Two distinct lipid transporters together regulate invasive filamentous growth in the human fungal pathogen *Candida albicans*

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**Abstract**

Flippases transport lipids across the membrane bilayer to generate and maintain asymmetry. The human fungal pathogen *Candida albicans* has 5 flippases, including Drs2, which is critical for filamentous growth and phosphatidylserine (PS) distribution. Furthermore, a drs2 deletion mutant is hypersensitive to the antifungal drug fluconazole and copper ions. We show here that such a flippase mutant also has an altered distribution of phosphatidylinositol 4-phosphate [PI(4)P] and ergosterol. Analyses of additional lipid transporters, i.e., the flippases Dnf1-3, and all the oxysterol binding protein (Osh) family lipid transfer proteins, i.e. Osh2-4 and Osh7, indicate that they are not critical for filamentous growth. However, deletion of Osh4 alone, which exchanges PI(4)P for sterol, in a drs2 mutant can bypass the requirement for this flippase in invasive filamentous growth. In addition, deletion of the lipid phosphatase Sac1, which dephosphorylates PI(4)P, in a drs2 mutant results in a synthetic growth defect, suggesting that Drs2 and Sac1 function in parallel pathways. Together, our results indicate that a balance between the activities of two putative lipid transporters regulates invasive filamentous growth, via PI(4)P. In contrast, deletion of OSH4 in drs2 does not restore growth on fluconazole, nor on papuamide A, a toxin that binds PS in the outer leaflet of the plasma membrane, suggesting that Drs2 has additional role(s) in plasma membrane organization, independent of Osh4. As we show that *C. albicans* Drs2 localizes to different structures, including the Spitzenkörper, we investigated if a specific localization of Drs2 is critical for different functions, using a synthetic physical interaction approach to restrict/stabilize Drs2 at the Spitzenkörper. Our results suggest that the localization of Drs2 at the plasma membrane is critical for *C. albicans* growth on fluconazole and papuamide A, but not for invasive filamentous growth.

**Author summary**

*Candida albicans* is a human opportunistic fungal pathogen that diverged from *Saccharomyces cerevisiae* ~250 million years ago, in which a switch from budding growth to
Filamentous hyphal growth is associated with virulence. We sought here to understand how membrane expansion in these hyperpolarized filamentous cells is regulated by lipid transport mechanisms, and in particular flippases that transport phospholipids across lipid bilayers, thus generating membrane lipid asymmetry. Deletion of one of these flippases results in cells defective for hyphal invasive growth, cell wall integrity and virulence, as well as increased sensitivity to the most commonly used antifungal drug fluconazole. We show that this flippase localizes to different cellular compartments and our results suggest that the plasma membrane localization is critical for growth on fluconazole, but not for invasive filamentous growth and cell wall integrity. Strikingly, our data reveal that a genetic interaction between homologs of two different lipid transporters, previously identified in *S. cerevisiae* budding growth, regulates *C. albicans* hyphal growth. An attractive possibility that merits investigation is whether this is a conserved feature of the fungal kingdom.

**Introduction**

Polarized growth is an essential process that is regulated, in particular, by cooperative interactions between key establishment proteins and specific lipids at the plasma membrane (PM). For instance in the baker’s yeast *Saccharomyces cerevisiae*, dynamic nanoclusters of the Rho-GTPase Cdc42 are regulated by multivalent interactions between its sole activator Cdc24, the scaffold protein Bem1 and anionic lipids, including PS [1–3]. Similarly, in the fission yeast *Schizosaccharomyces pombe*, the localization and function of the two essential Rho-GTPases, Cdc42 and Rho1, depend on polarized PS distribution [4]. The importance of anionic lipids, and PS in particular, for cellular function and signaling encompasses kingdoms from fungi, to mammals [5] to plants [6]. In addition, PS has been reported to be critical for virulence in a broad spectrum of microbial pathogens (reviewed in [7,8]). In particular, in the human fungal pathogen *Candida albicans*, the PS synthase Cho1, which is conserved in fungi but does not have an ortholog in Humans, is required for virulence in a murine candidiasis model [9]. A cho1 deletion mutant also exhibits increased exposure of β(1,3)-glucan via up-regulation of cell wall MAPK cascades, facilitating its detection by innate immune cells [10]. PS is synthesized in the endoplasmic reticulum (ER) and its cellular distribution is regulated both by lipid transfer proteins (LTPs), which function at contact sites between the ER and the target cellular compartments, and lipid transporters, such as flippases that establish PS asymmetry between membrane leaflets (reviewed in [11]).

Lipid flippases are P4-ATPases, only found in eukaryotes, where they are similar in domain structures from fungi to Humans (reviewed in [12]). These proteins actively transport phospholipids from the external/luminal to cytoplasmic membrane leaflets and play an important role in polarized growth (reviewed in [13]). There are 14 P4-ATPases in Humans and only 5 in the yeast *S. cerevisiae* (Dnf1-3, Drs2, Neo1), with most of them regulated by non-catalytic subunits from the Cdc50/Lem3 family. For example, in *S. cerevisiae*, Cdc50 forms a functional complex with Drs2 [14,15] and very recently, the cryo-electron microscopy structure of different conformations of this complex was resolved at 2.8 to 3.7 Å [16]. This complex, which is well characterized, both in vitro and in vivo, primarily transports PS [17,18]. The role of lipid flippases on membrane curvature and in trafficking has been extensively studied in *S. cerevisiae*, as well as in mammals (reviewed in [19]). In *S. cerevisiae*, Neo1, which is the sole essential flippase [20], localizes to the Golgi and endomembranes, Drs2 and Dnf3 to the trans-Golgi network (TGN) and Dnf1-2 to the PM [17,21,22]. A recent study indicates that Dnf3 can also...
be found at the PM in a cell-cycle dependent fashion, where it regulates, together with Dnf1-2, *S. cerevisiae* pseudohyphal growth [23]. Furthermore, in response to pheromone, it was shown that Dnf1, Dnf2 and Dnf3 localize to the schmoo tip, while Drs2 remains at the Golgi [24]. Interestingly, in the filamentous fungi *Aspergillus nidulans* and *Fusarium graminearum*, it was shown that the Drs2 homolog, DnfB, localizes primarily to a cluster of vesicles at the hyphal apex, called the Spitzenkörper (SPK), which is characteristic of fungi that grow in a filamentous form [25,26]. Together, these data indicate that flippases can localize to different compartments in specific conditions of polarized growth.

LTPs and more specifically oxysterol-binding protein (OSBP)-related proteins (ORPs), are also important for membrane lipid composition, via non-vesicular traffic (reviewed in [27]), including for PS distribution (reviewed in [11]). LTPs can bind specific ligands such as PI(4)P, PS and sterol. For example, in *S. cerevisiae*, there are 7 oxysterol-binding homology (Osh) proteins, among which Osh6/Osh7 transports PS from cortical ER (cER) and PM [28], in counter-exchange with PI(4)P [29]. On the other hand, another Osh protein, Kes1, which is homolog to Osh4 and transports sterol in counter-exchange with PI(4)P [30], was proposed to act antagonistically with Drs2 to regulate the sterol distribution between PM and internal membranes in *S. cerevisiae* [31].

Filamentous fungi are highly polarized organisms, in which the role of flippases has been investigated. In *A. nidulans*, the homologs of Dnf1 and Drs2, *i.e.* DnfA and DnfB, regulate growth and PS asymmetry [25], while in *Maganopore grisea*, MgAPT2, one of the 4 aminophospholipid translocase (APT) encoding genes related to Drs2, is required for plant infection but not filamentous growth [32]. In *F. graminearum*, flippases play redundant as well as distinct roles in vegetative growth, where FgDnfA is critical, stress response, reproduction and virulence [26,33,34]. In the human fungal pathogen *C. albicans*, deletion of either Dnf1 or Dnf2 results in moderate increase sensitivity to copper [35]. The *drs2* deletion mutant is hypersensitive to copper [35], as well as to the antifungal drug fluconazole [36]. Deletion of Drs2 also results in altered PS distribution and impaired filamentous growth [36], and that of its Cdc50 subunit in altered filamentous growth and reduced virulence in a murine candidiasis model [37]. Here, we sought to determine more specifically the role of Drs2 in morphogenesis and whether PS asymmetry is critical for this process.

Our results show that in response to serum, of the four flippases Dnf1-3 and Drs2, only Drs2 is critical for filamentous growth, with Dnf2 having a minor role in this process. Filamentous growth was largely restored in the *drs2* mutant upon deletion of the LTP Osh4, but not by that of other Osh proteins (Osh2, Osh3 and Osh7). Furthermore, our results demonstrate that the distribution of PS and of the phosphatidylinositol phosphate PI(4)P, which are both altered in the *drs2* mutant, is restored upon deletion of *OSH4*. These data indicate that the requirement for flippase activity across the lipid bilayer during filamentous growth can be specifically bypassed by lipid exchange between membrane compartments.

