Phosphatidylserine translocation at the yeast trans-Golgi network regulates protein sorting into exocytic vesicles

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ABSTRACT Sorting of plasma membrane proteins into exocytic vesicles at the yeast trans-Golgi network (TGN) is believed to be mediated by their coalescence with specific lipids, but how these membrane-remodeling events are regulated is poorly understood. Here we show that the ATP-dependent phospholipid flippase Drs2 is required for efficient segregation of cargo into exocytic vesicles. The plasma membrane proteins Pma1 and Can1 are missorted from the TGN to the vacuole in Δdrs2Δ cells. We also used a combination of flippase mutants that either gain or lose the ability to flip phosphatidylserine (PS) to determine that PS flip by Drs2 is its critical function in this sorting event. The primary role of PS flip at the TGN appears to be to control the oxysterol-binding protein homologue Kes1/Osh4 and regulate ergosterol subcellular distribution. Deletion of KES1 suppresses plasma membrane–missorting defects and the accumulation of intracellular ergosterol in Δdrs2Δ mutants. We propose that PS flip is part of a homeostatic mechanism that controls sterol loading and lateral segregation of protein and lipid domains at the TGN.

INTRODUCTION

The trans-Golgi network (TGN) serves as a major sorting station for the delivery of post-Golgi cargo to different subcellular locations (Gu et al., 2001; Papanikou and Glick, 2014). Cargo proteins traveling through the TGN are sorted into exocytic vesicles destined for the plasma membrane or are sorted into vesicles targeted to the endosomal system. Sphingolipid synthesis occurs in the Golgi, and lipids are also sorted at the TGN to produce exocytic vesicles enriched in sphingolipid and sterol relative to the TGN donor membrane (Klemm et al., 2009). Whereas many of the cargoes leaving the TGN for endosomes are sorted by coat-dependent mechanisms (Robinson, 2004), exocytic vesicles enriched in sterol and sphingolipids do not associate with any known coat (Klemm et al., 2009; Surma et al., 2012). Lipid-dependent protein sorting (“lipid raft”–based sorting) has been proposed to explain how specific cargoes are sorted into these vesicles; however, the mechanisms and proteins involved in this sorting process are not well understood (Simons and van Meer, 1988; Simons and Ikonen, 1997; Ikonen and Simons, 1998; Graham and Burd, 2011; Surma et al., 2012). Several candidate proteins have been proposed to be involved in the sorting of cargo into exocytic vesicles, including the budding yeast oxysterol-binding protein (OSBP) homologue Kes1/Osh4 (hereafter referred to as Kes1), which was previously identified in a screen for proteins involved in efficient exocytosis of plasma membrane proteins (Proszynski et al., 2005). OSBP and Kes1 have been proposed to mediate the unidirectional transport of endoplasmic reticulum (ER) sterol to the TGN, where it is exchanged for Golgi phosphatidylinositol-4-phosphate (PI(4)P), thereby enriching sterol at the TGN (or directly at exocytic vesicles; Im et al., 2005; Mesmin et al., 2013; von Filseck et al., 2015). The cycle is completed by delivery of PI(4)P to the ER, where it is converted to phosphatidylinositol (PI) by the phosphatase Sac1 (Mesmin et al., 2013). Genetic studies have implicated KES1 as a negative regulator of TGN function in exocytosis (Jiang et al., 1994; Fang et al., 1996; Li et al., 2002; Alfaro et al., 2011) and shown that...
Ke1 acts in a mutually antagonistic manner with the phospholipid flippase Drs2 (Muthusamy et al., 2009). However, the effect of the relationship between the OSBP homologue Kes1 and the flippase Drs2 on the sorting of proteins into exocytic vesicles, in which ergosterol is highly enriched, is unclear.

There are five yeast flippases in the type IV P-type ATPase family: Neo1, Dnf1, Dnf2, Dnf3, and Drs2. This essential group of flippases generate and maintain plasma membrane asymmetry with phosphatidylserine (PS) and phosphatidylethanolamine (PE) primarily in the cytosolic leaflet and phosphatidylcholine and sphingolipids primarily in the outer (extracellular) leaflet (Kato et al., 2002; Pomorski et al., 2003; Saito et al., 2004; Chen et al., 2006). The mechanism of phospholipid recognition and translocation has been studied extensively with Dns and Dnf1 (Baldridge and Graham, 2012, 2013; Baldridge et al., 2013), which are heterodimers with their β subunits Cdc50 and Lem3, respectively (Saito et al., 2004; Chen et al., 2006; Furuta et al., 2007; Takahashi et al., 2011). Drs2 localizes to the TGN and appears to be the primary PS flippase in the cell, but it is also capable of flipping PE (Chen et al., 1999; Hua et al., 2002; Natarajan et al., 2004; Liu et al., 2008; Baldridge et al., 2013). In contrast, Dnf1 dynamically translocates between the plasma membrane, endosomes, and TGN and preferentially flips lysophosphatidylcholine and lysophosphatidylethanolamine (Hua et al., 2002; Pomorski et al., 2003; Riekhof et al., 2007; Baldridge et al., 2013).

