Asymmetric distribution of phosphatidylserine is generated in the absence of phospholipid flippases in *Saccharomyces cerevisiae*

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**Abstract**
In eukaryotic cells, phosphatidylserine (PS) is predominantly located in the cytosolic leaflet of the plasma membrane; this asymmetry is generated by an unknown mechanism. In this study, we used the PS-specific probe mRFP-Lact-C2 to investigate the possible involvement of type 4 P-type ATPases, also called phospholipid flippases, in the generation of this asymmetry in *Saccharomyces cerevisiae*. PS was not found in the trans-Golgi Network in wild-type cells, but it became exposed when vesicle formation was compromised in the sec7 mutant, and it was also exposed on secretory vesicles (SVs), as reported previously. However, flippase mutations did not reduce the exposure of PS in either case, even at low levels that would only be detectable by quantitative analysis of mRFP-Lact-C2 fluorescence in isolated SVs. Furthermore, no reduction in the PS level was observed in a mutant with multiple flippase mutations. Because PS was not exposed in a mutant that accumulates ER or cis/medial-Golgi membranes, Golgi maturation seems to be a prerequisite for PS translocation. Our results suggest that an unknown mechanism, possibly a protein with flippase-like activity, acts in conjunction with known flippases to regulate PS translocation.

**Introduction**
Phospholipids in the eukaryotic plasma membrane are unevenly distributed between the cytosolic and exoplasmic leaflets. Phosphatidylcholine (PC) is mainly located in the exoplasmic leaflet, whereas phosphatidylserine (PS) and phosphatidylethanolamine (PE) are mainly located in the cytosolic leaflet (Daleke 2003). In apoptotic cells, this phospholipid asymmetry is disrupted, and exposure of PS to the exoplasmic leaflet is recognized as an “eat me” signal (Ravichandran and Lorenz 2007). Changes in phospholipid asymmetry are also involved in the regulation of cell polarity through the control of Cdc42p and its associated factors (Saito et al. 2007; Fairn et al. 2011a; Das
Phospholipid flippases are candidate factors responsible for generating PS asymmetry in the plasma membrane. Flippases are type 4 P-type ATPases that are believed to translocate phospholipids from the exoplasmic to cytosolic leaflet (flip) (Daleke 2007; Lenoir et al. 2007; Tanaka et al. 2011; Sebastian et al. 2012). In budding yeast, there are five flippases: Drs2p, Dnf1p, Dnf2p, Dnf3p, and Neo1p. Drs2p, Dnf1/2p, and Dnf3p form complexes with Cdc50p, Lem3p, and Crf1p non-catalytic subunits, respectively, and these interactions are required for ER exit, proper localization, function, and activity of the flippases (Saito et al. 2004; Furuta et al. 2007; Lenoir et al. 2009; Takahashi et al. 2011; Puts et al. 2012). Therefore, defects in drs2Δ, dnf1Δ dnf2Δ, and dnf3Δ mutants are phenocopied by cdc50Δ, lem3Δ, and crf1Δ mutants, respectively (Saito et al. 2004; Furuta et al. 2007).

Cdc50p–Drs2p resides primarily in the TGN and endosomes (Chen et al. 1999; Saito et al. 2004), and its ATP-dependent PS flippase activity has been demonstrated in isolated Golgi membranes (Natarajan et al. 2004), isolated SVs (Alder-Baerens et al. 2006), and in an in vitro reconstitution system (Zhou and Graham 2009). Cdc50p–Drs2p has been implicated in the formation of clathrin-coated vesicles from the TGN (Gall et al. 2002; Liu et al. 2008), and work by our group showed that Cdc50p–Drs2p is involved in the endocytic recycling pathway, in which endocytosed proteins are transported to the TGN via early endosomes to be recycled back to the plasma membrane, in conjunction with Lem3p–Dnf1/2p and Crf1p–Dnf3p (Furuta et al. 2007). In the absence of these flippases, the Snc1p v-SNARE accumulates in enlarged early endosome-derived membranes due to defects in vesicle formation. We recently showed that Drs2p physically interacts with the F-box protein Rcy1p, which is specifically required for the endocytic recycling pathway (Hanamatsu et al. 2014).

Lem3p–Dnf1/2p is mainly localized to the plasma membrane (Kato et al. 2002; Pomorski et al. 2003), but like Cdc50p–Drs2p, this complex is also processed through the endocytic recycling pathway via early endosomes and the TGN (Saito et al. 2004; Liu et al. 2007). PS translocation by Lem3p–Dnf1/2p has been implicated in the sorting of Tat2p tryptophan transporter at the TGN (Hachiro et al. 2013). Crf1p–Dnf3p is localized to early endosome/TGN and plays a redundant role with Cdc50p–Drs2p and Lem3p–Dnf1/2p in growth and endocytic recycling; consistent with this, the crf1Δ and dnf3Δ single mutants do not exhibit a discernible phenotype (Hua et al. 2002; Pomorski et al. 2003; Furuta et al. 2007). Neo1p is different from other flippases in that it does not associate with a Cdc50 family member (Saito et al. 2004) and is independently essential for viability (Hua et al. 2002). Neo1p is involved in membrane trafficking from the cis-Golgi to the ER (Hua and Graham 2003), as well as within the endosomal-Golgi system (Wicky et al. 2004). Although Neo1p has not been demonstrated to have flippase activity, it is clear that the function of Neo1p in the endocytic recycling pathway is redundant with that of Cdc50p–Drs2p (Takeda et al. 2014).

All five yeast flippases are mainly or partially localized to endosomal/TGN membranes, suggesting that they may be involved in PS flipping at the TGN, and thus involved in generation of the plasma membrane PS asymmetry. In this study, we examined the contribution of flippases to the development of PS asymmetry in yeast, using mRFP-tagged Lact-C2 (mRFP-Lact-C2) as a probe for endogenous PS. Our results suggest that PS translocation occurs at the TGN concomitantly with the formation of SVs, even in the absence of flippases.