### Results

**Drs2 has a unique role in *C. albicans***

In *A. nidulans*, the Drs2 homolog DnfB, is not critical for hyphal growth [25]. In contrast, in *C. albicans*, Drs2 is required for filamentous growth, whereas budding growth is largely unaffected (mean doubling time of 90 min, compared to 80 min in the wild-type strain) [36]. We investigated the importance of other flippases, *i.e.* Dnf1, Dnf2 and Dnf3, using loss of function mutants that we generated (S1A Fig). As illustrated in Fig 1A, a *drs2* deletion mutant was not invasive on serum-containing media. In contrast, deletion of *DNF1* or *DNF3* did not alter serum-induced invasive growth, while deletion of *DNF2* resulted in reduced invasive growth,
which was further reduced in a double dnf1dnf2 deletion mutant. Over-expression of DNF2 restored invasive growth in the dnf2 and dnf1 dnf2 mutants, but not in the drs2 mutant, suggesting that Dnf2 and Drs2 do not functionally overlap in this invasive growth process. Upon serum-induced hyphal growth in liquid media (Fig 1B–1D), the dnf1, dnf2 and dnf3 deletion mutants produced hyphae similar to the wild-type cells, with hyphal formation in the dnf1
dnf2 cells somewhat reduced. The average hyphal filament length at 90 min was, nonetheless, slightly reduced in dnf2 cells as well as in dnf1 dnf2 (14 ± 3 μm and 13 ± 3 μm, respectively) compared to the WT, dnf1 and dnf3 cells (20 ± 4 μm, 18 ± 4 μm and 20 ± 4 μm, respectively). These results are in agreement with very recent data, which also show that both a dnf2 and a dnf1 dnf2 mutant exhibit reduced invasive filamentous growth in response to the nutrient poor spider media, another inducer of filamentous growth [38]. These data indicate that, in contrast to the drs2 deletion mutant, dnf1, dnf2 and dnf3 deletion mutants undergo filamentous growth similar to the wild-type cells, albeit with a reduced efficiency for dnf2. Furthermore, Fig 2A and 2B shows that the dnf1-3 mutants also grew similar to the wild-type cells in the presence of the cell wall perturbants calcofluor white (CFW) and congo red (CR), the antifungal drugs, caspofungin (Caso) and fluconazole (FCZ), and the detergent sodium dodecyl sulfate (SDS), with only the dnf2 mutant growth somewhat reduced on 0.05% SDS. In contrast, the drs2 mutant was hypersensitive to SDS, in addition to CFW and FCZ [36]. The growth defect of drs2 cells on FCZ could result from a mislocalization of multi-drug transporters, such as Cdr1 [39]. Fig 2C shows that, although a Cdr1-GFP fusion protein was detected at the PM in drs2 cells, 79 ± 8% of the cells had internal Cdr1 signal, in contrast to wild-type and complemented cells, suggesting that PM targeting of MDR is partially impaired in drs2 cells. Together, these data indicate that Drs2 has a unique role in C. albicans.

**Drs2 is critical for hyphal extension after septin ring formation**

In response to serum, drs2 cells appear to initiate filamentous growth, although they were unable to form hyphae [36] and invasive colonies (Fig 1A), and reintroduction of a copy of DRS2 in this mutant restored invasive growth (Fig 3A). We examined this mutant in a murine model for systemic candidiasis and Fig 3B shows that the drs2 mutant causes no lethality, compared to the wild-type and complemented strains, at 16 days post injection. At the transcriptional level, hyphal growth is controlled by the hyphal specific cyclin HGC1, which is further regulated by the transcription factor UME6 [40]. Both hgc1 and ume6 deletion mutants are defective in hyphal extension and attenuated for virulence in a mouse infection model [41,42]. Quantitative RT-PCR analyses in Fig 3C show that, upon serum exposure, HGC1 and UME6 were up-regulated in the drs2 mutant compared to budding cells (>100-fold for HGC1 and 40-fold for UME6), with a level of induction of HGC1 slightly reduced compared to that of the wild-type cells. Similarly, ECE1, which encodes the peptide toxin candidalysin and whose expression correlates with cell elongation [43], HWP1, which encodes a hyphal cell wall protein associated with hyphal development [44] and ALS3, which encodes an agglutinin-like (Als) adhesin [45], were all up-regulated in the drs2 mutant upon serum exposure, with levels slightly reduced (3- to 5-fold) compared to the control cells. Only the induction of SAP6, which encodes a secreted acid protease [46], was substantially reduced (~10-fold) in drs2. Together, these data indicate that the drs2 mutant hyphal growth defect is unlikely due to the modest reduction of HGC1 induction.

To further characterize the drs2 mutant at the molecular level, we used time-lapse microscopy to follow cells expressing fluorescent reporters for different cellular compartments. Fig 4A illustrates representative time courses of hyphal growth both in WT and drs2 cells expressing a fluorescently tagged septin Cdc10 [47]. Measurements of the diameters of filament compartments, apical (pre-septum) and distal (post-septum) to the septin ring (Fig 4B), show that, while it remained constant in wild-type cells (1.8 ± 0.2 μm), the diameter increased in the apical compartments of drs2 cells by about 40% (to 2.5 ± 0.3 μm). This increase was associated with a reduced extension rate (on average 0.09 μm/min in drs2 cells, compared to 0.34 μm/min in WT cells; Fig 4C). These data are consistent with a defect in polarized growth in the
drs2 mutant after the first septin ring forms. In agreement with this, we observed that, upon filament extension, active Cdc42, visualized with the CRIB (Cdc42 Rac1 Interactive Binding domain) reporter [48], became depolarized in drs2 cells and the SPK (visualized with the myosin light chain Mlc1, [49,50]), was not maintained at the filament tip following cell division,

Fig 2. Drs2 has a unique role in C. albicans. (A). The drs2 mutant has specific increased susceptibility to fluconazole and calcofluor white. Serial dilutions of indicated strains, as in Fig 1, were spotted on YEPD media (Ctrl) containing 25 μg/ml calcofluor white (CFW), 400 μg/ml Congo red (CR), 125 ng/ml caspofungin (Caso) or 5 μg/ml fluconazole (FCZ). Images were taken after 2 days at 30°C. Similar results were observed in 2 independent experiments. (B). The drs2 mutant has increased susceptibility to SDS. Serial dilutions of indicated strains, grown as in 2A, were spotted on YEPD media (Ctrl) containing 0.01% or 0.05% SDS. (C). PM targeting of the multidrug ABC transporter Cdr1 is altered in drs2 cells. Central z-sections of representative wild-type (WT, PY6393), drs2/drs2 (drs2, PY6395) and drs2/drs2 + pDRS2DRS2 (drs2 + DRS2, PY6646) cells expressing Cdr1-GFP are shown.

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Fig 3. Drs2 is critical for virulence in a mouse model of systemic candidiasis. A) Reintroduction of DRS2 complements the invasive growth defect of the drs2 mutant. The indicated strains WT (PY4861), drs2/drs2 (drs2, PY3375), and drs2/drs2 + pDRS2DRS2 (drs2 + DRS2, PY5042), were grown as in Fig 1A and 1B) The drs2 mutant has attenuated virulence in a mouse model of hematogenously disseminated candidiasis. Balb/C mice (n = 10) were injected with an inoculum (5 x 10^5 cells) of the indicated strains and the survival was assessed. C) Hyphal specific gene are induced in drs2 cells upon serum exposure. mRNA and cDNA were prepared from the indicated strains grown 120 min in the presence of serum. HGCl, ECE1, HWP1, ALS3 and SAP6 transcripts were determined by qRT-PCR using primer pairs described in [89], and UME6 transcripts were determined using UME6.pTm/UME6.mTm (89 bp) primer pair. Bars indicate the mean ± SD (n = 3 determinations). Similar results were observed in an additional biological replicate. NAD-linked Glyceraldehyde-3-phosphate dehydrogenase (TDH3) transcript levels were used for normalization.

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compared to the control cells (Fig 4D–4E). Furthermore, comparison of the wild-type and drs2 cells expressing both reporters for active Cdc42 and the Spitzenkörper, indicates that depolarization of Cdc42 occurred prior to the SPK delocalization from the filament tip (S1 and S2 Movies). Together, these data indicate that, subsequent to septin ring formation, the drs2 mutant is unable to redirect or reinitiate growth to the apex, which ultimately results in growth arrest and/or pseudohyphal growth.

Drs2 localizes to the hyphal tip

In S. cerevisiae, Drs2 localizes to the late Golgi during budding [20] and mating [24], while its homolog in A. nidulans, DnfB, localizes within the SPK core [25]. To examine the distribution of Drs2 in C. albicans, we generated a strain that expresses a functional DRS2-GFP fusion (Fig 5A). Given that the homolog of Dnf1-2 in A. nidulans, DnfA, also localizes to the Spitzenkörper, albeit to the outer layer macrovesicles compared to DnfB [25], we also generated a strain that expressed a functional DNF2-GFP fusion (Fig 5A). Fig 5B shows that Drs2 was restricted to the apical region of the hyphal tip, as well as in internal structures, likely Golgi cisternae by analogy with S. cerevisiae [20]. Dnf2 was essentially localized at the tip crescent, similar to its localization in S. cerevisiae at bud tips and mating projections [24]. Both Dnf2 and Drs2 co-localized with a PM marker (a prenylated RFP fusion, RFP-CtRac1, [51]), although Drs2 localized to a more restricted region of the hyphal tip than Dnf2 (Fig 5C). Drs2 also partially co-localized with the SPK marker, Mlc1 (Fig 5D), which could be due to the proximity (less than 100 nm) of this structure to the PM [52]. These data indicate that Drs2 and Dnf2 have distinct distributions at the filament apex.

