, Q9L183; ALA11, Q9SFA5; ALA12, P57792; Saccharomyces cerevisiae: DnSp2, P39524; DnEp1, P32660; DnEp2, Q12765; DnEp3, Q12764; Neop1, P41250; Homo sapiens: ATP8A1, Q9Y2QO; ATP8A2, Q9NT12; ATP8B1, O43520; ATP8B2, Q9P189; ATP8B3, O66423; AT8B4, Q8TF62; ATP9A, O75110; ATP9B, O43861; ATP10A, O60312; ATP10B, O94823; ATP10D, Q9P241; ATP11A, Q98196; ATP11B, Q9Y2G3; ATP11C, Q8NB49.

Statistical analysis

All assays were performed in triplicates and data are presented as the mean± s.e.m., unless otherwise stated. For all assays except for the evaluation of the proportion of pseudohyphal colonies containing specific cell numbers, statistics were performed using a two-way ANOVA analysis with strains and biological replicates as the two variables followed by pairwise comparisons with Tukey adjusted P-values. P<0.01 was considered significant. For the distribution of pseudohyphal colony sizes within a population, pairwise comparisons were made with multinomial regression with strains and biological replicates as variables and P-values adjusted with Holm’s method.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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Data availability

All raw data from flow cytometry analysis, immunoblot scans and phenotypic characterization experiments can be found at Figshare.org under doi:10.6084/m9.figshare.8293730.v1

Supplementary information

Supplementary information available online at https://jcs.biologists.org/lookup/doi/10.1242/jcs.235994.supplemental