**Experimental Procedures**

**Media and genetic techniques**

Unless otherwise specified, strains were grown in rich medium (YPDA: 1% yeast extract (BD Difco, Franklin Lakes, NJ), 2% bacto-peptone (BD Difco), 2% glucose...
Strains and plasmids

Yeast strains used in this study are listed in Table 1. PCR-based procedures were used to construct gene deletions and gene fusions with the GALI promoter and mRFP1 (Longtine et al. 1998). The sec6–4 (AN56-2D), sec7–1 (SF821-8A), sec12–4 (MYB10-11D), and sec23–1 (MYB8-20C) mutants were kind gifts from Dr. Akihiko Nakano (The University of Tokyo). The gea1–4 gea2Δ mutant was a kind gift from Dr. Catherine L. Jackson (Institut Jacques Monod). The sec6–4 and sec7–1 mutations were introduced into the YEF473 genetic background by three serial backcrosses. The myo2–12 mutant in the YEF473 genetic background was from our laboratory stock (Yamamoto et al. 2010). All constructs produced by the PCR-based procedure were verified by colony-PCR amplification to confirm the replacement occurred at the expected locus. The plasmids used in this study are listed in Table 2. The GFP-tagged Lact-C2 plasmid (pRS416-GFP-Lact-C2) (Yeung et al. 2008) was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). pRS416-mRFP1-Lact-C2 was constructed by subcloning the coding region of mRFP1 to the HindIII–BglII gap of pRS416-GFP-Lact-C2. The KpnI–SacI mRFP1-Lact-C2 fragment was transferred to the integrating vector pRS306 to yield pRS306-mRFP1-Lact-C2 (pKT2108). pRS306-mRFP1-Lact-C2-AA (Lact-C2-W26A, W33A, F34A) (pKT2131) was constructed by subcloning the coding region of mRFP1 to the HindIII–BglII gap of pRS306-GFP-Lact-C2-AA (pKT1995) (Takeda et al. 2014). The URA3::mRFP1-Lact-C2 (YKT1843) and URA3::mRFP1-Lact-C2-AA (YKT1918) strains were constructed by integrating linearized pKT2108 and pKT2131 into the URA3 locus, respectively. Schemes detailing construction of plasmids and DNA sequences of nucleotide primers are available upon request.

Determination of mRFP-Lact-C2 fluorescence of isolated SVs

SVs were isolated using a previously described protocol (Harsay and Bretscher 1995) with minor modifications. Unless otherwise specified, chemicals and reagents were purchased from Wako Pure Chemical Industries Ltd. Briefly, cells were grown at 25 or 30°C to early to mid-logarithmic phase (OD600 of 0.5–0.7) in 0.5 L of YPD or SD, followed by further incubation at 37°C for 2 h to allow accumulation of SVs. The cells (~500 OD600 units) were then collected and converted to spheroplasts in spheroplast wash buffer (1.4 mol/L sorbitol [Sigma], 50 mmol/L KPi, at pH 7.4, 10 mmol/L sodium azide) containing 90 μg mL−1 Zymolyase 100T (Seikagaku Corp., Tokyo, Japan) at 37°C for 1 h. After spheroplasting efficiency was estimated by measuring OD600 units: spheroplasts were washed with spheroplast wash buffer and lysed with 20 strokes in a Dounce glass homogenizer with a tight pestle (Wheaton Industries, Millville, NJ) in lysis buffer (0.8 mol/L sorbitol; 10 mmol/L triethanolamine, adjusted to pH 7.2 with acetic acid; 1 mmol/L ethylenediaminetetraacetic acid) containing protease inhibitors (1 μg mL−1 aprotinin, 1 μg mL−1 leupeptin, 1 μg mL−1 pepstatin [Peptide institute inc., Osaka, Japan], 2 mmol/L benzamidine [Sigma], and 1 mmol/L phenylmethylsulfonyl fluoride). To prevent nonspecific binding of mRFP-Lact-C2 to SVs, lysis buffer containing 1 mol/L NaCl was added to cell lysates to a final concentration of 0.1 mol/L NaCl. A 700g spin for 10 min yielded the pellet (P1) and supernatant (S1) fractions. The S1 fraction was spun at 13,000g for 20 min to generate P2 and S2. The S2 fraction was centrifuged at 100,000g for 1 h in a 55.2Ti rotor (Beckman Coulter, Fullerton, CA) to generate membrane pellets (P3). For gradient fractionation, an 11 mL 15–30% continuous Nycodenz (Sigma) gradient was created in lysis buffer containing 0.1 mol/L NaCl. The P3 membrane pellets were resuspended in 1 mL of lysis buffer containing 0.1 mol/L NaCl, adjusted to 35% Nycodenz, and loaded on the bottom of the gradient using a 10-cm needle. Gradients were centrifuged in a P40ST rotor (Hitachi, Tokyo, Japan) at 100,000g for 16 h, and 0.5-mL fractions were manually collected from the bottom of the tube. Fluorescence intensity of mRFP-Lact-C2 was measured using an FP-6500 spectrofluorometer (Jasco Corp., Tokyo, Japan) at 590 nm (excitation, 530 nm; emission bandwidth, 10 nm; excitation bandwidth, 10 nm; Response, 1.0 sec; Gain, high) and normalized to the OD600 equivalent of the spheroplasted
Table 1. Saccharomyces cerevisiae strains used in this study.