Residues defining these differences in substrate specificity have been mapped in Drs2 and Dnf1, and it is possible to tune their substrate preference through targeted mutagenesis. For example, substitution of QQ in the first transmembrane segment of Drs2 for GA (Dnf1 residues) disrupts the ability of Drs2 to flip PS without altering its ability to flip PE (Baldridge and Graham, 2013). Conversely, a N550S point mutation in Dnf1 allows it to flip PS in addition to its normal substrates (Baldridge and Graham, 2013; Baldridge et al., 2013). Surprisingly, Dnf1-N550S, but not wild-type (WT) Dnf1, can replace the function of Drs2 in vivo, indicating that PS flip is the crucial function of Drs2 (Baldridge et al., 2013; Xu et al., 2013).

PS flip by Drs2 was shown to be critical for TGN-associated trafficking pathways (Natarajan et al., 2004; Xu et al., 2013). Drs2 is required for bidirectional transport between the TGN and early endosomes, as well as for the formation of one class of exocytic vesicle; however, loss of Drs2 does not appear to perturb the rate of protein secretion (Chen et al., 1999; Gall et al., 2002; Hua et al., 2002; Liu et al., 2008). Consistent with Drs2's TGN localization, studies by the Grinstein lab using the PS probe Lact-C2 in mammalian cells suggest that PS remains primarily in the luminal leaflet of the ER and Golgi but gets flipped to the cytosolic leaflet at the TGN (Fain et al., 2011; Hankins et al., 2015). This change in PS sidedness to the cytosolic leaflet is maintained when TGN-derived vesicles fuse with the plasma membrane, where PS is highly enriched. This finding suggests that the TGN is a major site of PS translocation and also that mammalian cells have PS flippase(s) at the TGN that function similarly to Drs2.

In this study, we investigated how the TGN-localized flippase Drs2 and the OSBP homologue Kes1 are involved in the lipid-based sorting of plasma membrane proteins into exocytic vesicles. To do this, we looked at the distribution of two plasma membrane proteins: the plasma membrane ATPase Pma1 and the arginine transporter Can1. Pma1 and Can1 have been proposed to traffic from the Golgi to the plasma membrane through a lipid-based (coat-independent) mechanism (Bagnat et al., 2000, 2001; Opekarská et al., 2005; Surma et al., 2012). However, once at the plasma membrane, these proteins partition into distinct membrane compartments called the membrane compartments of Pma1 (MCP) and Can1 (MCC; Malinská et al., 2003; Grossmann et al., 2007; Spira et al., 2012; Ziolkowska et al., 2012). We examined the distribution of these proteins both at the plasma membrane and throughout the cell to study the influence of transverse PS asymmetry on exocytic trafficking and lateral compartmentalization of the plasma membrane.

**RESULTS**

**Compartmentalization of the plasma membrane is significantly perturbed in cho1Δ cells but not drs2Δ cells**

To first determine whether plasma membrane organization is perturbed in drs2Δ cells, we examined the segregation of Pma1-red fluorescent protein (RFP) and Can1-green fluorescent protein (GFP) into their respective compartments at the cell surface (Figure 1). In WT cells, Pma1-RFP and Can1-GFP colocalized little (Pearson's coefficient = -0.0044 ± 0.023) as previously reported (Malinská et al., 2003; Spira et al., 2012). There was a small increase in colocalization of these proteins in drs2Δ cells (Pearson's coefficient = 0.030 ± 0.025) but not significantly so. This result suggests that PS asymmetry is not critical for the normal segregation of MCP and MCC at the plasma membrane. Because we did not see a strong defect when PS asymmetry was disrupted, we also examined whether the absence of PS would have an effect on compartmentalization. To do this, we also imaged cho1Δ cells, which are unable to synthesize PS because they lack the PS synthase and must be supplemented with choline to be viable (Atkinson et al., 1980). There was a significant increase in

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**FIGURE 1:** Plasma membrane compartmentalization is significantly perturbed in cho1Δ cells but not drs2Δ cells. Cells expressing Pma1-RFP and Can1-GFP were grown to mid log phase and imaged on a DeltaVision Elite Imaging system. Optical sections were collected every 200 nm. Images were deconvolved using the DeltaVision software SoftWoRx. Arrowheads denote regions of colocalization. Colocalization of the two markers was determined using the ImageJ plug-in Colocalization Finder on deconvolved images. Values represent mean ± SEM (10 cells, one-way analysis of variance [ANOVA], ***p < 0.001). Scale bar, 5 μm.
degraded in drs2Δ cells as detected by Western blotting with anti-GFP antibody (Figure 2C).