The drs2 mutant is altered for PI(4)P distribution

Drs2 is a P4-ATPase that flips PS selectively across the lipid bilayer in vitro and in vivo in S. cerevisiae [17,18]. In C. albicans, using the reporter Lactadherin C2 (LactC2) [53–55], we observed that the distribution of PS was altered during hyphal growth in the drs2 mutant, as the reporter was visible in an intracellular punctate pattern [36]. To determine the impact of the DRS2 deletion on the distribution of other lipids, shown to be critical for hyphal growth, such as the phosphatidylinositol phosphates PI(4)P and PI(4,5)P2 [56,57], as well as ergosterol [58], we used specific fluorescent reporters. The distribution of PI(4,5)P2 appears to be similar in wild-type cells and drs2 filamentous cells (Fig 6A), yet the distribution of PI(4)P was substantially less polarized in the mutant, compared to the wild-type or complemented strains (Fig 6B). This depolarization is further illustrated by the graph in Fig 6B, which shows the relative concentration of PI(4)P, as a function of filament length. In contrast, PI(4)P at the Golgi was largely unaffected in drs2, compared to WT cells, both during filamentous growth (Fig 6C), and budding growth (mean of 7.1 ± 2.8 Golgi cisternae per cell in drs2 cells compared to 7.8 ± 2.4 in the wild-type control, Fig 6D). These data indicate that deletion of DRS2 results in altered distribution of PM PI(4)P, but not PI(4,5)P2.

Using filipin staining, it was shown that membrane sterols are highly concentrated at the apex during C. albicans hyphal growth, with such a polarization not observed in budding and pseudohyphal cells [58]. We examined sterol distribution, using both filipin staining and the genetically encoded biosensor D4H. This biosensor consists of the Domain 4 of perfringolysin O, a toxin produced by Clostridium perfringens, used to monitor sterols in vivo [59–62]. Fig 7A shows that sterols are highly concentrated at the apex of WT hyphal cells, irrespective of the reporter used. In contrast, in the drs2 mutant, the D4H reporter preferentially labeled internal structures and reintroduction of a copy of DRS2 restored the ergosterol distribution to the PM.
Fig 4. Drs2 is critical for maintaining polarized filament extension. A) Both cell morphology and filament extension rate are altered in the drs2 mutant, after septin ring formation. Time lapse of wild-type (WT, PY5613) and drs2/drs2 (drs2, PY5615) cells expressing Cdc10-mScarlet, incubated in the presence of serum. Images were taken every 10 min and merges between DIC and sum projections of 21 z-sections are shown. Bars are 5 μm. B) Graphs represent the filament diameter before (open circles) and after (solid circles) the first septin ring, as a function of the times from the first image in which the septin ring is observed in wild-type (blue) and drs2 (magenta) cells. Means ± SD of 25–50 cells are shown. ***, P < 0.0001; ns, not significant. C) The graph shows the filament extension rate in wild-type (blue) and drs2 (magenta) cells. Means ± SD of 30–60 cells are shown. D) and E) Polarized growth is altered in the drs2 mutant. D) Time lapse of wild-type (WT, PY2263) and drs2 cells (drs2, PY4972) expressing CRIB-GFP, incubated in the presence of serum. Images were taken every 10 min and maximum projections of 21 z-sections are shown. E) Time lapse of wild-type (WT, PY5349) and drs2/drs2 cells (drs2, PY5218) expressing Mlc1-mScarlet, incubated in the presence of serum. Images were taken every 5 min and sum projections of 23 z-sections are shown.
Deletion of OSH4 in the drs2 mutant restores invasive filamentous growth and plasma membrane PI(4)P distribution

LTPs can bind specific ligands such as PI(4)P, PS and sterol and, in S. cerevisiae, it was shown that Osh6/Osh7 transports PS in counter-exchange with PI(4)P [29], while Kes1 transports sterol in counter-exchange with PI(4)P [30]. C. albicans has 4 Osh proteins (Osh2-4 and Osh7), with Osh4 and Osh7 sharing ~60% identity with their S. cerevisiae counterparts, however their function is unknown in this organism. Note that the genes encoding OSH7 (called OBPA and OBPALPHA) are located at the mating-type-like locus (MTL), similar to those encoding the Golgi phosphatidylinositol kinase Pik1 (PIKA and PIKALPHA).

To investigate the importance of Osh proteins in C. albicans invasive hyphal growth, we first generated osh4 and osh7 deletion mutants, as well as drs2 osh4 and drs2 osh7 double deletion mutants (S1B Fig), and examined these mutants for invasive hyphal growth in response to serum. While deletion of OSH4 or OSH7 alone did not alter invasive growth, deletion of OSH4, but not OSH7, in the drs2 mutant restored invasive growth to a level similar to that of WT cells (Fig 8A). In serum-containing liquid media, we also observed a rescue of hyphal growth upon deletion of OSH4 in the drs2 mutant (Fig 8B). In this drs2 osh4 mutant, hyphal length was only slightly reduced compared to that of the wild-type and osh4 cells (17 ± 3 μm, compared to 19 ± 1 μm for WT and osh4 cells). To confirm the specificity of the OSH4 deletion in restoring invasive growth in the drs2 mutant, we also generated drs2 osh2 and drs2 osh3 double deletion mutants (S2A Fig). Candida albicans OSH3 has been reported to be important for invasive growth in nutrient poor media (Spider media), but not in the presence of serum [63]. Similarly, we observed that mutants deleted for either OSH2 or OSH3 alone exhibited invasive growth in the presence of serum (S2B Fig). In contrast to OSH4 deletion, absence of either OSH2 or OSH3 did not rescue the drs2 invasive growth defect, although colonies from the drs2 osh3 mutant were somewhat crenelated compared to the control strains (S2B Fig).

Furthermore, while the osh2 and osh3 mutants formed hyphae similar to those of the wild-type (83 ± 5%, 73 ± 9% and 86 ± 6% for the WT, osh2 and osh3 strains, respectively), the percent of hyphae in the drs2 osh2 and drs2 osh3 mutants was similar to that of drs2 (8 ± 6% and 21 ± 7%, respectively, compared to 19 ± 8% in drs2) (S2C Fig). These data indicate that, while Osh proteins (Osh2-4 and Osh7) are not required per se for serum-induced invasive filamentous growth, deletion of OSH4 specifically bypasses the drs2 requirement in this process. Notably, OSH4 expression was not significantly altered in drs2 cells, compared to the WT cells (S1C Fig).

To better characterize the mechanisms underlying the rescue of hyphal growth in the drs2 osh4 mutant, we examined whether deletion of OSH4 also restored the lipid distribution in the drs2 mutant. Fig 9A shows that the distribution of PI(4)P was polarized in the drs2 osh4 double mutant, similar to that in wild-type and osh4 cells. Similarly, deletion of OSH4 in the drs2 mutant significantly restored PS distribution, as internal punctae were absent in drs2 osh4 cells, similar to the wild-type, complemented and osh4 strains (Fig 9B). Quantification of the LactC2 signal in the drs2 mutant shows that the fraction of PS was reduced at the PM and increased internally, compared to wild-type and osh4 cells, resulting in a decreased ratio of PM to intracellular signals (Fig 9B). Deletion of OSH4 in drs2 significantly increased this ratio, with an increase in PM signal, as well as a decrease in internal signal (Fig 9B). In contrast, the overall distribution of ergosterol appears similar in drs2 and drs2 osh4, with the presence of a number of internal punctae, compared to wild-type and osh4 cells (Fig 9C). The ergosterol tip
Fig 5. Drs2 localizes preferentially to the filament apex. A) The Drs2-GFP and Dnf2-GFP fusions are functional. The indicated strains, WT (PY4861), drs2/DRS2GFP (PY4665) and dnf2/DNF2GFP (PY5746) were grown as in Fig 1A and 1B and C) Drs2 and Dnf2 localize differently to the filament apex. Sum projections and zoom images of Max projections of 16 z-sections of representative cells expressing Drs2-GFP and Dnf2-GFP after 90 min serum induction are shown (B). Central z-sections and merge of representative cells expressing mScarlet-CtRac1, together with either Drs2-GFP (PY6241) or Dnf2-GFP (PY6239), after 90 min serum induction are shown (C). D) Drs2 partially overlaps with Mlc1. Central z-section and merge of representative cells expressing Drs2-GFP together with Mlc1-mScarlet (PY4928), after 90 min serum induction are shown. Bars are 5 μm.