| Strain | Genotype | Reference or source |
|--------|----------|---------------------|
| AN56-2D | MATα sec6-4 ura3-52 leu2-3,112 trp1-289 his3/4 | Gift from Dr. Akihiko Nakano |
| SF821-8A | MATα sec7-1 ura3-52 leu2-3,112 trp1-289 his4-580 | Gift from Dr. Akihiko Nakano |
| MBY10-11D | MATα sec12-4 ura3-52 leu2-3,112 trp1-289 his3/4 | Gift from Dr. Akihiko Nakano |
| MBY6-4D | MATα sec21-1 ura3-52 leu2-3,112 trp1-289 his3/4 | Gift from Dr. Akihiko Nakano |
| MBY8-20C | MATα sec23-1 ura3-52 leu2-3,112 trp1-289 his3/4 | Gift from Dr. Akihiko Nakano |
| CYO62-10-2 | MATα ura3-52 leu2-3,112 hisΔ200 lys2-801 ade2-101 gea1-4 gea2Δ: HIS3 | Peyroche et al. (2001) |
| YEF473 | MATα lys2-810 lys2-810 ura3-52 his3Δ200/trp1Δ-63 trp1Δ-63 leu2Δ-1 | Longtine et al. (1998) |
| YKT38 | MATα lys2-801 ura3-52 his3Δ200/trp1Δ-63 leu2Δ-1 | Misu et al. (2003) |
| YKT249 | MATα cdc50Δ::HIS3MX6 | Misu et al. (2003) |
| YKT1678 | MATα sec6-4 URA3::mRFP1-Lact-C2 | This study |
| YKT1843 | MATα URA3::mRFP1-Lact-C2 | This study |
| YKT1845 | MATα cho1Δ::hphMX4 URA3::mRFP1-Lact-C2 | This study |
| YKT1846 | MATα sec6-4 cho1Δ::hphMX4 URA3::mRFP1-Lact-C2 | This study |
| YKT1847 | MATα lem3Δ::TRP1 crf1Δ::hphMX4 URA3::mRFP1-Lact-C2 | This study |
| YKT1848 | MATα sec6-4 lem3Δ::TRP1 crf1Δ::hphMX4 URA3::mRFP1-Lact-C2 | This study |
| YKT1849 | MATα cdc50Δ::HIS3MX6 URA3::mRFP1-Lact-C2 | This study |
| YKT1850 | MATα sec6-4 cdc50Δ::HIS3MX6 URA3::mRFP1-Lact-C2 | This study |
| YKT1660 | MATα KanMX6::PGAL1-3HA-NEO1 | Takeda et al. (2014) |
| YKT1894 | MATα sec6-4 KanMX6::PGAL1-3HA-NEO1 | This study |
| YKT1851 | MATα KanMX6::PGAL1-3HA-NEO1 URA3::mRFP1-Lact-C2 | This study |
| YKT1852 | sec6-4 KanMX6::PGAL1-3HA-NEO1 URA3::mRFP1-Lact-C2 | This study |
| YKT1103 | MATα KanMX6::PGAL1-3HA-CDC50 lem3Δ::TRP1 crf1Δ::hphMX4 | This study |
| YKT1853 | MATα HIS3MX6::PGAL1-3HA-CDC50 lem3Δ::TRP1 crf1Δ::hphMX4 | This study |
| YKT1854 | MATα sec6-4 HIS3MX6::PGAL1-3HA-CDC50 lem3Δ::TRP1 crf1Δ::hphMX4 | This study |
| YKT1855 | MATα sec6-4 HIS3MX6::PGAL1-3HA-CDC50 lem3Δ::TRP1 crf1Δ::hphMX4 | This study |
| YKT1856 | MATα KanMX6::PGAL1-3HA-CDC50 lem3Δ::TRP1 crf1Δ::hphMX4 | This study |
| YKT1857 | MATα sec7-1 URA3::mRFP1-Lact-C2 | This study |
| YKT1858 | MATα sec7-1 cho1Δ::hphMX4 URA3::mRFP1-Lact-C2 | This study |
| YKT1859 | MATα sec7-1 cdc50Δ::HIS3MX6 URA3::mRFP1-Lact-C2 | This study |
| YKT1860 | MATα sec7-1 lem3Δ::TRP1 crf1Δ::hphMX4 URA3::mRFP1-Lact-C2 | This study |
| YKT1670 | MATα SEC7-mRFP1::TRP1 | This study |
| YKT1149 | MATα cdc50Δ::HIS3MX6 SEC7-mRFP1::TRP1 | This study |
| YKT1861 | MATα KanMX6::PGAL1-3HA-NEO1 SEC7-mRFP1::TRP1 | This study |
| YKT1862 | MATα HIS3MX6::PGAL1-3HA-CDC50 lem3Δ::TRP1 crf1Δ::hphMX4 | This study |
| YKT1863 | MATα SEC7-mRFP1::KanMX6 | This study |
| YKT1918 | TRP1::PGAL1-3HA-PMA1 URA3::mRFP1-Lact-C2 | This study |
| YKT1919 | MATα URA3::mRFP1-Lact-C2-AAA | This study |

Only relevant genotypes are described.

1YKT strains are isogenic derivatives of YEF473.

cells. For quantitative determination of total phospholipid phosphates in each fraction, lipids were extracted (Bligh and Dyer 1959), and colorimetric assays were performed (Rouser et al. 1970). Fraction densities were determined by measuring refractive indices on a refractometer (PAL-1; ATAGO Co. Ltd., Tokyo, Japan).

Immunoblot analysis

Immunoblot analysis was performed as described previously (Misu et al. 2003). For SDS-PAGE of Pma1p, samples were heated at 37°C for 15 min before loading. Rabbit anti-RFP (MBL, Nagoya, Japan) and anti-Pma1p
Table 2. Plasmids used in this study.

| Plasmid                        | Characteristics         | Reference or source |
|-------------------------------|-------------------------|---------------------|
| pKT1444 [pRS416 GFP-SNC1 pm]  | Prm1-GFP-SNC1 pm URA3 CEN | Lewis et al. (2000) |
| pKT1491 [pRS315 GFP-SNC1 pm]  | Prm1-GFP-SNC1 pm LEU2 CEN | This study          |
| pKT1563 [pRS416 mRFP1-SNC1]   | Prm1-mRFP1-SNC1 URA3 CEN | Furuta et al. (2007) |
| pKT1568 [pRS315 mRFP1-SNC1]   | Prm1-mRFP1-SNC1 LEU2 CEN | Takeda et al. (2014) |
| pKT1749 [pRS416 GFP-Lact-C2]  | GFP-Lact-C2 URA3 CEN    | Yeue et al. (2008)  |
| pKT1755 [pRS416 mRFP1-Lact-C2]| mRFP1-Lact-C2 URA3 CEN  | This study          |
| pKT1995 [pRS306-GFP-Lact-C2-AAA] | GFP-Lact-C2-AAA URA3    | This study          |
| pKT2108 [pRS306-mRFP1-Lact-C2]| mRFP1-Lact-C2 URA3     | This study          |
| pKT2131 [pRS306-mRFP1-Lact-C2-AAA] | mRFP1-Lact-C2-AAA URA3 | This study          |