Drs2 primarily flips PS and to a lesser extent PE, and so to distinguish which substrate is important for Pma1-GFP trafficking, we used two flippase mutants that specifically lose (Drs2-[QQ→GA]) or gain (Dnf1-N550S) the ability to flip PS (Baldridge and Graham, 2013). Dnf1-N550S, but not Drs2-[QQ→GA], was able to restore WT-like Pma1 localization to the plasma membrane in drs2Δ cells (Figure 2, A and B). This restoration was not due to adding an extra copy of DNF1, as drs2Δ DNF1 cells mislocalized Pma1-GFP comparably to drs2Δ cells. This finding suggests that PS flip is critical for normal localization of Pma1 at the plasma membrane. Consistently, Pma1 also mislocalized to the vacuole in cho1Δ cells (∼30% reduction in plasma membrane fluorescence), which are unable to synthesize PS. The same results were observed for Can1 (Figure 3, A–C), although the vacuolar mislocalization defect was stronger for Can1-GFP (∼40% reduction in plasma membrane fluorescence; Figure 3, A and B) than Pma1-GFP (∼25% reduction in plasma membrane fluorescence). These observations suggest that PS translocation across the TGN membrane is required for efficient sorting of Pma1-GFP and Can1-GFP to the plasma membrane.

Drs2 and Kes1 work antagonistically in the sorting of Pma1 and Can1

We observed the same antagonistic relationship between drs2Δ and kes1Δ in sorting plasma membrane proteins that has been described previously for ergosterol homeostasis (Muthusamy et al., 2009). Pma1 and Can1 mislocalized to the vacuole in both drs2Δ and kes1Δ
Ergosterol is enriched at the plasma membrane, particularly in the MCC, and is required for correct sorting of Can1 and other plasma membrane permeases (Umebayashi and Nakano, 2003; Malinska et al., 2004; Grossmann et al., 2006, 2007). Therefore we next tested the influence of PS flippases on the cellular distribution of ergosterol to determine whether PS flip is Drs2’s critical role in sterol homeostasis.

We first used the fluorescent sterol-binding compound filipin, a polyene antibiotic (Norman et al., 1972; Drabikowski et al., 1973), to observe sterol distribution throughout the cell. Filipin primarily stains the plasma membrane of WT and drs2Δkes1Δ cells, but we observed ~20% reduction in filipin staining at the plasma membrane in drs2Δ and kes1Δ single mutants (Figure 4, A and B), consistent with previous findings (Muthusamy et al., 2009). Transformation of drs2Δ single mutants but had WT-like distribution in drs2Δkes1Δ cells (Figures 2, A–C, and 3, A–C). These results indicate that the activities (or lack of activities) of these two proteins must be matched to facilitate normal trafficking of Pma1 and Can1 to the plasma membrane. Of interest, kes1Δ cells did not suppress the cho1Δ defect in Pma1 and Can1 mislocalization (Figure 3, D and E), indicating that the presence of PS is required for their efficient plasma membrane localization, and this deficiency cannot be overcome by disrupting KES1.

**Role of PS in ergosterol enrichment at the plasma membrane**

Previously our laboratory showed that Drs2 and Kes1 act antagonistically to regulate sterol distribution between the plasma membrane and internal membranes (Muthusamy et al., 2009).
Of interest, cho1Δ cells had highly variable filipin staining patterns, ranging from a more severe defect than drs2Δ to WT-like, even within the same culture. On average, filipin staining of the plasma membrane was reduced in cho1Δ cells, but it was not statistically different from that in WT cells (Figure 4, A and B). The cho1Δ cells were cultured and imaged under a variety of different conditions, but we were unable to pinpoint the source of variability in the filipin staining pattern. Another potential issue with using the drs2-[QQ → GA] mutant or WT DNF1, which do not recognize PS, failed to correct this defect, and the cells displayed an equivalent 20% reduction in plasma membrane ergosterol. In contrast, WT DRS2 or DNF1-N550S, which can flip PS, completely restored plasma membrane ergosterol in drs2Δ cells. The sterol content of drs2Δ cells is similar to that in WT cells (Fei et al., 2008), and so these results suggest that PS translocation at the Golgi is required for normal sterol enrichment at the plasma membrane.
filipin staining as a readout for ergosterol localization is that filipin and other sterol-binding toxins may bind to other molecules or have nonlinear responses to sterol level (Maxfield and Wüsten, 2012).