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Fig 6. Plasma membrane PI(4)P distribution is altered in the drs2 mutant. A) PM PI(4,5)P₂ distribution is not altered in the drs2 mutant. Wild-type (PY1206) and drs2/drs2 cells (drs2, PY4050) expressing GFP-(PH<sup>PLCδ1</sup>)₂-GFP were incubated for 60 or 90 min, respectively, in the presence of serum. Sum projections (21 z-sections) and central z-sections of representative cells are shown. B) PM PI(4)P distribution is altered in the drs2 mutant. The indicated cells, WT (PY5619), drs2/drs2 (drs2, PY5568) and drs2/drs2 + pDRS2/DRS2 (drs2 + DRS2, PY6407) expressing GFP-(PH<sup>OSH2 H340R</sup>)₂-GFP were incubated for 90 min in the presence of FCS. Sum projections of (21 z-sections) of representative cells are shown. The graph illustrates the means ± the SEM of the relative concentration of PM PI(4)P as a function of filament length, normalized to the maximal signal for each cell (n = 25–60 cells). C) and D) The number of Golgi cisternae is not substantially affected in the drs2 mutant. DIC and maximum projections (21 deconvolved z-sections) of representative WT (PY2578) and drs2/drs2 cells (drs2, PY3873) expressing FAPP1<sup>E50A,H54A</sup>-GFP incubated in the presence of serum are shown (C). The number of Golgi cisternae per cell was determined in budding cells of the indicated strains from maximum projections of deconvolved images (21 z-sections). (D) Bars indicate the mean ± the SD of 3 independent biological samples (n = 100 cells and ~700–800 cisternae for each strain). * P < 0.05.

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polarization, determined as the apical versus sub-apical D4H signal, was also similar in these two mutants and not substantially different from that of wild-type cells. Notably, the ergosterol tip polarization was significantly reduced in the \textit{osh4} mutant alone, which exhibits hyphal growth similar to wild-type cells, suggesting that there is not a direct correlation between ergosterol tip polarization and hyphal growth. Together, these data indicate that Drs2 \textit{per se} is not required for hyphal invasive growth, but rather that a balance in the activities of Drs2 and Osh4 regulate this process, likely via regulation of the PM PI(4)P gradient.

Deletion of both \textit{DRS2} and \textit{SAC1} results in a synthetic growth defect

To further examine the importance of the PM PI(4)P gradient, we generated mutants deleted for the lipid phosphatase Sac1, which dephosphorylates PI(4)P, and is responsible for regulating PM PI(4)P, both in yeast and mammalian cells [64,65]. In \textit{C. albicans}, Sac1 is critical for the steep PM PI(4)P gradient [56] and hyphal growth maintenance [56,66]. In \textit{S. cerevisiae}, deletion of either \textit{KES1} or \textit{SAC1} is synthetically lethal in cells largely devoid of ER-PM contact

Fig 7. Distribution of ergosterol is altered in the \textit{drs2} mutant. A) The ergosterol reporters Filipin and D4H localize similarly at the apex of the filament in wild-type cells. Top panel: Wild-type cells (PY4861) were induced serum prior to staining with filipin, as described [58]. Images were taken with a wide-field fluorescence microscope. Bottom panel: Wild-type cells expressing mScarlet-D4H together with Trq-CtRac1 (PY6237) were induced with serum as in Fig 1B and images were taken with a spinning disk confocal microscope and central z-section, as well as merge images, are shown. B) Ergosterol distribution is altered in the \textit{drs2} mutant. Images of \textit{drs2/drs2} cells (\textit{drs2}, PY6083) and \textit{drs2/drs2 + pDRS2DRS2} cells (\textit{drs2 + DRS2}, PY6218) expressing mScarlet-D4H were taken as in Fig 6A bottom panel.

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sites [67], and cold-sensitive growth of a drs2 deletion mutant is partially suppressed by deletion of either SAC1 or KES1 [31]. As deletion of OSH4 restored PI(4)P polarized distribution in drs2, as well as hyphal growth, we investigated the effect of SAC1 deletion in the drs2 mutant and RT-PCR confirmed the absence of DRS2 and/or SAC1 in the respective mutants (Fig 10A). Fig 10B and 10C show that budding growth was altered in such a drs2 sac1 mutant, with a doubling time of 270 min compared to 90 min for WT, drs2 and sac1 strains. Furthermore, in serum-containing liquid media, while the sac1 mutant formed filamentous cells, albeit shorter than the wild-type cells (Fig 10D; [56,66]), the drs2 sac1 mutant was unable to form filamentous cells, even after 270 min incubation. Together, these data show that DRS2 and SAC1 show a synthetic negative interaction in C. albicans, indicating that they operate in parallel pathways.

**Drs2 is important for plasma membrane organization**

In addition to a defect in filamentous hyphal growth, the drs2 mutant also exhibits a hypersensitivity to CFW and FCZ, compared to wild-type cells (Fig 2A). We investigated whether a balance in the activities of Drs2 and Osh4 is also critical for growth on these compounds, and, while deletion of OSH4 in the drs2 mutant rescued the growth defect on CFW, it did not rescue...
Fig 9. Deletion of OSH4 rescues PI(4)P distribution in the drs2 mutant. A) PI(4)P distribution is restored in the drs2 mutant upon OSH4 deletion. Indicated cells expressing GFP-(PH\^{OSH2[H340R]})\_2-GFP, WT (PY5619), drs2/drs2 osh4/osh4 (drs2 osh4, PY5630) and osh4/osh4 (osh4, PY5626) were incubated as in Fig 6B, with sum projections of representative cells shown. The graph illustrates the means ± the SEM of the relative concentration of PM PI(4)P as a function of filament length, normalized to the maximal signal for each cell (n = 100 cells). B) PS distribution is partially restored in the drs2 mutant upon OSH4 deletion. Indicated cells expressing GFP-LacC2, WT (PY3239), drs2/drs2 osh4/osh4 (drs2 osh4, PY5134), drs2/drs2 osh4/osh4 (drs2 osh4, PY5364) and osh4/osh4 (osh4, PY5336) were incubated as in Fig 6B, with sum projections of representative cells shown. The graphs represent the relative PM and internal PS fractions, as well as the ratio of the signal at the PM over the internal signal for the indicated strains (n = 30–50 cells each), and bars mean ± the SD. ****, P < 0.0001; ***, P < 0.001; **, P < 0.01; ns, not significant. C) OSH4 deletion does not substantially restore ergosterol distribution in the drs2 mutant. Images of the indicated strains expressing mScarlet-D4H, WT (PY6037), drs2/drs2 osh4/osh4 (drs2 osh4, PY6286) and osh4/osh4 (osh4, PY6076) were taken as in Fig 7B. The graph represents the ergosterol tip polarization, i.e. the ratio of apical to sub-apical D4H signals, in the indicated strains.

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that on FCZ (Fig 11A). Furthermore, as the *drs2* mutant was reported to be hypersensitive to copper ions [35], we investigated whether deletion of *OSH4* could restore growth in this condition. Fig 11B shows that both *drs2* and *drs2 osh4* mutants were similarly reduced for growth on
CuSO$_4$, compared to wild-type and $osh4$ cells, indicating that Drs2 has specific roles that are not linked to Osh4 activity.

As shown above, using the LactC2 reporter, we observed that the distribution of PS was altered during hyphal growth in the $drs2$ mutant [36]. Papuamide A (PapA) is a depsipeptide toxin that binds PS in the outer leaflet of the PM [68]. In *C. albicans* the cho1 deletion mutant, which lacks the PS synthase, is less sensitive to PapA than WT cells [69]. Fig 11C shows that the $drs2$ mutant was hypersensitive to PapA, compared to the wild-type strain, reflecting increased PS in the PM outer leaflet. Interestingly, the $drs2$ $osh4$ mutant did not grow on PapA, similar to $drs2$, while the $osh4$ mutant grew similar to the wild-type (Fig 11C).
results indicate that in this *drs2 osh4* mutant, the PM PS bilayer asymmetry is not re-established.

**The distinct roles of Drs2 can be associated with different localizations**

Our data indicate that while filamentous growth was restored in the *drs2* mutant by OSH4 deletion, growth on fluconazole and papuamide A was not. On the other hand, Fig 5 shows that, in *C. albicans*, Drs2 localizes to different structures, including the SPK. Hence, we investigated whether a specific localization of Drs2 is critical for distinct functions, by using a synthetic physical interaction (SPI) approach to stabilize Drs2 at secretory vesicles, which accumulate at the SPK [50]. To generate a mutant with Drs2 restricted/stabilized at the SPK, we used a strain expressing a GFP nanobody (GNB) fused to one copy of Mlc1-iRFP, together with Drs2GFP as the sole expressed copy of *DRS2*. Fig 12A (bottom panel) shows that, in such a mutant, Drs2 and Mlc1 co-localized predominantly at the SPK, with the Drs2 signal strikingly increased in the strain expressing Mlc1-GNB, compared to a control strain (top panel), consistent with Drs2 stabilized at this structure. In this strain expressing Mlc1-GNB, Drs2 was not detected along the PM, compared to what we observed in the absence of the GNB construct (Figs 5 and 12A, top panel). In such a mutant expressing Mlc1-GNB, invasive filamentous growth was not altered (Fig 12B), with 85% of the cells forming hyphae in serum-containing liquid media after 90 min. In contrast, this mutant did not grow on fluconazole or papuamide A (Fig 12C). These data indicate that different roles of Drs2 are associated with its distinct cellular localizations, and suggest that *C. albicans* sensitivity to papuamide A is associated with Drs2 localization at the PM.