Microscopic observations

Cells were observed using a Nikon ECLIPSE E800 microscope (Nikon Instec, Tokyo, Japan) equipped with an HB-10103AF superhigh-pressure mercury lamp and a 1.4 numerical aperture 100× Plan Apo oil immersion objective lens (Nikon Instec) with appropriate fluorescence filter sets (Nikon Instec) or differential interference contrast optics. Images were acquired using a cooled digital camera (Hamamatsu Photonics, Hamamatsu, Japan) and AQUA-COSMOS software (Hamamatsu Photonics). GFP- or mRFP-tagged proteins were observed in living cells, which were grown from early to mid-logarithmic phase, harvested, and resuspended in SD medium. Cells were immediately observed using a GFP bandpass (for GFP) or G2-A (for mRFP) filter set. Treatment with LAT-A (Wako Pure Chemical Industries Ltd.) was performed at 100 μmol/L by addition of a suitable volume of 20 mmol/L stock in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries Ltd.) to the medium, as described (Ayscough et al. 1997).

Results

PS translocation occurs during vesicle formation from the TGN

In wild-type cells, mRFP-Lact-C2 was localized to the plasma membrane, but not to any other organelle including the TGN, implying that PS translocation occurs during or after vesicle formation on the TGN (Yeung et al. 2008; Fairn et al. 2011a). To determine at what stage of the secretory pathway PS translocation takes place, we observed mRFP-Lact-C2 in temperature-sensitive secretory pathway mutants (Fig. 1A). Vesicle transport through the secretory pathway was monitored using GFP-Snc1p-pm, a plasma membrane-localized mutant v-SNARE containing point mutations that inhibit endocytosis (Lewis et al. 2000).

Sec6p is a subunit of exocyst that is a conserved protein complex required for tethering and fusion of SVs on the plasma membrane (Guo et al. 1999). The sec6-4 mutation leads to accumulation of post-Golgi plasma membrane-targeted SVs at the restrictive temperature (Novick et al. 1980). When sec6-4 cells were shifted to 37°C for 1 h, GFP-Snc1p-pm was localized to SVs accumulated in buds (64.9%, n = 111 cells), and mRFP-Lact-C2 was colocalized with them (98.5%, n = 133 structures) (Fig. 1B). This mRFP-Lact-C2 signal disappeared upon deletion of CHO1, which encodes a unique PS synthase (Lettis et al. 1983). We also examined a mutant that accumulates SVs due to a defect in polarized transport, not that in the exocyst complex. Myo2p, a yeast class V myosin, is required for the actin-based transport of SVs to the polarized site, and loss of the Myo2p function leads to accumulation of SVs throughout cells (Govindan et al. 1995; Schott et al. 1999; Jin et al. 2011). In myo2-12 cells at 37°C, GFP-Snc1p-pm was diffusely localized to the cytoplasm, and mRFP-Lact-C2 exhibited a similar localization pattern (20.0%, n = 120 cells). SEC7 encodes a guanine nucleotide exchange factor for Arf small GTPases, and the sec7-1 mutant is defective in formation of SVs from the TGN (Novick et al. 1980; Achstetter et al. 1988). In sec7-1 cells at 37°C, GFP-Snc1p-pm accumulated in internal membrane structures (76.5%, n = 115 cells) identified as enlarged TGN membranes (Novick et al. 1980; Achstetter et al. 1988), with which mRFP-Lact-C2 was also associated (98.2%, n = 111 structures). These results are consistent with previous observations (Fairn et al. 2011a). We also examined early secretory pathway mutations (sec12-4, sec21-1, sec23-1) that block exit from the ER (Novick et al. 1980). When these mutants were incubated at 37°C, GFP-Snc1p-pm accumulated in the ER (more than 87.6%, n > 100 cells), but mRFP-Lact-C2 was not colocalized with these membranes (less than 9.0%, n > 100 cells) (Fig. 1C).
### Flippase-Independent PS Translocation

**Flippase-Independent PS Translocation**

![Diagram](image)

**Defective in the fusion of SVs with the PM**
- **sec6-4**

**Defective in the transport of SVs to the polarized site**
- **myo2-12**

**Defective in the formation of SVs from the Golgi**
- **sec7-1**

**Defective in the transport from the ER**
- **sec12-4, sec21-1, sec23-1**

### Table A

|          | 30°C | 37°C |
|----------|------|------|
| DIC      |      |      |
| WT       |      |      |
| cho1Δ    |      |      |
| sec6-4   |      |      |
| sec6-4 cho1Δ | |      |
| myo2-12  |      |      |
| sec7-1   |      |      |
| sec7-1 cho1Δ | |      |

### Table B

|          | 25°C | 37°C |
|----------|------|------|
| DIC      |      |      |
| sec12-4  |      |      |
| sec21-1  |      |      |
| sec23-1  |      |      |
Taken together, these results suggest that PS translocation occurs during the formation of SVs from the TGN.

**Golgi cisternal maturation may be involved in PS flipping**

It is likely that PS flipping occurs concurrently with SV formation from the TGN. However, PS was flipped in the TGN even when SV formation was blocked in the sec7-1 mutant, suggesting that PS translocation is regulated independently of SV formation. Because mRFP-Lact-C2 did not localize to the ER membrane in mutants defective in ER exit, PS flipping may be specific to the TGN membrane. Therefore, we examined localization of mRFP-Lact-C2 in two mutants defective in membrane traffic in Golgi compartments, pik1 and gea1 gea2.

