A functional genomic screen in *Saccharomyces cerevisiae* reveals divergent mechanisms of resistance to different alkylphosphocholine chemotherapeutic agents

Jaquelin M. Garcia,1,† Michael J. Schwabe,1,‡ Dennis R. Voelker2 and Wayne R. Riekhof1,*

1School of Biological Sciences, University of Nebraska—Lincoln, Lincoln, NE 68588, USA
2Department of Medicine, National Jewish Health, Denver, CO 80206, USA

*Corresponding author: Email: wriekhof2@unl.edu
†Present address: Division of Biology and Biomedical Sciences, Washington University, St. Louis, MO 63108, USA
‡Present address: Department of Surgery, Creighton University School of Medicine, Omaha, NE 68131, USA

Abstract

The alkylphosphocholine (APC) class of antineoplastic and antiprotozoal drugs, such as edelfosine and miltefosine, are structural mimics of lyso-phosphatidylcholine (lyso-PC), and are inhibitory to the yeast *Saccharomyces cerevisiae* at low micromolar concentrations. Cytotoxic effects related to inhibition of phospholipid synthesis, induction of an unfolded protein response, inhibition of oxidative phosphorylation, and disruption of lipid rafts have been attributed to members of this drug class, however, the molecular mechanisms of action of these drugs remain incompletely understood. Cytostatic and cytotoxic effects of the APCs exhibit variability with regard to chemical structure, leading to differences in effectiveness against different organisms or cell types. We now report the comprehensive identification of *S. cerevisiae* titratable-essential gene and haploid nonessential gene deletion mutants that are resistant to the APC drug miltefosine (hexadecyl-O-phosphocholine). Fifty-eight strains out of ~5600 tested displayed robust and reproducible resistance to miltefosine. This gene set was heavily enriched in functions associated with vesicular transport steps, especially those involving endocytosis and retrograde transport of endosome derived vesicles to the Golgi or vacuole, suggesting a role for these trafficking pathways in transport of miltefosine to potential sites of action in the endoplasmic reticulum and mitochondrion. In addition, we identified mutants with defects in phosphatidylinositol-4-phosphate synthesis (TetO::STT4) and hydrolysis (sac1Δ), an oxysterol binding protein homolog (osh2Δ), a number of ER-resident proteins, and multiple components of the eisosome. These findings suggest that ER-plasma membrane contact sites and retrograde vesicle transport are involved in the interorganelle transport of lyso-PtdCho and related lyso-phospholipid-like analogs to their intracellular sites of cytotoxic activity.

Keywords: *Saccharomyces*; lipid; phosphatidylcholine; alkylphosphocholine; miltefosine; antifungal; drug resistance; transport

Introduction

The alkylphosphocholine (APC) class of drugs are structural analogs of lyso-phosphatidylcholine (lyso-PC), and have been extensively investigated as antineoplastic agents (Gajate et al. 2012; Gajate and Molinillo 2014). They also act as effective antiprotozoal compounds, with potent activity against *S. cerevisiae* and other apicomplexan parasites (D’Alessandro et al. 2002; Santa-Rita et al. 2004; Verma et al. 2007; Aichelburg et al. 2008; Machado et al. 2010). *Leishmania donovani* has been investigated with regard to genetic mechanisms leading to drug resistance, and miltefosine-resistant strains have been identified in which a P2-ATPase (lipid flipase) at the plasma membrane is defective, and thus the resistant strain fails to import the toxic compound (Pérez-Victoria et al. 2006; Seifert et al. 2007; Weingartner et al. 2010). The APC’s are also active against fungal pathogens such as *Candida albicans* and *Cryptococcus neoformans* (Widmer et al. 2006; Vila et al. 2015), and the yeast *Saccharomyces cerevisiae* has served as a model for studies on drug resistance and elucidation of the molecular mechanism(s) of action of these drugs (Hanson et al. 2003; Cuesta-Marbán et al. 2013; Czyz et al. 2013). Although the body of work on APC resistance is extensive, particularly on mechanisms of action of edelfosine and miltefosine (Figure 1A), an understanding of the precise mechanism(s) of action of the APC’s remains incomplete, and the intracellular location of targets and mechanisms of action of these drugs appear to vary from compound to compound and between organisms and cell types.