To try to resolve the variability issues with the cho1Δ cells and potential issues intrinsic to filipin staining, we used a second approach to monitor subcellular sterol distribution. We examined cells containing dehydroergosterol (DHE), a fluorescent, naturally occurring sterol that closely mimics ergosterol and is able to functionally replace ergosterol in yeast (Georgiev et al., 2011). Cells were grown under hypoxic conditions for 36 h in medium supplemented with DHE to allow for sterol uptake, which effectively replaces >95% of the ergosterol with DHE (Georgiev et al., 2011). Consistent with the filipin staining, DHE was primarily at the plasma membrane in WT but displayed increased localization to internal structures in drs2Δ cells (Figure 4, C and D). Quantitatively, filipin and DHE both indicated ~20% reduction in plasma membrane–localized sterol in drs2Δ relative to WT. Therefore filipin appears to provide an accurate measure of ergosterol distribution in drs2 mutants. Unlike the filipin staining, cho1Δ cells were uniformly WT-like, which may be due to differences in growth conditions between the two assays (i.e., growth under aerobic conditions for the filipin stain vs. hypoxic conditions for DHE uptake).

For a third approach to further investigate changes in ergosterol distribution in cho1Δ cells, we lysed cells grown under aerobic conditions and separated the plasma membrane from the ER and other internal organelles by fractionation through a sucrose gradient. Ergosterol was quantified in each membrane fraction, and by this assay, 55% of ergosterol in WT cells are at the plasma membrane (fraction 7). Plasma membrane ergosterol of drs2Δ cells was reduced to 50%, although this was not statistically different from WT. This lack of statistical significance may be due to the sucrose gradient assay not being sensitive enough to pick up on the relatively small defect observed by filipin staining and DHE fluorescence (the expected result was ~45% ergosterol in the drs2Δ plasma membrane fraction). Surprisingly, cho1Δ cells displayed a significant depletion of plasma membrane ergosterol (30% of ergosterol in plasma membrane fraction) relative to WT, with a concomitant increase in fractions 1 and 2 corresponding to ER and internal organelles (Figure 4, E and F).

The reason for these discrepancies in the three approaches is not clear, but they may reflect differences in sterol distribution under different growth conditions or the ability of different assays to reproducibly detect small differences in sterol distribution. However, we can conclude that PS is not absolutely required to enrich ergosterol at the plasma membrane because all cho1Δ cells lack PS and there are always cells in the population that are indistinguishable from WT when imaged with filipin or DHE. The PS and phosphatidylinositol synthases compete for the same precursor (CDP-diacylglycerol; Kelley et al., 1988), and perhaps increased synthesis of phosphatidylinositol in cho1Δ cells can partially compensate for the complete lack of PS. Disruption of DR52 does not substantially alter total cellular PS or PI levels (Chen et al., 2006) but instead more subtly perturbs transbilayer PS distribution. In contrast to cho1Δ, the drs2 mutants display a fairly uniform reduction of PM ergosterol as assayed by filipin or DHE and clearly perturb sterol homeostasis.

**Pma1 and Can1 are missorted from the TGN to the vacuole in drs2Δ and cho1Δ cells**

We next sought to determine the route by which Pma1 and Can1 travel to the vacuole in the drs2Δ and cho1Δ mutants. The observed vacuolar mislocalization phenotype could be due to either increased endocytosis at the plasma membrane or misrouting of newly synthesized Pma1 and Can1 from the TGN to the vacuole through the endosomal system. To differentiate these two possibilities for Pma1, we used the actin polymerization inhibitor latrunculin A (Lat A) to block endocytosis (Huang and Chang, 2011). As a positive control, we also imaged GFP-Snc1, an exocytic vesicle–soluble N-ethylmaleimide–sensitive factor attachment protein receptor that constantly cycles between the TGN, plasma membrane, and early endosomes (Lewis et al., 2000; Galan et al., 2001).

In WT cells, Snc1 localizes to the bud plasma membrane because endocytosis is the slowest step of Snc1 recycling. In contrast, Snc1 accumulates intracellularly in the early endosomes of drs2Δ and cho1Δ cells, which have defects in Snc1 recycling (Hua et al., 2002; Furuta et al., 2007; Xu et al., 2013). However, because newly synthesized Snc1 travels to the plasma membrane before arrival in the early endosomes, it will accumulate at the plasma membrane when endocytosis is blocked. After 3 h of Lat A treatment, we found that GFP-Snc1 accumulated significantly more at the plasma membrane in all of the strains, indicating that Lat A was effectively blocking endocytosis (Figure 5, A and B). In contrast, Lat A treatment did not shift the distribution of Pma1-GFP to the plasma membrane in drs2Δ and cho1Δ cells (Figure 5, A and C). This observation suggests that Pma1-GFP is missorted directly from the TGN rather than being endocytosed from the plasma membrane.