The phosphatidylinositol 4-kinase Pik1p, a binding partner of Sec7p in the late Golgi (Gloor et al. 2010), is required (like Sec7p) for anterograde transport from the TGN to the plasma membrane (Hama et al. 1999; Walch-Solimena and Novick 1999; Audhya et al. 2000). Phosphatidylinositol 4-phosphate (PI(4)P), synthesized at the Golgi by Pik1p, is important for recruitment of a regulator of Rab family small GTPases and clathrin adaptors (Santiago-Tirado and Bretscher 2011). When Pik1p was depleted for 9 h, GFP-Snc1p-pm accumulated throughout the cells (88.8%, n = 107 cells). mRFP-Lact-C2 extensively colocalized with GFP-Snc1p-pm, but 11.0% of the cells (n = 100 cells) had mRFP-Lact-C2-negative, GFP-Snc1p-pm-containing structures that were hardly detected in sec7-1 mutant cells (Fig. 2, arrowheads). Thus, Pik1p may be involved in the regulation of PS translocation, but it is also possible that these mRFP-Lact-C2-negative membranes are not TGN membranes: instead, given that PI(4)P was shown to be also involved in retrograde Golgi trafficking (Wood et al. 2009), they may be early or medial-Golgi membranes.

Gea1p and Gea2p are Arf1p nucleotide exchange factors that are required for Golgi-to-ER retrograde transport and intra-Golgi transport (Peyroche et al. 2001; Spang et al. 2001). In the gea1-4 gea2Δ temperature-sensitive mutant, early and late Golgi enzymes colocalize to a few ring-like structures, suggesting that these structures are derived from early/ medial-Golgi membranes (Peyroche et al. 2001). In gea1-4 gea2Δ cells, we observed that GFP-Snc1p-pm localized to these structures, as well to the ER (Fig. 2, arrowheads; 42.0%, n = 200 cells). mRFP-Lact-C2 was only rarely localized to these structures (6.4%, n = 173 structures).

Taken together, our results suggest that PS flipping is restricted to TGN membranes. Thus, we conclude that Golgi maturation is a prerequisite for PS translocation.

**Flippase mutations do not reduce PS exposure on accumulated SVs and TGN membranes**

We next investigated the contribution of flippases to PS exposure on accumulated SVs and TGN membranes. In the lem3Δ crf1Δ mutant, both GFP-Snc1p-pm and mRFP-Lact-C2 were exclusively localized to the plasma membrane, as in the wild type (Fig. 3A). The sec6-4 lem3Δ crf1Δ mutant accumulated GFP-Snc1p-pm in buds (66.9%, n = 133 cells) that colocalized with mRFP-Lact-C2 (98.6%, n = 139 structures) as in sec6-4 cells. Similarly, as in sec7-1 cells, the sec7-1 lem3Δ crf1Δ mutant accumulated GFP-Snc1p-pm membrane structures (62.3%, n = 130 cells) that colocalized with mRFP-Lact-C2 (96.8%, n = 125 structures). These results suggest that Lep3p-Dnf1/2p and Crf1p-Dnf3p are not essential for PS flipping in the TGN or/and SVs.

We next examined cdc50Δ cells and found that this mutant accumulated low levels of GFP-Snc1p-pm, which seemed to be accumulated early endosome/TGN membranes as described below, near polarized sites such as the bud tip or cytokinesis site, both at 30°C (45.8%, n = 155 cells) and 37°C (45.5%, n = 168 cells) (Fig. 3B). mRFP-Lact-C2 was almost entirely colocalized with these structures of GFP-Snc1p-pm, both at 30°C (100%, n = 136 structures) and 37°C (96.6%, n = 117 structures). Likewise, the cdc50Δ mutation did not affect the exposure of PS on the accumulated SVs and TGN membranes.
Figure 2. PS flipping is restricted to the TGN membrane. Localization of GFP-Snc1p-pm and mRFP-Lact-C2 was examined in mutants defective in Golgi membrane trafficking. sec7-1 mRFP1-Lact-C2 (YKT1857) cells carrying pRS315-GFP-SNC1 pm (pKT1491) were incubated in SD-Leu at 37°C for 1 h. To deplete Pik1p, PGAL1-3HA-PIK1 mRFP1-Lact-C2 (YKT1863) cells carrying pRS315-GFP-SNC1 pm (pKT1491) were incubated in SD-Leu at 30°C for 9 h. gea1-4 gea2Δ (CY62-10-2) cells cotransformed with pRS315-GFP-SNC1 pm (pKT1491) and pRS416-mRFP1-Lact-C2 (pKT1755) were incubated in SD-Leu-Ura at 37°C for 1 h. Arrowheads indicate GFP-Snc1p-pm-positive and mRFP-Lact-C2-negative membrane structures. Bar, 5 μm.
Figure 3. Localization of GFP-Snc1p-pm and mRFP-Lact-C2 in flippase-defective secretory mutant cells. (A) and (B) Localization of GFP-Snc1p-pm and mRFP-Lact-C2 in lem3Δ crf1Δ and sec50Δ mutants. Cells were incubated in SD-Leu medium at 30°C (control) or 37°C for 1 h. The strains used were lem3Δ crf1Δ (YKT1847), sec6-4 lem3Δ crf1Δ (YKT1848), sec7-1 lem3Δ crf1Δ (YKT1860), cdc50Δ (YKT1849), sec6-4 cdc50Δ (YKT1850), and sec7-1 cdc50Δ (YKT1859), all carrying URA3::mRFP1-Lact-C2 and pRS315-GFP-SNC1 pm (pKT1491). Bar, 5 μm. (C) Localization of GFP-Snc1p-pm with Sec7p-mRFP or mRFP-Snc1p in the cdc50Δ mutant. (Left panel) SEC7-mRFP1 (YKT1670) and cdc50Δ SEC7-mRFP1 (YKT1149) cells, both carrying pRS416-GFP-SNC1 pm (pKT1444), were incubated in SD-Ura medium at 30°C. (Right panel) Wild-type (YKT38) and cdc50Δ (YKT249) cells, both carrying pRS416-GFP-SNC1 pm (pKT1444) and pRS315-mRFP1-SNC11 (pKT1568), were incubated in SD-Leu-Ura medium at 30°C. Regions labeled with small characters are twofold enlarged to compare GFP and mRFP signal patterns. Bar, 5 μm.
to the Neo1p-depleted cells described above. This localization pattern did not significantly change after the shift to 37°C: the GFP-Snc1p-pm structures (99.1%, n = 113 cells) were randomly localized, rather than restricted to the bud, and they colocalized with mRFP-Lact-C2 (88.9%, n = 144 punctate structures), suggesting that Neo1p depletion may affect SV formation.