A main mechanism of drug resistance, however, is clearly attributable to the loss of active transport of the APC’s into cells, which occurs via P2-family ATPase mediated internalization at the plasma membrane (Hanson et al. 2003; Riekhof and Voelker 2009). In the extensively studied *L. donovani* system, disruption of the miltefosine transporter, LdMT (Pérez-Victoria et al. 2006; Seifert et al. 2007) or its noncatalytic β-subunit, LdRos3 (Pérez-Victoria et al. 2006) leads to drug resistance, similar to loss of the homologous *S. cerevisiae* proteins Dnf2p or Lem3p,
in APC treated cells, a corresponding defect in regulation of intracellular pH, and increased sensitivity to low extracellular pH. The edelfosine-resistant gene deletion mutant set these studies identified was enriched in genes encoding proteins with roles in endocytosis and endosomal transport, as well as in intracellular pH regulation.

We now report the comprehensive identification of miltefosine-resistant S. cerevisiae haploid deletion and titratable-essential gene strains. While there is overlap with the edelfosine-resistant mutant strains, we identified a substantial number of new mutant strains as miltefosine-resistant, suggesting that the mechanism(s) by which these compounds exert their cytotoxic effects are different, and that the mechanisms of fungal resistance to these drugs is not conserved across this drug class. In addition, we identified an oxysterol-binding protein homolog (Osh2p) as being necessary for miltefosine sensitivity, and suggest that its presence at membrane contact sites is necessary for efficient APC dissemination to sites of action within the cell. We further show that proper Osh2p localization is likely to be dependent on the content and localization of phosphatidylinositol-4-phosphate, as judged by mislocalization of Osh2p-GFP in TetO::STT4 and sac1Δ strains.

Materials and methods

Strains and growth conditions

All media components were from Fisher Scientific or Sigma-Aldrich. Routine growth and screening was conducted on YPD (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) or YPGro (2% v/v glycerol instead of glucose) solidified with 1.5% w/v agar. Miltefosine was prepared as a 10 mg/ml stock solution in water, filter sterilized, and stored in frozen aliquots until just prior to use. Doxycycline was prepared as a 10 mM stock solution in ethanol and added to media (10 μM final concentration) as needed.

The MATα deletion collection, constructed in parental strain BY4742 (Baker-Brachmann et al. 1998), and the tetracycline-repressible essential gene collection, constructed in strain R1158 (Mnaimneh et al. 2004), were purchased from Invitrogen. Additional osh1Δ, osh2Δ, osh3Δ, and osh1Δ osh2Δ osh3Δ strains in the SEY6210 background were provided by Tim Levine (University College London.) Initial screens were conducted by thawing 96-well glycerol stock plates, mixing with a stainless steel 96-pin tool (Dan-Kar model MC-96; Fisher Scientific), and dilution into 150 μl of sterile YPD medium in a 96-well plate. Approximately 3 μl of culture was transferred by each pin under these conditions, giving a ~50 fold dilution in the recipient plate. The diluted cultures were then pinned to solid YPD medium with or without 4 μg/ml (9.8 μM) miltefosine (Avanti Polar Lipids, Alabaster, AL, USA), incubated at 30°C for up to 7 days, and visually monitored daily for growth of miltefosine-resistant patches. Cultures from the essential doxycycline-repressible (“Tet-off”) promoter collection (Mnaimneh et al. 2004) were screened similarly, except that an additional series of plates were used for the screen, which included 10 μg/ml doxycycline to affect repression of the essential library gene, as previously described (Wishart et al. 2005).