Given that deletion of OSH4 restored filamentous growth in a *drs2* mutant, but not growth on fluconazole or papuamide A, we investigated the effect of recruiting Osh4 to the SPK in these growth conditions. In *S. cerevisiae*, it was shown that the majority of Osh4 was cytoplasmic, with some punctae concentrated in small buds and mother–daughter necks [70]. In *C. albicans* hyphal cells, we did not detect a distinct localization of Osh4 (Fig 13A, top left panel), similar to what was reported for the *A. nidulans* Osh4 homolog, OshC [71]. However, in cells expressing both Mlc1-GNB and Osh4-GFP, as the sole expressed copy of *OSH4*, Osh4 co-localized with Mlc1 at the SPK, indicating that Mlc1-GNB recruited this LTP (Fig 13A). Such a recruitment of Osh4 in a wild-type strain background did not alter filamentous growth, yet it restored filamentous growth in liquid media and partially restored that on solid media in a *drs2* strain background (Fig 13B). In contrast, growth on papuamide A was not restored (Fig 13C). Together, these data indicate that recruitment of Osh4 to the SPK has a similar affect as deleting OSH4 in *drs2* cells for filamentous growth.

**Discussion**

Our results show that Drs2 is critical for *C. albicans* invasive filamentous growth and that a *drs2* deletion mutant exhibits increased sensitivity to calcofluor white and fluconazole, as well as papuamide A. The *drs2* mutant also has an altered PS, PI(4)P and ergosterol distribution, but not that of PI(4,5)P₂. The requirement for Drs2 in invasive filamentous growth, as well as growth in the presence of the chitin binding dye calcofluor white, can be specifically bypassed by the deletion of the lipid transfer protein Osh4, but not by that of other Osh proteins. Deletion of *OSH4* in *drs2* also restores the PI(4)P distribution and significantly the PS distribution, but not growth on papuamide A. Given that papuamide A binds PS in the outer leaflet of the PM, this indicates that the level of PS in the PM outer leaflet remains altered. In *C. albicans*, Drs2 localizes to different compartments, *i.e.* the Golgi, the Spitzenkörper and the PM. Such a localization of Drs2 is reminiscent of that of ChsB and SynA in *A. nidulans* [72], suggesting
Fig 12. Stabilization of Drs2 at the Spitzenkörper alters growth on papuamide A and fluconazole. A) Synthetic physical interaction (SPI) between Drs2 and Mlc1 stabilizes Drs2 at the Spitzenkörper. Representative images (central z-sections) of cells expressing Drs2-GFP, as a sole copy of Drs2, with either Mlc1-RFP (PY4928) or Mlc1-iRFP-GNB (PY5885) are shown; iRFP stands for near-infrared fluorescent protein and GNB for GFP nanobody. Right panels are enlargement of merge images. B) Stabilization of Drs2 at the SPK does not alter invasive filamentous growth. The indicated strains, WT (PY4861), drs2/DRS2GFP MLC1-iRFP-GNB (PY5885) and DRS2/DRS2 MLC1-iRFP-GNB (PY5385) were grown on agar-containing serum media and images were taken after 6 days (top panels) or grown in the presence of serum for 90 min (bottom panels). The percent of filamentous cells was 85–86% for the wild-type, drs2/DRS2GFP MLC1-iRFP-GNB and DRS2/DRS2 MLC1-iRFP-GNB strains; 2 experiments, n ~ 100 cells. C) Stabilization of Drs2 at the SPK alters growth on fluconazole and papuamide A. Serial dilutions of WT (PY4861), drs2/drs2 (PY3375), drs2/DRS2GFP (PY4665), drs2/DRS2GFP MLC1-iRFP-GNB (PY5885) and DRS2/DRS2 MLC1-iRFP-GNB (PY5385) strains spotted on YE PD media (Ctrl) containing either 2.5 μg/ml fluconazole (FCZ) or 2 μg/ml papuamide A (PapA) and images were taken after 2 days.

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Fig 13. The recruitment of Osh4 to the Spitzenkörper in drs2 phenocopies drs2 osh4. A) Synthetic physical interaction between Osh4 and Mlc1 recruits Osh4 to the Spitzenkörper. Representative images (Max projections) of cells expressing Osh4-GFP, as a sole copy of Osh4, without (PY5875) or with Mlc1-iRFP-GNB, in the wild-type (PY5931) or the drs2 deletion (PY6447) strains are shown. B) Recruitment of Osh4 at the SPK restores invasive filamentous growth in a drs2 deletion strain. Indicated strains, wild-type (PY4861), drs2/drs2 (PY3375), drs2/drs2 osh4/OSH4GFP MLC1-iRFP-GNB (PY6447) and DR52/DR52 osh4/OSH4GFP MLC1-iRFP-GNB (PY5931) were grown on agar-containing YEPD with serum and images were taken after 6 days (top panels) or grown in the presence of serum for 90 min (bottom panels). At 90 min,
Role of Drs2 in plasma membrane organization

In *S. cerevisiae*, it was shown that the *DRS2/DNF1-3* genes have some redundancy during budding growth [20]. Similarly, in the filamentous fungus *A. nidulans*, a double deletion of the Dnf1 and Drs2 homologs (DnfA and DnfB, respectively) is lethal [25,73], indicating that these flippases are redundant, in particular for growth. Yet, they appear to also have unique functions and/or localizations in different fungi. For example, in contrast to the case in *C. albicans*, deletion of the Drs2 homolog DnfB alone does not drastically alter *A. nidulans* hyphal growth [25]. With respect to localization, Drs2 is associated to the Golgi apparatus in *S. cerevisiae*, even in shmoos [24], while its homolog in *A. nidulans* DnfB localizes to the SPK [25]. We show here that CaDrs2 localizes to the filament apex (SPK and PM), in addition to the Golgi apparatus, and our data indicate that these distinct localizations are associated with different Drs2 functions. Indeed, upon stabilization of Drs2 at secretory vesicles that accumulate at the SPK, in which this flippase is no longer detected at the PM, cells are similar to that of the wild-type for invasive filamentous growth and calcofluor white sensitivity, yet unable to grow on papuamide A. Furthermore, deletion of Osh4, which restores hyphal growth and calcofluor white sensitivity, does not restore *drs2* growth on papuamide A, hence the level of PS in the PM external leaflet. Copper binds PS with high affinity and a *drs2* mutant is hypersensitive to copper, linking PS and copper sensitivity [35]. Given that *drs2 osh4* grows similar to *drs2* on copper and papuamide A, we suggest that Drs2 is required for regulating PS asymmetry, via its ability to flip PS across the PM.

Role of lipids in invasive filamentous growth

A *drs2* deletion mutant is defective in invasive filamentous growth in response to serum, and deletion of *OSH4* can specifically overcome these defects. Upon serum exposure, the *drs2* cells exhibit an alteration in the PM lipid distribution, i.e. PS, PI(4)P and ergosterol, which is at least partially compensated by *OSH4* deletion, raising the question of the respective contribution of these lipids to hyphal growth.

Sterol-rich membrane domains, have been visualized, by using filipin, at the tips of mating projections in *S. cerevisiae* [74], at cell poles and division site in *S. pombe* [75], as well as at the hyphal tips of *C. albicans* [58] and *A. nidulans* [76], where they are thought to contribute to polarized growth. In *S. cerevisiae* *drs2* cells, 20% of ergosterol appears to mislocalize to internal membranes, as assessed by filipin staining and DHE fluorescence, and deletion of KES1 suppresses the accumulation of intracellular ergosterol in this *drs2* mutant [77]. Our data, using the D4H reporter for free ergosterol within the cell, indicates that 96 ± 7% and 78 ± 12% of cells had multiple internal punctae in the *drs2* and *drs2 osh4* mutants, respectively, compared to 1 ± 2% and 9 ± 6% in wild-type and *osh4* cells, respectively, indicating that removal of Osh4 did not substantially suppress internal ergosterol accumulation in *C. albicans* *drs2* cells. Polarization of ergosterol to the growth tip also appears similar in the *drs2* and *drs2 osh4* mutants, at a level intermediate between the wild-type and *osh4* cells. Whether this polarized ergosterol signal is associated with the tip crescent and/or the SPK is unclear. In *S. pombe*, ergosterol...
associated D4H internal signals, which are enriched upon Arp2/3 inhibition, correspond to membrane-enclosed compartments, referred to as sterol-rich compartments [62]. Both this ergosterol movement to internal structures and the anterograde transport appear to be independent of vesicular trafficking [62]. Although the mechanism of sterol transfer is still to be determined, it is unlikely that the filamentous growth defect in \textit{drs2} directly results from the observed alteration of ergosterol distribution.