In some of the Neo1p-depleted cells, GFP-Snc1p-pm also accumulated in ER-like structures (~15% at 30°C, Fig. 4C, arrows and ~35% at 37°C, data not shown), consistent with the previous electron microscopic observation that some ER-like membranes accumulated in the neo1 mutant (Hua and Graham 2003). As in the early sec mutants, mRFP-Lact-C2 did not colocalize with these structures.

Taken together, our results suggest that PS exposure on accumulated SVs and TGN membranes is independent of flippases.

**Measurement of mRFP-Lact-C2 fluorescence in isolated SVs**

Although mRFP-Lact-C2 appeared to be localized on accumulated SVs or TGN membranes in the absence of flippases, subtle changes in PS concentrations on the surfaces of these vesicles/membranes would not be detectable by fluorescence microscopy. Therefore, to quantitatively analyze PS on the surface of SVs, we isolated SVs from cells expressing mRFP-Lact-C2 in the sec6-4 background. SVs were purified by subcellular fractionation followed by Nycodenz density gradient centrifugation, based on the procedures described previously (Harsay and Bretscher 1995). The authors who developed these procedures showed that the sec6-4 mutant accumulates two classes of SVs that differ with regard to both density and cargo proteins: low-density SVs (LDSVs) contain a plasma membrane H+-ATPase (Pma1p) activity, whereas high-density SVs (HDSVs) contain the soluble secreted enzymes such as invertase. Isolation of SVs was confirmed by estimating total phospholipid phosphates and Western blotting of Pma1p (Fig. 5A). Consistent with a previous report that HDSVs are obtained at much lower yields than LDSVs (Alderbaersens et al. 2006), we detected only a single peak of phospholipids corresponding to LDSVs (Fig. 5C and D). mRFP-Lact-C2, detected by Western blotting and measurement of mRFP fluorescence intensity using a spectrofluorometer, cofractionated with Pma1p and total phospholipid phosphates (Fig. 5A). This mRFP-Lact-C2 peak was not detected in sec6-4 cho1Δ cells (Fig. 5B), indicating that mRFP-Lact-C2 specifically bound to PS on LDSVs.

Because mRFP-Lact-C2 fluorescence intensity was highly correlated with the amount of total phospholipid phosphates, we concluded that mRFP-Lact-C2 fluorescence could be used as a quantitative measure of PS on LDSVs. HDSVs were not analyzed here, because their amount was not sufficient to give a peak of total
phospholipids that is required for calculation of relative PS content.

**Flippase mutations do not reduce PS on isolated LDSVs**

Using the method described above, we isolated LDSVs from flippase mutants carrying sec6-4, and then measured the mRFP-Lact-C2 fluorescence intensity and total phospholipid phosphates (Fig. 6A). To quantitatively assess PS content on the surface of LDSVs, we calculated the ratio of mRFP-Lact-C2 fluorescence to total phospholipid phosphates (referred to as Lact/Phospholipid) in the peak and four neighboring fractions (Fig. 6B). A mutant version of mRFP-Lact-C2, mRFP-Lact-C2-AAA, which is deficient in PS binding (Yeung et al. 2008), was included as a negative control in addition to sec6-4 cho1Δ (Fig. 6A, B, and C). Lact/Phospholipid was clearly lower in the sec6-4 cho1Δ and sec6-4 mRFP-Lact-C2-AAA mutants than in the sec6-4 mutant.

The sec6-4 lem3Δ crf1Δ mutant exhibited a peak of mRFP-Lact-C2 comparable to the phospholipid level in sec6-4. In denser fractions, Lact/Phospholipid was higher in this mutant than in the sec6-4 mutant. In the sec6-4 cdc50Δ mutant, no marked reduction in the phospholipid level was observed, suggesting that LDSVs were almost normally produced in this mutant. Lact/Phospholipid was slightly higher in this mutant than in the sec6-4 mutant. These results are essentially consistent with the microscopic observations shown Figure 3. On the basis of these findings, we concluded that in the absence of Lem3p–Dnf1/2p and Crf1p–Dnf3p or

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**Figure 5.** Quantitative analysis of PS on isolated LDSVs by measurement of mRFP-Lact-C2 fluorescence intensity. Cells were grown at 30°C or shifted to 37°C for 2 h. LDSVs were isolated from the cells by subcellular fractionation followed by Nycodenz gradient fractionation. Relative fluorescence intensity of mRFP-Lact-C2 was measured using a spectrofluorometer, and total phospholipid phosphates were determined. Pma1p and mRFP-Lact-C2 were detected by Western blotting using antibodies against Pma1p and RFP, respectively. The SV-enriched fraction from sec6-4 cells in (A) was loaded as a positive control (PC) for Western blotting in (C) and (D). The strains used were sec6-4 (YKT1844) (A and D), wild type (WT) (YKT1843) (C), and sec6-4 cho1Δ (YKT1846) (B), all carrying mRFP1-Lact-C2 at the genomic URA3 locus.
Figure 6. Loss of Lem3p/Crf1p or Cdc50p does not decrease the level of PS in the cytosolic face of LDSVs. (A) Fractionation profile of mRFP-Lact-C2 and total phospholipid phosphates in flippase mutants. Cells were grown in YPDA medium at 30°C and shifted to 37°C for 2 h, whereas $PGAL1-3HA-NEO1$ sec6-4 cells were incubated in YPDA medium at 30°C for 8.5 h, followed by a shift to 37°C for 2 h. SVs were isolated and fractionated by Nycodenz density gradient as in Figure 5. The strains used were sec6-4 (YKT1844), sec6-4 cho1Δ (YKT1846), sec6-4 lem3Δ crf1Δ (YKT1848), sec6-4 cdc50Δ (YKT1850), and sec6-4 $PGAL1-3HA-NEO1$ (YKT1852), all carrying mRFP1-Lact-C2 at the genomic $URA3$ locus, and sec6-4 (AAA) (YKT1919) carrying mRFP1-Lact-C2-AAA at the genomic $URA3$ locus. (B) Lact/Phospholipid was calculated as the ratio of relative fluorescence intensity of mRFP-Lact-C2 to total phospholipid phosphates in the peak and adjacent four fractions. Data shown are means ± SD of three independent experiments. (C) Localization of GFP-Snc1p-pm and mRFP-Lact-C2-AAA. Cells were incubated in SD-Leu medium at 30°C or 37°C for 1 h. The strains used were mRFP1-Lact-C2-AAA (YKT1918) and sec6-4 mRFP1-Lact-C2-AAA (YKT1919), both carrying pRS315-GFP-SNC1 pm (pKT1491). Bar, 5 μm.
Cdc50p–Drs2p, PS is flipped during or after SV formation.