To determine whether Can1 was missorted at the level of the TGN or plasma membrane, we used an endocytosis-resistant Can1-GFP mutant (Can1end-GFP). Can1end-GFP has three N-terminal lysine residues that have been mutated to arginines (K42R/K45R/K47R). These mutations prevent the protein from undergoing ubiquitin-mediated endocytosis, such that once Can1end-GFP reaches the plasma membrane, it should remain there (Charles Lin and Scott Emr, unpublished observations). Can1-GFP and Can1end-GFP were both mislocalized in drs2Δ and cho1Δ to the same extent, indicating that Can1 is missorted from the TGN to the vacuole (Figure 5, D and E). To ensure that Can1end-GFP is indeed endocytosis resistant, we induced Can1 endocytosis with 5 mM arginine (Supplemental Figure S1). There was a stark decrease in Can1-GFP, but not Can1end-GFP, fluorescence at the plasma membrane in the presence of the high arginine concentration, confirming that Can1end-GFP is endocytosis resistant. These results indicate that TGN missorting, not endocytosis, is the route by which Can1-GFP is mislocalized to the vacuole in drs2Δ and cho1Δ cells.

**Missorting of Pma1 and Can1 is not due to drs2Δ AP-1/Rcy1 trafficking defects**

We next examined whether the missorting of Pma1 and Can1 is due to previously described trafficking defects caused by disruption of Drs2 flipase activity. Drs2 is required for AP-1/clathrin trafficking between the TGN and early endosomes (Chen et al., 1999; Liu et al., 2008) and the Rcy1 recycling pathway from the early endosomes to TGN (Hua et al., 2002; Furuta et al., 2007). However, disrupting these two pathways by deleting an AP-1 subunit (apl4Δ) or rcy1Δ had no effect on Pma1 localization (Figure 6, A and C). Can1 localized normally in apl4Δ cells, but there was a small increase in Can1 localization to puncta in rcy1Δ cells (Figure 6, B and D). However, the Can1 trafficking defect was very mild in comparison to drs2Δ (Figure 3, A and D) and was phenotypically distinct. Therefore it is unlikely that disruption of the Rcy1 recycling pathway is the cause of Pma1 and Can1 missorting to the vacuole in drs2Δ cells. In addition, we examined whether disrupting other TGN-associated trafficking pathways would affect Pma1 and Can1 sorting. We disrupted the GGA pathway (gga1,2Δ) from the TGN.
mislocalize to internal membranes in \( \text{drs2} \Delta \) cells as assessed by filipin staining and DHE fluorescence (Figure 4, A–D). These defects are specific to loss of PS flip because Drs2-[QQ → GA], which loses the ability to flip PS without altering the ability to flip PE (Baldridge and Graham, 2013), causes mislocalization of Pma1-GFP, Can1-GFP, and ergosterol to the same extent as \( \text{drs2} \Delta \). In contrast, Dnf1-N550S, which gains the ability to flip PS without altering transport of its normal substrates, fully restores efficient sorting of Pma1-GFP, Can1-GFP, and ergosterol localization to the plasma membrane in \( \text{drs2} \Delta \) cells. However, Pma1-RFP and Can1-GFP that arrive at the plasma membrane of \( \text{drs2} \Delta \) cells segregate normally into distinct membrane compartments, despite aberrantly exposing PS in the outer (extracellular) leaflet (Figure 1; Chen et al., 2006). Thus, lateral compartmentalization of the plasma membrane does not depend on PS transverse asymmetry.