Miltefosine-resistant strains were identified and subcultured for further studies. Resistant strains were colony-purified from patches on the uninhibited miltefosine-free YPD replica plates, and single colonies were picked and grown to saturation in YPD.

A 10-fold dilution series was prepared in 96-well plates and

Figure 1 Overlap between previous and current screens for APC resistance. (A) Structures of APC compounds are discussed in the text in comparison with lyso-PC.; (B) Venn diagram illustrating the limited overlap in gene sets between the current study and previous studies on edelfosine resistance.; (C) Physical and genetic interaction network of genes identified as being important for both miltefosine and edelfosine resistance, with additional network neighbors from the miltefosine-only screen described in this work.

respectively, which leads to edelfosine and miltefosine resistance in yeast (Hanson et al. 2003). A whole-genome nonessential gene knockout screen for edelfosine resistant and hypersensitive mutants of S. cerevisiae was reported in a pair of studies (Cuesta-Marbán et al. 2013; Czyz et al. 2013). This work showed that edelfosine and miltefosine treatment causes loss of the proton efflux pump Pma1p from detergent-resistant subdomains of the plasma membrane. This leads to an apparent mislocalization of Pma1p...
pinned to YPD (4 μg/ml miltefosine) or YPGro (1 μg or 4 μg/ml miltefosine) and growth assessed after 48 or 96 hours, respectively. Strains that showed reproducible miltefosine resistance were thus identified, and categorized based on degree of sensitivity with glucose or glycerol as carbon sources.

Bioinformatic analyses
Primary and secondary screening led to the identification of 58 genes which, when deleted or repressed, led to a reproducible miltefosine-resistant growth phenotype. Gene ontology (GO) analysis was performed with YeastMine tools available at the Saccharomyces Genome Database website (www.yeastgenome.org), and gene set analysis was conducted with YeastNet version 3.0 at www.inetbio.org/yeastnet (Kim et al. 2014).

Microscopy
Strain BY4742 and the isogenic sac1Δ:KanMX deletion strain were transformed to uracil prototrophy with plasmid pTS312, a URA3 CEN plasmid expressing a C-terminal GFP fusion of Osh2p, which was a gift of Christopher Beh (Simon Fraser University). Cultures were grown overnight in SC -Ura and imaged on an Evos FL inverted microscope with GFP fluorescence cube.

Results and discussion
Screening for miltefosine resistant strains identifies a network of 58 highly connected genes enriched in membrane and trafficking functions
Table 1 provides a list of the genes that were identified as showing reproducible resistance to 4 μg/ml miltefosine on YPD, and that rescreened as positive after colony purification of original stocks and assessment with a fivefold serial dilution spot-test. Relevant molecular structures are shown in Figure 1A. Figure 2 shows a typical re-screening result for a subset of 6 mutants from the haploid MATα BY4742 based mutant collection. These initial results demonstrated that the miltefosine-resistance phenotype is tractable in the context of a genome-wide deletion screen conducted on solid medium. Figure 5 shows a result typical of the essential-gene Tet-off screening phenotype (TetO::SST74), in which we selected strains that showed robust growth in the presence of miltefosine when doxycycline was present (gene repressed), and weak or absent growth in its absence (gene expressed normally).

When screening mutant libraries for strains with alterations in a phenotype of interest, it is useful to assess whether the screen has identified mutants in a subset of genes with related functions, or whether the screen has identified a set of diverse genes with little in common with regard to function or localization. We approached this question by assessing the connectivity of the identified gene set with regard to systems-based epistasis screens, protein–protein interaction studies, and other measures, such as shared protein domain architectures and co-citation indices. We also analyzed the enrichment of GO terms associated with membrane assembly, protein trafficking, and transmembrane-perturbing agent, GO components and processes as a way to infer functional or regulatory interactions among these subsets of genes.