In \textit{C. albicans}, the phosphatidylinositol 4-kinase \textit{stt4} deletion mutant, in which PM PI(4)P is no longer detectable, and the PS synthase \textit{cho1} mutant, which has little to no PS, exhibit defective filamentous growth and increased sensitivity to the antifungal drug caspofungin [9,78]. Both these mutants have increased exposure of cell wall β(1,3)-glucan and, given that 50% of PS is still detectable at the PM of \textit{stt4}, we proposed that PM PI(4)P levels are associated with virulence by masking of cell wall β(1–3)-glucan [78]. In contrast to these mutants, the \textit{drs2} mutant grows similar to the wild-type cells on caspofungin and congo red, making it unlikely that the glucan synthase pathway is affected. As PM PS and PI(4)P levels are not drastically altered compared to the \textit{cho1} and \textit{stt4} deletion mutants, why the virulence is altered in \textit{drs2} is unclear. Similar to the \textit{drs2} mutant, the ratio of mean PM PS to internal PS decreases in a \textit{C. albicans} \textit{stt4} mutant, with increased internal PS levels compared to wild-type cells [78], yet, as for \textit{stt4}, our results are inconsistent with a correlation between PS distribution and filamentous growth defect in the \textit{drs2} mutant. Indeed, while invasive filamentous growth was restored upon deletion of \textit{Osh4}, the PM to internal PS ratio was only partially restored, and deletion of \textit{Osh4} did not restore growth on papuamide A. Rather, PM PI(4)P appears to be critical. In \textit{S. cerevisiae}, the cold-sensitive growth of a \textit{drs2} deletion mutant is partially suppressed by deletion of either the \textit{Osh4} homolog, \textit{Kes1}, or the PI(4)P phosphatase \textit{Sac1} [31]. In \textit{C. albicans}, deletion of \textit{Sac1} results in cells with altered PM PI(4)P gradient, as well as filamentous growth [56,66]. While the apical PI(4)P gradient as well as filamentous growth are restored upon deletion of \textit{Osh4} in \textit{drs2} cells, deletion of \textit{Sac1} substantially affects growth in \textit{drs2}, presumably by increasing the level of PM PI(4)P. Together, these results strongly suggest that the PM PI(4)P gradient, rather than the level of PI(4)P, is critical for filamentous growth.

Role of Drs2 in cell wall integrity and fluconazole sensitivity

Lipid transport through vesicular and non-vesicular trafficking is well established [79] and PS flipping activity of Drs2 is critical for the vesicular transport of a number of PM proteins. For example, ScDrs2 is required at the Golgi for efficient segregation of cargo into exocytic vesicles, as the PM proteins Pma1 and Can1 are missorted to the vacuole in \textit{drs2} cells, and deletion of \textit{KES1} suppresses this defect [77]. Hence, it is likely that the hypersensitivity of the \textit{drs2} mutant both for calcofluor white and fluconazole results from an alteration of membrane traffic in specific pathways. Interestingly, the \textit{drs2} mutant exhibits increased sensitivity to calcofluor white but not to caspofungin, suggesting that the chitin synthase pathway is selectively altered. Consistently, it was proposed that PS translocation to cytosolic leaflet of the Golgi by a flippase is critical for the \textit{S. cerevisiae} chitin synthase (Chs3) trafficking [80]. The growth defect of \textit{drs2} on fluconazole may result from a mislocalization of multi-drug transporters [39] and, consistent with such a scenario, we observe that ~ 80% of the \textit{drs2} cells had internal Cdr1 signal (Fig 2C). It would be interesting to investigate the importance of this flippase in pathogenic non \textit{albicans} \textit{Candida} species, known for their resistance to the antifungal drug, such as \textit{C. glabrata} or \textit{C. auris} [81].

Altogether our results are consistent with the notion that in \textit{C. albicans} Drs2 down-regulates \textit{Osh4} activity, similar to \textit{S. cerevisiae} [77], as wild-type filamentous growth and cell wall integrity were rescued in \textit{drs2}, either by deleting or targeting \textit{Osh4} to the SPK. Furthermore,
our data suggest that Osh4 does not function together with Drs2 at the SPK for filamentous
growth, as targeting Osh4 to this organelle has the same effect as deleting Osh4. Strikingly, our
data show that the genetic interaction between homologs of two different lipid transporters
identified in S. cerevisiae budding growth [31], occurs during hyphal growth, a distinct growth
mode in filamentous fungi. C. albicans diverged from S. cerevisiae ~ 250 million years ago
[82], hence an attractive possibility is that such an interaction is a conserved feature of the fun-
gal kingdom, and filamentous fungi, with a large diversity in size and growth rate, represent
useful models to understand how membrane expansion is regulated by lipid transport
mechanisms.

**Materials and methods**

**Ethics statement**

Animal procedures were approved by the Bioethical Committee and Animal Welfare of the
Instituto de Salud Carlos III (CBA2014_PA51) and of the Comunidad de Madrid (PROEX
330/14) and followed the current Spanish legislation (Real Decreto 53/2013) along with Direc-
tive 2010/63/EU.

**Growth conditions**

Yeast extract-peptone dextrose (YEPD) or synthetic complete (SC) medium was used and
strains were grown at 30˚C, unless indicated otherwise. Filamentous growth induction was
carried out as described previously either with 50% serum in liquid media and agar plates, or
75% on agarose pads for microscopy [83]. For filipin staining experiments, 10% of serum was
used. Growth on YEPD plates containing Congo red, calcofluor white, caspofungin or flucona-
azole was examined as described [84]. Copper sensitivity was investigated as described [30].
Congo red, calcofluor white, filipin, hygromycin and fluconazole were from Fluka, Sigma-
Aldrich, Saint Quentin Fallavier, France. Papuamide A was from University British Columbia.

**Strains and plasmids**

Strains and oligonucleotides used are listed in Tables 1 and 2, respectively. All strains were
derived from BWP17 [85]. The deletion mutants were generated by homologous recombina-
tion. Each copy was replaced by either HIS1, URA3, ARG4, SAT1 or HYGB, using knockout
cassettes generated by amplification of pGem-HIS1, pGem-URA3, pGem-CdARG4, pFa-
ARG4, pFa-SAT1 and pBH1S [85–88] with primer pairs DNF1.P1/DNF1.P2, DNF2.P1/DNF2.
P2, DNF2.P3/DNF2.P4 DNF3.P1/DNF3.P2, OSH4.P1/OSH4.P2, OSH7a.P1/OSH7a.P2,
OSH7a.P1/OSH7a.P2, OSH2.P1/OSH2.P2, OSH3.P1/OSH3.P2 and SAC1.P1/SAC1.P2. The
ds2Δ/drs2Δ osh4Δ/osh4Δ, drs2Δ/drs2Δ osh2Δ/osh2Δ, drs2Δ/drs2Δ osh3Δ/osh3Δ, drs2Δ/drs2Δ
osh7AΔ/osh7AΔ and drs2Δ/drs2Δ sac1Δ/sac1Δ strains were generated from the drs2Δ/drs2Δ
strain (PY3310 [36]) and the dnf1Δ/dnf1Δ dnf2Δ/dnf2Δ strain from the dnf2Δ/dnf2Δ strain
(PY5804).

Plasmid pDUP3-pADH1DNF2 was constructed by amplification from gDNA of the DNF2
ORF, using primers with a unique Ascl site at the 5’ end (DNF2.P5) and a unique MluI site at
the 3’ end (DNF2.P6) and pDUP3-pDRS2DRS2 by amplification from pExp-pDRS2DRS2 [36]
of the DRS2 ORF together with 1 kb upstream and downstream, using primers with a unique
SpeI site at the 5’ end (DRS2.P1) and a unique NotI site at the 3’ end (DRS2.P2). The fragments
were subsequently cloned into pDUP3-SAT1 [89], yielding to pDUP3-pADH1DNF2 and
pDUP3-pDRS2DRS2, respectively. To visualize Drs2, Dnf2 and Osh4, GFPγ was inserted by
homologous recombination at the 3’ end of DRS2, DNF2 or OSH4 ORF in strains heterozygous
Table 1. Strains used in the study.