**DISCUSSION**

The experiments presented here show that PS flip by Drs2 at the yeast TGN regulates the lipid-based sorting of proteins into exocytic vesicles. Pma1 and Can1 populate nonoverlapping compartments of the plasma membrane, but partitioning into microdomains rich in ergosterol and sphingolipids is believed to concentrate both proteins into exocytic vesicles at the TGN (Klemm et al., 2009; Surma et al., 2012). Evidence of Drs2's involvement as a sorting factor in this pathway is that disrupting \( \text{DRS2} \) causes 25 and 40% of Pma1 and Can1 to missort to the vacuole (Figures 2, A and B, and 3, A and B), respectively. In addition, 20% of ergosterol appears to mislocalize to internal membranes in \( \text{drs2} \Delta \) cells as assessed by filipin staining and DHE fluorescence (Figure 4, A–D). These defects are specific to loss of PS flip because Drs2-[QQ → GA], which loses the ability to flip PS without altering the ability to flip PE (Baldridge and Graham, 2013), causes mislocalization of Pma1-GFP, Can1-GFP, and ergosterol to the same extent as \( \text{drs2} \Delta \). In contrast, Dnf1-N550S, which gains the ability to flip PS without altering transport of its normal substrates, fully restores efficient sorting of Pma1-GFP, Can1-GFP, and ergosterol localization to the plasma membrane in \( \text{drs2} \Delta \) cells. However, Pma1-RFP and Can1-GFP that arrive at the plasma membrane of \( \text{drs2} \Delta \) cells segregate normally into distinct membrane compartments, despite aberrantly exposing PS in the outer (extracellular) leaflet (Figure 1; Chen et al., 2006). Thus, lateral compartmentalization of the plasma membrane does not depend on PS transverse asymmetry.

Consistent with plasma membrane compartmentalization being unperturbed, the mislocalization of Pma1-GFP and Can1-GFP in
**Missorting of Pma1 and Can1 is not due to disrupting other TGN-associated trafficking pathways.** Cells expressing (A) Pma1-GFP or (B) Can1-GFP were grown to mid log phase at 30°C and imaged. Percentage of PM-localized (C) Pma1-GFP and (D) Can1-GFP were calculated by dividing plasma membrane fluorescence by total fluorescence with WT set to 100%. Values represent mean ± SEM (n = 2, total of 100 cells, one-way ANOVA. **p < 0.01). Scale bar, 5 μm.

**FIGURE 6:**

Missorting of Pma1 and Can1 is not due to disrupting other TGN-associated trafficking pathways. Cells expressing (A) Pma1-GFP or (B) Can1-GFP were grown to mid log phase at 30°C and imaged. Percentage of PM-localized (C) Pma1-GFP and (D) Can1-GFP were calculated by dividing plasma membrane fluorescence by total fluorescence with WT set to 100%. Values represent mean ± SEM (n = 2, total of 100 cells, one-way ANOVA. **p < 0.01). Scale bar, 5 μm.

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In budding yeast are significantly enriched for ergosterol relative to the TGN membrane from which they were formed (Zinser et al., 1974; Graham, 2004). In addition, the PS head group carries a negative charge and also has an intrinsic positive curvature due to its conical shape that should accentuate membrane bending toward the cytosol (Fuller et al., 2003). Consistent with this prediction, localization of a biosensor for membrane curvature and charge (+ALPS-GFP) to TGN and early endosomal membranes requires PS flip by Drs2 (Xu et al., 2013). The induction of curvature by PS flip may be sufficient to facilitate sorting of lipids or proteins into subdomains of the TGN that give rise to exocytic vesicles (Roux et al., 2005; Sorre et al., 2009; Ryu et al., 2014).

Another potential effect of unidirectional PS flip is that over-crowding the cytosolic leaflet could provide a driving force to push lipids that can spontaneously flip-flop, such as ergosterol, to the lumenal leaflet. Freeze-fracture electron microscopy studies of filipin-stained mammalian cells indicate that cholesterol moves in bulk across the bilayer to the lumenal leaflet during secretory granule formation (Sheetz and Singer, 1974; Graham, 2004). In addition, the PS head group carries a negative charge and also has an intrinsic positive curvature due to its conical shape that should accentuate membrane bending toward the cytosol (Fuller et al., 2003). Consistent with this prediction, localization of a biosensor for membrane curvature and charge (+ALPS-GFP) to TGN and early endosomal membranes requires PS flip by Drs2 (Xu et al., 2013). The induction of curvature by PS flip may be sufficient to facilitate sorting of lipids or proteins into subdomains of the TGN that give rise to exocytic vesicles (Roux et al., 2005; Sorre et al., 2009; Ryu et al., 2014).

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Based on the present study, Drs2 and Kes1 are required for efficient sorting of Pma1 and Can1 to the plasma membrane. In the absence of PS flip or Kes1 activity, the TGN still buds exocytic vesicles.
vesicles that are delivered normally to the plasma membrane; however, Pma1 and Can1 are not efficiently concentrated into these exocytic vesicles and likely spill into vesicles targeted to the endosomal system. Genetically, DRS2 and KES1 appear to antagonize each other’s activity (Muthusamy et al., 2009). Single mutants carrying drs2Δ or kes1Δ mutations display the same defect in Pma1 and Can1 localization, but the drs2Δ kes1Δ double mutant is indistinguishable from WT cells. This observation may seem to diminish the significance of flippases and OSBP homologues in this pathway, as they are dispensable; however, Drs2 and Kes1 are both members of large protein families with some degree of functional redundancy (seven OSBP homologues and five flippases are expressed in budding yeast). For instance, kes1Δ will suppress drs2Δ phenotypes, but it does not suppress the lethality caused by deletion of DRS2 and the DNF genes (drs2Δ dnf1,2,3Δ; Muthusamy et al., 2009).