The miltefosine resistant gene deletion set shows little overlap with a previously described edelfosine resistance screen
As noted in Figure 1, approximately 264 deletion mutant strains were previously identified as showing some degree of edelfosine resistance (Cuesta-Marbán et al. 2013; Czyz et al. 2013). Our current study identified 58 mutants showing resistance to miltefosine, and of those, only 10 mutants were shared between the edelfosine and miltefosine resistance sets, although this may be a slight underestimate given that the edelfosine resistance screen did not encompass the essential gene titratable-promoter collection. Figure 1C shows a composite genetic and physical interaction map of edelfosine-miltefosine resistant shared genes and their directly interacting partners in the miltefosine resistance set, and identifies the t-SNARE TLG2 as a key hub in the interaction diagram for the shared edelfosine-miltefosine resistant gene set.

A tlg2Δ strain was identified along with dnf2Δ, lem3A, and other components of endosome-plasma membrane recycling in a screen for strains resistant to the lantibiotic peptide Ro 09-0198 (Takagi et al. 2012). This work also showed that an EGFP-tagged Dnf2p reporter was mislocalized in the tlg2Δ background, suggesting that the Ro-0198 sensitive phenotype of tlg2Δ is due to mislocalization of Dnf2p from the plasma membrane to endosomes. Disruption or mislocalization of this lipid flippase would thus result in the accumulation of phosphatidyl-ethanolamine on the outer leaflet of the plasma membrane, which is the ligand for this cytolytic peptide. Taken together with our results, this suggests that localization and proper function of the Dnf2p-Lem3p complex is dependent on Tlg2p and a small cadre of proteins to which it is functionally linked, and that the genes in the intersection of the Venn diagram of Figure 1B and in the interaction diagram of Figure 1C encompass the core components necessary for flippase function, activity, and localization at the plasma membrane. We thus propose that while the core components of lysophospholipid uptake (the flippase core components and factors regulating its proper
localization and function) are broadly conserved as determinants of APC sensitivity, individual members of this drug class exert their cytotoxic effects by interacting with multiple and variable intracellular targets after their import via the flippase.

Disruption of phosphatidylinositol-4-phosphate homeostasis and the oxysterol-binding protein homolog Osh2p alters miltefosine sensitivity

The proteins disrupted in a subset of functionally related miltefosine-resistant mutants (osh2Δ, sac1Δ, and TetO-STT4) are