Neo1p-depleted sec6-4 cells, in which Neo1p was depleted for 8.5 h at 30°C followed by a shift to 37°C for 2 h, exhibited a different pattern: total phospholipids were highest in lighter fractions but gradually decreased toward denser fractions. As described for Figure 4B, the Neo1p-depleted sec6-4 cells seem to be deficient in SV formation, because they accumulated GFP-Snc1p-pm in aberrant membrane structures, rather than in buds like sec6-4 cells. Thus, abnormal vesicles or membranes other than SVs might also be obtained by fractionation of the Neo1p-depleted sec6-4 cells. This abnormal fractionation pattern was not caused by cell death, because about 80% of the cells were alive before cell collection (Fig. S1A). Because Lact/Phospholipid was higher in all fractions in Neo1p-depleted sec6-4 than in the sec6-4 single mutant (data not shown), PS may be present in the cytosolic leaflet of these membranes in the absence of Neo1p. As noted above, however, we cannot exclude the possibility that disrupted membranes were also obtained by fractionation of the Neo1p-depleted sec6-4 cells.

**PS is still exposed on accumulated endosomal/TGN membranes in the simultaneous absence of all known flippases**

Because it was possible that Lem3p–Dnfl1/2p and Crf1p–Dnfl3p compensated for the lack of PS flippase activity in the absence of Cdc50p–Drs2p, and vice versa, we investigated a mutant lacking all these flippases. Because the cdc50Δ mutation is synthetically lethal with the lem3Δ crf1Δ mutations (Saito et al. 2004), we created the P_GAL1-CDC50 lem3Δ crf1Δ mutant. To quantitatively examine whether the Cdc50p-depleted lem3Δ crf1Δ mutations would affect the PS level on LDSVs, we tried to perform the SV fractionation assay on the Cdc50p-depleted sec6-4 lem3Δ crf1Δ cells. However, when the culture was shifted to 37°C for 2 h after Cdc50p was depleted for 4 h at 30°C in YPDA medium, about 50% of the cells died (Fig. S1B). We then attempted the same treatment in synthetic (SD) medium, and found that the cell viability remained high (86.5%) under these conditions (Fig. S1C). Therefore, we performed SV fractionation on the cells grown in SD medium. SVs could be recovered from sec6-4 cells, as in cells grown in YPDA medium, but the sec6-4 cho1Δ mutant exhibited a higher background (4.6-fold) of mRFP-Lact-C2 fluorescence intensity than the cells grown in YPDA medium (Fig. 7A), possibly because LDSVs from SD-grown cells contain more PS-independent substance(s) that bind to mRFP-Lact-C2. Nonetheless, Lact/Phospholipid in sec6-4 cells (20.3 ± 3.7 in the peak fraction of total phospholipid phosphates) was significantly higher than that in sec6-4 cho1Δ cells (13.6 ± 1.9). Surprisingly, LDSVs were not recovered from Cdc50p-depleted sec6-4 lem3Δ crf1Δ cells (Fig. 7A), raising the possibility that Cdc50p–Drs2p, Lem3p–Dnfl1/2p, and Crf1p–Dnfl3p function redundantly in the generation of LDSVs.

We next examined the localization of GFP-Snc1p-pm and mRFP-Lact-C2 in the Cdc50p-depleted lem3Δ crf1Δ cells. As shown in Figure 7B, these cells accumulated GFP-Snc1p-pm near the polarized growth site (50.4%, n = 133 cells), and mRFP-Lact-C2 colocalized with those structures (98.1%, n = 108 structures). In 84.5% of these cells (n = 116 cells), some Sec7p-mRFP dots also clustered to the polarized site, as in cdc50Δ cells, and appeared to partially colocalize with the GFP-Snc1p-pm structures. These results suggest that Cdc50p–Drs2p, Lem3p–Dnfl1/2p, and Crf1p–Dnfl3p may function cooperatively in the transport of GFP-Snc1p-pm from endosomal/TGN membranes, including LDSV formation. Importantly, PS was still exposed on the cytosolic face of these membranes in the absence of these flippases.