Our data suggest that it is critical to balance the activity of Drs2 and Kes1 during TGN membrane remodeling events to ensure correct sorting of proteins and lipids. Biochemical experiments support this idea: Drs2 PS flippase activity is twofold higher in Golgi membranes purified from kes1Δ cells relative to WT cells (Muthusamy et al., 2009). Addition of recombinant Kes1 to kes1Δ Golgi membranes inhibited Drs2 to WT levels with IC50 = 100 fM (Muthusamy et al., 2009). Inhibition was likely caused by the ability of Kes1 to extract PI4P from the TGN, which is a stimulator of Drs2 activity (Natarajan et al., 2009; Zhou et al., 2013). Conversely, high concentrations of PS inhibit the ability of Kes1 to exchange sterol for PI4P in transfer assays with liposomes, potentially by trapping Kes1 on the membrane and causing a futile cycle of exchange with the same membrane (Saint-Jean et al., 2011). Thus Drs2-catalyzed PS translocation at the TGN may attenuate Kes1’s ability to load ergosterol into the TGN. Therefore we suggest that Kes1 overloads the TGN with ergosterol in situations when Drs2 is absent, when Drs2 cannot recognize PS (Drs2-[QQ → GA]), or when Drs2 cannot flip PS because PS is absent (cho1Δ cells). The similar phenotypes of kes1Δ and drs2Δ single mutants imply that overloading or underloading the TGN with ergosterol disrupts the balance required for concentrating Pma1 and Can1 into microdomains for their selective incorporation into exocytic vesicles.

MATERIALS AND METHODS

Media, strains, and plasmids

Yeast strains were grown in synthetic minimal medium (SD) containing required nutrients and 2% glucose. For experiments involving cho1Δ, the SD medium was supplemented with 1 mM choline (Sigma-Aldrich, St. Louis, MO). All yeast transformations were performed by the lithium acetate method (Gietz and Schiestl, 2007). PCR-based genomic integration of fluorescent tags was carried out according to the method described by Janke et al. (2004). The strains and plasmids used in this study are listed in Supplemental Tables S1 and S2, respectively.

Western blot

Samples were prepared using a protocol modified from Chang and Slayman (1991). Cell pellets were resuspended in lysis buffer (10 mM Tris, pH 7.4, 0.3 M sorbitol, 0.1 M NaCl, and 5 mM MgCl2) containing 1 mM phenylmethylsulfonyl fluoride and 1× protease inhibitor cocktail (ThermoFisher Scientific, San Jose, CA). The cells were then lysed by vortexing for 6 min with 1-min pulses at 4°C. The beads were allowed to settle without centrifugation for 10 min at 4°C before removing the lysate supernatant. The lysates were then added 1:1 to Laemmli buffer and incubated at 37°C for 5 min. The samples were then subjected to SDS-PAGE and transferred to a polyvinylidene fluoride membrane. For the anti-GFP Western blot, an IR800CW secondary antibody was used, and the bands were imaged and quantified using an Odyssey CLx infrared imager and its software (LI-COR Biosciences, Lincoln, NE).

Vacuolar labeling with FM4-64

To confirm vacuolar localization of GFP-tagged Pma1 and Can1, mid log-phase (OD600 = 0.5–0.8) cells were labeled with the lipophilic dye FM4-64 (Life Technologies, Grand Island, NY) as described previously (Babu et al., 2012). Cells were labeled with 32 μM FM4-64 for 20 min in the dark at 30°C. The cells were then washed with 1× phosphate-buffered saline (PBS) and resuspended in SD medium for 30 min at 30°C. The cells were washed twice in 1× PBS before imaging.

Filipin staining

To examine the distribution of ergosterol, cells were stained with filipin (Sigma-Aldrich) using a protocol similar to the one described by Beh and Rine (2004). A 5-ml amount of mid log-phase (OD600 = 0.5–0.8) cells grown in SD medium was fixed with 4% formaldehyde for 10 min at 30°C with shaking. The cells were then harvested and washed twice with 10 ml of sterile water. The cells were resuspended in 1 ml of sterile water, 0.2 ml of which was mixed with 3 μl of freshly prepared, ice-cold 5 mg/ml filipin in ethanol. The cells were then incubated with filipin for 15 min at 30°C in the dark before harvesting the cells and imaging.