### Table 1 List of genes identified in the miltefosine-resistance screen

| Gene ID | Name | Previous ID | Description |
|---------|------|-------------|-------------|
| YAL009W | SPO7 | Yes | Subunit of Nem1p-Spo7p phosphatase holoenzyme |
| YAL039C | CYC3 | No | Cytochrome c heme lyase |
| YBR097W | VPS15 | No | Protein kinase involved in vacuolar protein sorting |
| YBR184W | | No | Putative protein of unknown function |
| YCR009C | RVS161 | No | Amphiplysins-like lipid raft protein |
| YCR067C | SED4 | No | ER-protein that stimulates Sar1p GTase activity |
| YDL019C | OSH2 | No | oxysterol-binding protein homolog family member |
| YDR027C | VPS54 | No | Golgi-associated retrograde protein complex |
| YDR028C | REG1 | No | Subunit of type 1 protein phosphatase Glc7p |
| YDR093W | DNF2 | Yes | Aminophospholipid translocase (flippase) |
| YDR095C | | No | Dubious ORF overlapping DNF2 |
| YDR097C | MSH6 | No | Required for mismatch repair in mitosis and meiosis |
| YDR126W | SWF1 | Yes | Palmitoyltransferase of SNARE proteins |
| YDR323C | PEP7 | No | Vesicle-mediated vacuolar protein sorting |
| YDR472W | TRS31 | No | Transport protein particle (TRAPP) I-III |
| YER031C | YPT31 | No | Rab family GTase |
| YFL047W | RGD2 | No | GTase-activating protein for Cdc42p and Rho5p |
| YGL011W | ERG4 | No | C-24(28) sterol reductase |
| YGL054C | ERV14 | No | COPII-coated vesicle protein |
| YGL095C | VPS45 | No | Protein of the Sec1p/Munc-18 family |
| YGL106W | MLC1 | No | Essential light chain for Myo1p |
| YGL158W | RCK1 | No | Protein kinase involved in oxidative stress response |
| YGR086C | PIL1 | No | Eiscosome core component |
| YGR141W | VPS62 | No | Vacular protein sorting (VPS) protein |
| YHL028W | WSC4 | No | Endoplasmic reticulum (ER) membrane protein |
| YHL031C | GOS1 | No | v-SNARE protein involved in Golgi transport |
| YHR012W | VPS29 | Yes | Subunit of retromer complex |
| YHR108W | GGA2 | No | Regulates Arf1p, Arf2p to facilitate Golgi trafficking |
| YJL154C | VPS35 | No | Endosomal subunit of retromer complex |
| YKL212W | SAC1 | No | PtdIns-4-phosphate phosphatase |
| YKR001C | VPS1 | No | Dynamin-like GTase required for vacuolar sorting |
| YKR019C | IRS4 | No | EH domain-containing protein |
| YKR020W | VPS51 | No | Golgi-associated retrograde protein complex |
| YKR068C | BET3 | No | Transport protein particle (TRAPP) complexes I-III |
| YLR039C | RIC1 | No | Retrograde transport to the cis-Golgi network |
| YLR082C | SRL2 | No | Protein of unknown function |
| YLR093C | NYY1 | No | v-SNARE component of vacuolar membrane |
| YLR305C | STT4 | No | Phosphatidylinositol-4-kinase |
| YLR360W | VPS38 | No | Vps34p phosphatidylinositol-3-kinase complex |
| YML052W | SUR7 | No | Plasma membrane component of eisosomes |
| YML082W | | Yes | Protein of unknown function |
| YMR032W | HOF1 | No | Regulates actin cytoskeleton organization |
| YNL051W | COG5 | No | Conserved oligomeric Golgi complex subunit |
| YNL058C | | No | Putative protein of unknown function |
| YNL082W | PMS1 | Yes | ATP-binding protein required for mismatch repair |
| YNL227C | JJI1 | No | Stimulates the ATPase activity of Ssa1p |
| YNL272C | SEC2 | No | Guanyl-exchange factor for small G-protein Sec4p |
| YNL323W | LEM3 | Yes | Beta-subunit of Lm3p flippase |
| YNR047W | FPK1 | No | Ser/Thr protein kinase activating Dnf2p |
| YNR049C | MSO1 | No | Lipid-interacting protein in SNARE assembly |
| YOL018C | TLG2 | Yes | Syntaxin-like t-SNARE |
| YOL107W | | No | Putative protein of unknown function |
| YOR311C | DGK1 | No | Phospholipase C |
| YPL001W | HAT1 | No | Guanylyl-exchange factor for small G-protein Sec4p |
| YPL028W | ERG10 | No | Acetyl-CoA C-acetyltransferase |
| YPR089W | | No | Protein of unknown function |
| YPR117W | | No | Putative protein of unknown function |
| YPR151C | SUE1 | No | Degradation of unstable forms of cytochrome c |
involved in the function and localization of the oxysterol binding protein homolog Osh2p. Osh2p contains an oxysterol-binding motif Osh2p. Osh2p contains an oxysterol-binding motif Osh2p. These miscellaneous motifs include Anykyrin-repeats which are likely to interact with other, currently unidentified, proteins (Beh et al. 2001), a pleckstrin-homology (PH) domain that interacts with PtdIns-4-P (Roy and Levine 2004), and a "two phenylalanines in an acidic tract" (FFAT) motif which interacts with the ER resident proteins Scs2p and Scs22p (Loewen et al. 2003; Kaiser et al. 2005; Loewen and Levine 2005).