| STRAIN       | RELEVANT GENEOTYPE                                 | REFERENCE |
|--------------|---------------------------------------------------|-----------|
| BWP17        | ura3Δ::imm434/ura3Δ::2imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG arg4Δ::hisG            | [65]      |
| PY1206       | Same as BWP17 with RP10::ARG4-pADH1-GFP-ΔHIS1::PH1::GFP                             | [57]      |
| PY2263       | Same as BWP17 with RP10::ARG4 pACT1-CRIBGFP                                               | [48]      |
| PY2578       | Same as BWP17 with RP10::pADH1-FAPP[56,54,58,59]GFP                                      | [56]      |
| PY3239       | Same as BWP17 with RP10::ARG4 -pACT1-GFP-yeLactC2                                        | [36]      |
| PY3310       | Same as BWP17 with drs2Δ::HIS1/ drs2Δ::URA3                                               | [36]      |
| PY3375       | Same as PY3310 with RP10::ARG4                                                            | [36]      |
| PY3873       | Same as PY3310 with RP10::pADH1-FAPP[56,54,58,59]GFP                                      | [56]      |
| PY3921       | Same as BWP17 with osh4Δ::URA3/ osh4Δ::HIS1                                               | This study|
| PY3945       | Same as BWP17 with osh3Δ::HIS1/ osh3Δ::URA3                                               | This study|
| PY3974       | Same as PY3921 with RP10::ARG4                                                            | This study|
| PY3977       | Same as PY3945 with RP10::ARG4                                                            | This study|
| PY3995       | Same as BWP17 with osh2Δ::HIS1/ osh2Δ::URA3                                               | This study|
| PY4002       | Same as PY3995 with RP10::ARG4                                                            | This study|
| PY4050       | Same as PY3310 with RP10::ARG4-pADH1-GFP-ΔHIS1::PH1::GFP                             | This study|
| PY4665       | Same as PY3303 (URA3) with DRS2:: DRS2::FPy-HIS1                                         | This study|
| PY4671       | Same as PY3310 with drs2Δ::HIS1/ drs2Δ::SAT1                                             | This study|
| PY4861       | ura3Δ::imm434/ura3Δ::3 imm434 his1::hisG/HIS1::hisG arg4::hisG/URA3::ARG4::arg4::hisG     | [83]      |
| PY4928       | Same as PY4665 with MLC1::MLC1::mScarlet-ARG4                                             | This study|
| PY4946       | Same as BWP17 with dnf1Δ::SAT1/ dnf1Δ::HYGB                                               | This study|
| PY4972       | Same as PY3310 with RP10::ARG4 pACT1-CRIBGFP                                             | This study|
| PY5003       | Same as PY3375 with NEUT5L::SAT1- pADHDNF2                                                | This study|
| PY5005       | Same as PY4861 with NEUT5L::SAT1- pADHDNF2                                                | This study|
| PY5042       | Same as PY3310 with RP10::ARG4-pDRS2::DRS2                                               | [36]      |
| PY5134       | Same as PY3310 with NEUT5L::SAT1-pACT1-GFP-yeLactC2                                      | This study|
| PY5218       | Same as PY4676 with MLC1::MLC1::mScarlet-ARG4                                             | This study|
| PY5246       | Same as BWP17 with osh7aΔ::SAT1/ osh7aΔ::HIS1                                             | This study|
| PY5256       | Same as PY5246 with RP10::URA3-ARG4                                                      | This study|
| PY5296       | Same as PY4671 with osh4Δ::URA3/ osh4Δ::HYGB                                             | This study|
| PY5297       | Same as PY4671 with osh7aΔ::HYGB/osh7aΔ::URA3                                           | This study|
| PY5336       | Same as PY3921 with RP10::ARG4- pACT1-GFP-yeLactC2                                      | This study|
| PY5349       | Same as PY4677 with MLC1::MLC1::mScarlet-ARG4                                             | This study|
| PY5364       | Same as PY5296 with RP10::ARG4- pACT1-GFP-yeLactC2                                      | This study|
| PY5385       | Same as BWP17 with MLC1::MLC1::miRFP670-GBN-URA3                                       | This study|
| PY5539       | Same as PY5296 with RP10::ARG4                                                           | [50]      |
| PY5568       | Same as PY3310 with RP10::ARG4- pADH1- GFP-[PH56][H340R]1::GFP                          | This study|
| PY5613       | Same as BWP17 with CDC10/CDC10::CDC10-mScarlet-ARG4 and NEUT5L::SAT1- pADH1::mRFP670::GFP| This study|
| PY5615       | Same as PY3310 with CDC10/CDC10::CDC10-mScarlet-ARG4 and NEUT5L::SAT1- pADH1::mRFP670::GFP| This study|
| PY5619       | Same as BWP17 with RP10::ARG4-pADH1- GFP-[PH56][H340R]1::GFP                          | [56]      |
| PY5626       | Same as PY3921 with RP10::ARG4- pADH1- GFP-[PH56][H340R]1::GFP                          | This study|
| PY5630       | Same as PY5296 with RP10::ARG4- pADH1- GFP-[PH56][H340R]1::GFP                          | This study|
| PY5677       | Same as BWP17 with DNF1::HIS1/ DNF2                                                        | This study|
| PY5741       | Same as BWP17 with dnf3Δ::URA3/ dnf3Δ::SAT1                                              | This study|
| PY5746       | Same as PY5677 with DNF2::DNF2::FPy-URA3                                                  | This study|
| PY5801       | Same as PY5741 with RP10::ARG4-pExp and his1Δ::HIS1                                      | This study|
| PY5804       | Same as BWP17 with dnf2Δ::HIS1/ dnf2Δ::ARG4                                              | This study|

(Continued)
Table 1. (Continued)

| STRAIN | RELEVANT GENEOTYPE | REFERENCE |
|--------|--------------------|-----------|
| PY5814 | Same as PY5804 with NEUT5L::URA3 | This study |
| PY5875 | Same as BWP17 with osh4Δ::URA3/ OSH4-GFPp-ARG4 | This study |
| PY5885 | Same as PY5835 with drs2Δ::CdARG4/DRS2-GFP-YSI | This study |
| PY5919 | Same as PY5814 with NEUT5L::SAT1-pADHDNF2 | This study |
| PY5922 | Same as PY5871 with NEUT5L::SAT1-pADHDNF2 | This study |
| PY5931 | Same as PY5835 with osh4Δ::HYG8/ OSH4-GFPp-ARG4 | This study |
| PY6037 | Same as BWP17 with RP10::ARG4- pACT1-mScarlet-D4H | This study |
| PY6076 | Same as PY3921 with RP10::ARG4- pACT1-mScarlet-D4H | This study |
| PY6083 | Same as PY3310 with RP10::ARG4- pACT1-mScarlet-D4H | This study |
| PY6213 | Same as PY6083 with NEUT5L:: pACT1-Turquoise-CtRAC1-SAT1 | This study |
| PY6218 | Same as PY6083 with NEUT5L::SAT1-pDRS2DRS2 | This study |
| PY6235 | Same as PY4946 with RP10::ARG4 | This study |
| PY6237 | Same as PY6037 with NEUT5L::ACT1-Turquoise-CtRAC1-SAT1 | This study |
| PY6239 | Same as PY5746 with NEUT5L:: mScarlet-CtRAC1-SAT1 | This study |
| PY6241 | Same as PY6665 with NEUT5L:: mScarlet-CtRAC1-SAT1 | This study |
| PY6286 | Same as PY6083 with osh4Δ::HYG8/ osh4Δ::SAT1 | This study |
| PY6350 | Same as PY4972 with drs2Δ::HISI/ drs2Δ::SAT1 | This study |
| PY6389 | Same as PY6350 with MLC1/MLC1-mScarlet-URA3 | This study |
| PY6393 | Same as PY4861 with CDR1/CDR1::CDR1-GFP-SAT1 | This study |
| PY6395 | Same as PY3375 with CDR1/CDR1::CDR1-GFP-SAT1 | This study |
| PY6400 | Same as PY5804 with dfs1Δ::URA3/ dfs1Δ::HYG8 | This study |
| PY6407 | Same as PY5568 with NEUT5L::SAT1-pDRS2DRS2 | This study |
| PY6408 | Same as PY4671 with osh4Δ::URA3/ osh4Δ::HYG8 | This study |
| PY6431 | Same as PY4671 with osh4Δ::URA3/ osh4Δ::ARG4 | This study |
| PY6432 | Same as PY4671 with sac1Δ::ARG4/ sac1Δ::HYG8 | This study |
| PY6436 | Same as BWP17 with sac1Δ::ARG4/ sac1Δ::HYG8 | This study |
| PY6447 | Same as PY4671 with osh4Δ::HYG8/ OSH4- GFPp-ARG4 and MLC1/MLC1-miRFP670-GNB-URA3 | This study |
| PY6450 | Same as PY2263 with MLC1/MLC1-mScarlet-ARG4 | This study |
| PY6646 | Same as PY5042 with CDR1/CDR1::CDR1-GFP-SAT1 | This study |

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for these genes, after amplification of GFPp from the plasmid pFA-GFPp-HISI [90], using the primers DRS2.P3/DRS2.P4, DNF2.P7/DNF2.P8 or OSH4.P3/OSH4.P4. pExp-pACT1CRIBGFP [48] was used to transform the WT (BWP17) and drs2/drs2 (PY3310) strains. Cdr1-GFP, PH-FAPP [E50A,H54A]GFP, GFP-(PH-[H340R]1)2-GFP and GFP-PHPLCδ1-PHPLCδ1-GFP expressing strains were generated as described [56,57]. pDUP5-mScarlet-CtRac1 [91] was used to transform the drs2/DRS2GFP (PY4665) and dfs2/DFS2GFP (PY5746) strains. Mlc1-mScarlet and Cdc10-mScarlet were generated by amplification of mScarlet-ARG4 from pFA-mScarlet-ARG4 [92], using the primer pairs MLC1.P1/MLC1.P2 and CDC10.P1/CDC10.P2, respectively. mTurquoise-CtRac1 (mTrq-CtRac1) was constructed by replacing mScarlet in pDUP5-pADH1-mScarlet-CtRac1 [91] with mTrq, and subsequently cloning mTrq-CtRac1 into pDUP3. To visualize the distribution of phosphatidylinerine, a fusion of GFP with the discoidin-like C2 domain of lactadherin (GFP-LactC2) was used, as previously described [36]. Strains expressing Mlc1-iRFP-GNB were generated by homologous recombination, using pFA-iRFP670-GNB-URA3, as in [50]. To visualize the distribution of ergosterol, we used filipin staining, as described [58], as well as the genetically encoded biosensor D4H [62]. D4H was amplified from plasmid pSM2244 [62], using primer pair D4H.P1 and D4H.P2 and cloned.
Table 2. Primer sequences.