Dehydroergosterol uptake assay

DHE (Sigma-Aldrich), a naturally occurring fluoresecence sterol (McIntosh et al., 2008), was used to visualize the distribution of ergosterol in living cells. Yeast strains were incubated with DHE under hypoxic conditions to allow for sterol uptake and imaged as described previously (Georgiev et al., 2011; Gatta et al., 2015). Briefly, yeast strains were inoculated from an overnight saturated liquid culture to OD400 ≈ 0.005 in 10 ml of synthetic medium supplemented with 20 μg/ml DHE, 0.5% ethanol, and 0.5% Tween-80. The cultures, together with BD GasPak EZ Anaerobe carbon sachets (BD Diagnostics, Franklin Lakes, NJ), were placed inside an anaerobic incubation chamber, and the chamber was then sealed and incubated at 30°C for 36 h. The sachets reduce oxygen levels to 0.7% (vol/vol), yielding hypoxic conditions. At the end of the hypoxic incubation, the chamber was opened, and cells were energy poisoned with ice-cold NaNO3/NaF (10 mM/10 mM) before imaging.

Sucrose gradient fractionation

Cell homogenates were analyzed by sucrose gradient fractionation as previously described (Georgiev et al., 2011; Gatta et al., 2015). Seven fractions were collected from the top of the gradient, and each fraction was immunoblotted against Dpm1 (ER marker) and Pma1 (plasma membrane marker) to verify that the fractionation was successful.

Actin depolymerization

Lat A (Cayman Chemical, Ann Arbor, MI) was used to block endocytosis through inhibition of actin polymerization. Mid log-phase (OD400 = 0.5–0.8) cells were harvested, resuspended in SD medium containing 10 μM Lat A, and incubated for 3 h at 30°C as described previously (Huang and Chang, 2011). Control cells were treated with an equal volume of ethanol.
Induction of Can1 endocytosis with arginine
Can1 endocytosis was triggered by the addition of arginine as described by Brach et al. (2011). Cells were grown to OD = 0.4, harvested, and resuspended in SD medium supplemented with 5 mM arginine. Cells were incubated for 3 h at 30°C before imaging.

Fluorescence microscopy
Images of Pma1-RFP and Can1-GFP distribution at the plasma membrane were taken with a DeltaVision Elite Imaging system (Applied Precision, Issaquah, WA). A Z-stack was acquired with 200-nm intervals, and the images were deconvolved in SoftWoRx (Applied Precision, Issaquah, WA). Images of DHE were taken with an inverted microscope (Leica, Deerfield, IL) equipped with a cooled charge-coupled device camera (Princeton Instruments, Trenton, NJ) and MetaMorph software (Universal Imaging, West Chester, PA). To visualize DHE, a specially designed filter cube (Chroma Technology, Brattleboro, VT) was used with a 355-nm (20-nm bandpass) excitation filter, a 365-nm long-pass dichromatic filter, and a 405-nm (40-nm bandpass) emission filter. All other images were taken with an Axioplan microscope (Carl Zeiss, Thornwood, NY) equipped with a Zeyla sCMOS 5.5-megapixel camera (Andor, Belfast, United Kingdom) and micromanager software (University of California, San Francisco, San Francisco, CA). Filipin was imaged using a 4′,6-diamidino-2-phenylindole filter set; GFP-tagged proteins were imaged using a GFP filter set; RFP-tagged proteins and FM4-64 were imaged using an RFP filter set. Images were merged and pseudocolored in ImageJ (National Institutes of Health, Bethesda, MD).

Data analysis
The percentage of filipin/Pma1/Can1/Snc1 localized to the plasma membrane (%PM-localized) was calculated by first outlining the outside of each cell using the freehand selection tool in ImageJ and measuring the total fluorescence of the cell (FluorTotal). Next, the area just within the plasma membrane was outlined using the freehand selection tool to give the total internal fluorescence excluding the plasma membrane (FluorPM). Next, the internal fluorescence was subtracted from the total to determine the fluorescence at the plasma membrane (FluorPM = FluorTotal – FluorPM). Finally, the fluorescence at the plasma membrane was divided by the total fluorescence of the cell to give %PM-localized fluorescence (FluorPM/FluorTotal = %PM-localized). All quantification was performed using raw images. To determine the degree of colocalization between Pma1-RFP and Can1-GFP at the plasma membrane, individual cells taken from one slice of a deconvoluted Z-stack were outlined using the freehand selection tool in ImageJ and copied into new images to produce two images, one in the GFP channel and one in the RFP channel. The ImageJ plug-in Colocalization Finder (rsb.info.nih.gov/ij/plugins/colocalization-finder.html) was then used to calculate Pearson’s coefficient for each cell.

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