The phosphatidylinositol (PtdIns) 4-kinase Stt4p (Yoshida et al. 1994; Cutler et al. 1997) generates PtdIns-4-P at the plasma membrane (Foti et al. 2001; Baird et al. 2008), and is involved in actin polymerization and endocytosis, as well as in transport of PtdSer from the ER to the site of Psd2p (Trotter et al. 1998). The pool of PtdIns-4-P generated by Stt4p is degraded by the phosphoinositide phosphatase Sac1p (Foti et al. 2001), which has additional functions in lipid trafficking and metabolism (Guo et al. 1999; Rivas et al. 1999; Hughes et al. 2000; Tahirovic et al. 2005; Riekhof et al. 2014). The strains sac1Δ, osh2A, and Teto::STT4 were all identified as miltefosine resistant (Figures 4 and 5), suggesting that the PtdIns-4-P cycle governed by Stt4p, and Sac1p might be involved in the correct localization of Osh2p, and that mislocalization of Osh2p might lead to a defect in miltefosine uptake and distribution to intracellular targets. This idea was tested by expressing a GFP-tagged form of Osh2p in a wild-type and sac1A background. As shown in Figure 6, Osh2p in the wild type is localized in a punctate pattern at the cell periphery, while the Sac1 mutant Osh2p is localized to intracellular structures and absent from the cell periphery.

**Summary**

Previous studies in S. cerevisiae (Cuesta-Marbán et al. 2013; Czyz et al. 2013) have identified a subset of genes that, when deleted, confer resistance to the APC analog edelfosine. While members of this drug class share superficial similarities to lysophosphatidylcholine in their structures, it has remained an open question as to the degree of overlap in their mechanisms of action. Our current work identifies the plasma membrane flippase Dnf2p and factors required for its correct localization and function as being shared between edelfosine, miltefosine, and likely other members of this drug class with regard to the specific and active transport of the compounds into the cell. However, unlike the edelfosine studies, we did not identify factors that regulate intracellular pH or alter the function of the plasma membrane proton pump Pma1p as having a role in altered miltefosine sensitivity. We did however identify a new subset of genes as determinants of wild-type miltefosine sensitivity, and chose to assess the roles of the PtdIns-4-P cycle and Osh2 localization in more detail. One interpretation of this data is that after the initial "flip" across the plasma membrane by Dnf2p/Lem3, miltefosine is transported to one or more sites of cytotoxic activity by nonvesicular transport.
routes that are dependent on the proper localization of the oxysterol-binding protein homolog Osh2p. Future work on this and other clusters of genes identified in this screen will provide additional insight into the mechanism(s) of action of miltefosine and other members of the APC drug class.

Data availability
All data and materials, including strains and plasmids, described in this work are freely available upon request.

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Table 2 Gene-ontology term enrichment