| PRIMER | SEQUENCE |
|--------|----------|
| DNFL.P1 | CAAGGTGACAACTCCAGTGGTACGCATTATGTTGACGACAAATCCGAGAAATGTTGAGAACTCTGAAAGGCTGAAAGTGGTACGACAGGGTCTAGTATAGGCTAGGTCAGGTC |
| DNFL.P2 | CGAAGCTGACCCTACTTTGACAGATTAGAACGCTAGTCATGCTGCTGACGCTAGGCTAGGCTAGGTCAGGTC |
| DNFL.P3 | CAGCTGCTGCTGACGCTAGGCTAGGCTAGGTCAGGTC |
| DNFL.P4 | CCTGCTGCTGACGCTAGGCTAGGCTAGGTCAGGTC |
| DNFL.P5 | GCGGCGCGCCAAGCTAGGCTAGGCTAGGTCAGGTC |
| DNFL.P6 | GGACAGAATATCCTCGGATAAGGAAATGTTGAGAACTCTGAAAGGCTGAAAGTGGTACGACAGGGTCTAGTATAGGCTAGGTCAGGTC |
| DNFL.P7 | GTAATGAGGGTGAATATCCTCGGATAAGGAAATGTTGAGAACTCTGAAAGGCTGAAAGTGGTACGACAGGGTCTAGTATAGGCTAGGTCAGGTC |
| DNFL.P8 | CCTGCTGCTGACGCTAGGCTAGGCTAGGTCAGGTC |
| DNFL.P9 | TTCGTTCTGCTGCTGACGCTAGGCTAGGCTAGGTCAGGTC |
| DNFL.P10 | GAGGCGGCAATCCTCGGATAAGGAAATGTTGAGAACTCTGAAAGGCTGAAAGTGGTACGACAGGGTCTAGTATAGGCTAGGTCAGGTC |
| DNFL.P11 | GCCATGCTGCTGACGCTAGGCTAGGCTAGGTCAGGTC |
| DNFL.P12 | GCTGCTGCTGACGCTAGGCTAGGCTAGGTCAGGTC |
| DNFL.P13 | CTGCTGCTGACGCTAGGCTAGGCTAGGTCAGGTC |
| DNFL.P14 | GCCATGCTGCTGACGCTAGGCTAGGCTAGGTCAGGTC |

(Continued)
into plasmid pExp-pACT1-mScarlet-CtRac1 [92], using Ascl and MluI unique restriction sites, yielding pExp-pACT1-mScarlet-D4H. This plasmid was linearized with Ncol and integrated into the RP10 locus. All other pExp plasmids were linearized with Stul and integrated into the RP10 locus. pDUP3 and pDUP5 plasmids were digested with NgoMIV to release the cassette to be integrated into the NEUTL5 locus.

Two independent clones of each strain were generated and confirmed by PCR. RT-PCR was also performed, where relevant, using the primers (GENE.pTm and GENE.mTm) listed in Table 2 or previously described [93] and RNA extraction was carried out using Master Pure yeast RNA extraction purification kit (Epicentre). All PCR amplified products were confirmed by sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Microscopy analyses

Colonies and cell morphology imaging were performed as described [83]. Briefly, plates were incubated for 3–6 days prior to imaging with a Leica MZ6 binocular (x20) and cells (budding or serum-induced) were imaged by differential interference contrast with a microscope Leica DMR, using an ImagingSource DMK 23UX174 sCMOS camera.

Time lapses and fluorescent images were obtained using a spinning disk confocal microscope (inverted IX81 Olympus microscope with a 100X objective and a numerical aperture 1.45) and an EMCCD camera (Andor technology, UK). Z-stacks (images of 0.4 μm sections) were acquired every 5 min, as described [56]. Maximum or sum intensity projections were generated from 21 z-sections with ImageJ software. Laser illuminations of 445 nm (Turquoise), 488 nm (GFP), 561 nm (mScarlet) and 640 nm (iRFP) were used. CRIBGFP distribution experiments were carried out as described [48]. For filipin analyses, widefield images were acquired on an inverted ZEISS Axio Observer Z1 microscope with a 100X (1.3 NA) objective. Huygens professional software version 18.04 (Scientific-Volume Imaging) was used to deconvolve z-stack images of cells expressing PH-FAPP1[E50A,H54A]-GFP. Bars are 5 μm.

Filament lengths and diameters were measured from DIC images, using ImageJ. Golgi cisternae were quantitated from deconvolved MAX projection images. Quantitation of PI(4)P distribution was performed on SUM projections, using the Matlab program HyphalPolarity [56]. Quantitation of PS PM and internal mean signals was performed on central z-sections, also using the Matlab program HyphalPolarity. Central Z sections are middle planes of the cells. Unless indicated otherwise, error bars represent the standard deviations. Statistical significance was determined with Student’s unpaired two-tailed t test.

Virulence assays

HDC was induced in 10 Balb/C mice (Charles Rivers, Italy) per group by injecting the lateral tail vein with an inoculum of 5 x 10^5 cells [94]. Animal body weight was monitored daily and

| PRIMER | SEQUENCE |
|--------|----------|
| SAC1.pTm | CCCATTCCACAGCAACAGATG |
| SAC1.mTm | TTCAACACCCGCTCCAGTGT |
| ACT1.pTm | AGTTGCCAGGATTTGCGGA |
| ACT1.mTm | ACATTGGGTTGAAACAGTGG |
| TDH3.pTm | ATCCCAACGAGACTGAGA |
| TDH3.mTm | GCAAGAGCTTTAGCAAGTG |

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animals were sacrificed by cervical dislocation when they had lost more than 20% of their weight.

Supporting information

**S1 Fig. Flippase deletion mutant strains verification.** A) DRS2 and DNF1-3 transcript levels. mRNA and cDNA were prepared from the indicated strains, as in Fig 1A. DRS2 and DNF1-3 transcripts were determined by RT-PCR, using DRS2.pTm/DRS2.mTm (62 bp), DNF1.pTm/DNF1.mTm (73 bp), DNF2.pTm/DNF2.mTm (106 bp) and DNF3.pTm/DNF3.mTm (104 bp) primer pairs, respectively. Actin (ACT1) transcript levels (ACT1.pTm/ACT1.mTm primer pair) were used for normalization. B) OSH4 and OSH7 transcript levels. mRNA and cDNA were prepared from the indicated strains, as in Fig 8A. Transcripts were determined as in S1A Fig, using OSH4.pTm/OSH4.mTm (69 bp), OSH7A.pTm/OSH7A.mTm (84 bp), OSH7α.pTm/OSH7α.mTm (65 bp) primer pairs, respectively. C) Deletion of DRS2 does not result in a significant alteration of OSH4 expression. mRNA and cDNA were prepared from the indicated strains grown without (0) or with serum for 120 min (120). OSH4 and DRS2 transcripts were determined by qRT-PCR using primer pairs OSH4.pTm/OSH4.mTm and DRS2.pTm/DRS2.mTm as above. Bars indicate the mean ± SD of 2 biological samples (n = 3 determinations each). ACT1 transcript levels were used for normalization.

(TIF)

**S2 Fig. Deletion of OSH2 and OSH3 do not recover invasive filamentous growth in the drs2 mutant.** A) DRS2 and OSH2-3 transcript levels. mRNA and cDNA were prepared from the indicated strains, and transcripts were determined as in S1A Fig, using OSH2.pTm/OSH2.mTm (74 bp), OSH3.pTm/OSH3.mTm (74 bp) primer pairs, respectively. B) Invasive growth is not restored in the drs2 mutant upon deletion of OSH2 or OSH3. The indicated strains, WT (PY4861), osh2/osh2 (osh2, PY3977), osh3/osh3 (osh3, PY4002), drs2/drs2 (drs2, PY3375), drs2/drs2 osh2/osh2 (drs2 osh2, PY6408) and drs2/drs2 osh3/osh3 (drs2 osh3, PY6431), were grown on agar-containing serum media and images were taken after 6 days. Similar results were observed in 2 independent experiments. C) Hyphal growth is not restored in the drs2 mutant upon deletion of OSH2 or OSH3. At 90 min, the percent of filamentous cells, was determined from 3 independent biological samples for the WT, drs2, osh2, osh3, drs2 osh2 and drs2 osh3 strains; n ~ 100 cells.

(TIF)

**S1 Movie. Active Cdc42 and Mlc1 distribution during filament extension in wild-type cells.** Time lapse of wild-type (PY6450) cells expressing Mlc1-mScarlet, together with CRIB-GFP incubated in the presence of serum. Images were taken every 10 min and maximum projections of 21 z-sections are shown.

(AVI)

**S2 Movie. Active Cdc42 delocalizes prior to Mlc1 upon filament extension in the drs2 mutant.** Time lapse of drs2/drs2 cells (PY6389) expressing Mlc1-mScarlet, together with CRIB-GFP incubated in the presence of serum. Images were taken every 10 min and maximum projections of 21 z-sections are shown.

(AVI)

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