| GO identifier | GO component (C) or process (P) | Total for term | Identified | Fold enrichment | P-value |
|---------------|---------------------------------|----------------|------------|-----------------|---------|
| GO: 0005794   | C: Golgi apparatus              | 214            | 17         | 8.2             | 6.92E-12|
| GO: 000139    | C: Golgi membrane               | 121            | 10         | 8.5             | 1.77E-07|
| GO: 0031201   | C: SNARE complex                | 25             | 5          | 20.7            | 3.26E-06|
| GO: 0005768   | C: endosome                     | 114            | 8          | 7.2             | 1.3E-05 |
| GO: 0016020   | C: membrane                     | 1718           | 31         | 1.9             | 5.54E-05|
| GO: 0010008   | C: endosome membrane            | 59             | 5          | 8.8             | 0.0002392|
| GO: 0000938   | C: GARP complex                 | 4              | 2          | 51.6            | 0.0005458|
| GO: 0034272   | C: phosphatidylinositol-3 kinase II | 4          | 2          | 51.6            | 0.0005458|
| GO: 0030906   | C: retromer complex, inner shell| 4              | 2          | 51.6            | 0.0005458|
| GO: 0030904   | C: retromer complex             | 5              | 2          | 41.3            | 0.0009041|
| GO: 0006810   | P: transport                    | 793            | 22         | 2.9             | 1.74E-06|
| GO: 0015031   | P: protein transport            | 389            | 17         | 4.5             | 7.43E-08 |
| GO: 0016192   | P: vesicle-mediated transport   | 140            | 10         | 7.4             | 6.98E-07|
| GO: 0006897   | P: endocytosis                  | 88             | 8          | 9.4             | 1.64E-06|
| GO: 0006896   | P: Golgi to vacuole transport   | 24             | 5          | 21.5            | 2.63E-06|
| GO: 0006233   | P: protein targeting to vacuole | 48             | 5          | 10.8            | 8.87E-05|
| GO: 0042147   | P: retrograde transport, endosome to Golgi | 19          | 5          | 27.2            | 7.4E-07 |
| GO: 0006914   | P: autophagy                    | 51             | 4          | 8.1             | 0.001407 |
| GO: 0032258   | P: CVT pathway                  | 38             | 4          | 10.9            | 0.0004564|
| GO: 0006906   | P: vesicle fusion               | 20             | 4          | 20.7            | 3.41E-05 |
| GO: 0006895   | P: Golgi to endosome transport  | 14             | 3          | 22.1            | 0.0002905|
| GO: 0006859   | P: lipid transport              | 21             | 3          | 14.8            | 0.001012|
| GO: 0045053   | P: protein retention in Golgi apparatus | 11          | 3          | 28.2            | 0.0001544|
| GO: 0000011   | P: vacuole inheritance          | 18             | 3          | 17.2            | 0.006335 |
| GO: 0006904   | P: vesicle docking involved in exocytosis | 10          | 3          | 31.0            | 9.84E-05|
| GO: 0048017   | P: inositol lipid-mediated signaling | 5            | 2          | 41.3            | 0.0009041|
| GO: 0060988   | P: lipid tube assembly          | 3              | 2          | 68.9            | 0.0002746|

Genes identified in the mutant screen were analyzed with GO-based gene-set enrichment analysis tools available at www.yeastgenome.org. P-values were calculated based on the hypergeometric test, and GO terms with P < 0.005 are included in the table.

Figure 5 The phosphatidylinositol-4-kinase/phosphatase cycle is a determinant of miltefosine sensitivity. The essential gene STT4, encoding the major PtdIns-4-kinase isoform in yeast, was identified in the essential-titratable gene collection. Inhibition of STT4 expression by including doxycycline in the growth media (10 μM) led to miltefosine resistance (4 μg/ml). The PtdIns-4-P phosphatase Sac1p, was also identified, and a sac1Δ strain was included in this series, and lem3Δ was included as a previously characterized miltefosine resistant positive control. Fifefold serial dilutions were prepared and pinned to solid media as described in the text.

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Author contributions
J.E.G. acquired funding, performed research, assisted with study design, performed data analysis, prepared figures, and provided feedback on the manuscript draft; M.J.S performed research, assisted with data analysis, and provided feedback on the manuscript draft; D.R.V. provided space and assistance for generating the conceptual framework that led to this study, and provided feedback on early stages of study design and on the manuscript.
draft; W.R.R. designed the study, acquired funding, performed and directed research, and wrote the manuscript.

**Conflicts of interest**
None declared.

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**Figure 6** Osh2p is mislocalized in a sac1Δ mutant background. Osh2-GFP expressed from a URA3 CEN plasmid under its native promoter was introduced into the BY4742 wild-type and isogenic sac1Δ mutant strains. Strains were grown in SC-Ura media overnight and images were acquired on an Evos-F1 inverted microscope with a GFP light cube using a 100× oil-immersion objective.
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