We showed previously that these flippases are involved in the endocytic recycling pathway, but not in the secretory pathway from the TGN to the plasma membrane (Furuta et al. 2007). In this study, we isolated and characterized the temperature-sensitive cdc50-11 lem3Δ crf1Δ mutant, which exhibited normal production of SVs but had defects in endocytic recycling of GFP-Snc1p from early endosome to the TGN. We speculated that a more severe defect in the early endosome-to-TGN pathway in the Cdc50p-depleted lem3Δ crf1Δ mutant than in the cdc50-11 lem3Δ crf1Δ mutant might cause a secondary defect in the secretory pathway. To test this idea, we examined the effect of inhibition of endocytosis on intracellular accumulation of GFP-Snc1p-pm to the TGN. Partial depletion of Cdc50p for 4 h in the P_GAL1-CDC50 lem3Δ crf1Δ mutant resulted in low-level accumulation of mRFP-Snc1p, but not GFP-Snc1p-pm (Fig. 7C). However, when endocytic transport was inhibited for 1 h with the actin polymerization inhibitor latrunculin-A (LAT-A) after 3 h depletion of Cdc50p, GFP-Snc1p-pm accumulated in intracellular structures with mRFP-Snc1p in 70.2% of cells (n = 114). In 75.9% of cells (n = 108 cells), some GFP-Snc1p-pm dots partially colocalized with Sec7p-mRFP structures (Fig. 7D). These results are consistent with our notion that inhibition of the endocytic pathway back to the TGN aggravated defects in the Cdc50p-depleted (4 h) lem3Δ crf1Δ mutant, resulting in a TGN defect. We confirmed that these GFP-Snc1p-pm structures colocalized with mRFP-Lact-C2 (98.3%, n = 116 structures) (Fig. 7D).
Finally, we examined the localizations of GFP-Snc1p-pm and mRFP-Lact-C2 in the Cdc50p- and Neo1p-depleted lem3Δ crf1Δ mutant. GFP-Snc1p-pm accumulated in almost all cells (93.5%, n = 124 cells), and mRFP-Lact-C2 colocalized with these membranes (97.3%, n = 111 cells) (Fig. 8). We previously showed that mRFP-Lact-C2 also colocalizes with GFP-Snc1p-containing membranes in this mutant (Takeda et al. 2014). We concluded that PS was still flipped in the accumulated endosomal/TGN membranes in the absence of all known flippases.

Discussion

In this study, we examined the possible involvement of flippases in the generation of plasma membrane PS asymmetry. Taken together with the results of a previous study (Fairn et al. 2011a), our findings suggest that PS has already been flipped in SVs before they fuse with the plasma membrane; that is, PS translocation seems to occur during or after SV formation from the TGN. Phospholipid flippases are candidates for the factors responsible for generating this PS asymmetry. Flippase activity toward PS has been demonstrated for Cdc50p-Drs2p (Natarajan et al. 2004; Alder-Baeren et al. 2006; Zhou and Graham 2009), and Lem3p-Dnf1/2p has also been implicated in PS translocation at the TGN (Hachiro et al. 2013). However, our microscopic observations suggested that none of the flippase mutations significantly changed PS distribution in the membranes we tested, including in the plasma membrane, in LDSVs accumulated in sec6-4 mutants, or in the enlarged TGN in sec7-1 mutants. We confirmed these results by quantitatively evaluating, for the first time, the amount of PS on the surface of isolated LDSVs.

We further examined the Cdc50p-depleted lem3Δ crf1Δ triple mutant, but again, this mutant exhibited localization of mRFP-Lact-C2 to GFP-Snc1p-pm–containing membranes. These membranes partially overlapped with Sec7p-mRFP, and the mutant was defective in LDSV production. On the other hand, we showed previously that the cdc50-11 lem3Δ crf1Δ mutant accumulated GFP-Snc1p on endosomal membranes due to defects in endocytic recycling (Furuta et al. 2007). The Cdc50p-depleted lem3Δ crf1Δ mutant accumulated both GFP-Snc1p-pm and mRFP-Snc1p in the same membranes, which probably represented early endosomes and TGN membranes (our unpublished results). Because Snc1p-pm is not endocytosed, it may have been transported to early endosomes from the TGN, where it accumulated. Together, the data led us to the conclusion that PS is exposed on the cytosolic leaflet in the TGN, as well as on early endosomes (Takeda et al. 2014).

We also investigated the possible involvement of Neo1p in PS translocation at the TGN. Neo1p-depleted cells accumulated GFP-Snc1p-pm in the membranes with which Sec7p-mRFP was partially localized, but mRFP-Lact-C2 also localized to these membranes. Finally, we showed that mRFP-Lact-C2 localized to the accumulated endosomal/TGN membranes in the Cdc50p- and Neo1p-depleted lem3Δ crf1Δ quadruple mutant. In summary, we did not find any evidence that flippases are involved in PS flipping during or after SV formation from the TGN.

Given the results described above, we hypothesize that a distinct protein with phospholipid translocase activity, in conjunction with flippases, is involved in PS translocation at the TGN (Fig. 9). This protein could be another type of ATP-dependent phospholipid translocase; indeed, such an activity has been detected in the plasma membrane by Stevens et al. (2008). Those authors demonstrated that 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)–labeled PS, a fluorescent PS analog commonly used to detect a flippase activity, but not NBD-PC or NBD-PE, translocated across the plasma membrane in an
ATP-dependent manner in the absence of Dnf1p, Dnf2p, Dnf3p, and Drs2p. This hypothetical translocase, or a related protein, may also be present in the TGN, like Dnf/Drs2 flippases, which are processed through the endocytic recycling pathway (Saito et al. 2004; Liu et al. 2007). Alternatively, this putative phospholipid translocase could be a scramblase-like protein that bidirectionally exchanges phospholipids across the bilayer in an energy-independent manner (Lhermusier et al. 2011). Because phospholipid translocases are elusive molecules whose activity is difficult to detect from isolated internal membranes such as the Golgi, a combination of genetic, cell biological, and biochemical approaches will be required to identify this protein.

PS flipping appears to occur concomitantly with vesicle formation, but its functional relevance is obscure. mRFP-Lact-C2 localized to isolated LDSVs, but not to TGN membranes in wild-type cells, suggesting that PS translocation occurs during or after vesicle formation. On the other hand, as demonstrated in the sec7-1 mutant, PS translocation could occur in the TGN membrane independently of SV formation. These results suggest that vesicle formation is not a prerequisite for PS flipping. Consistent with this idea, in mammalian cells PS translocation seems to occur prior to vesicle formation, because mRFP-Lact-C2 is localized to the TGN (Fairn et al. 2011b). Conversely, given that the cho1Δ mutant produced LDSVs, PS is not essential for vesicle formation from the TGN. Thus, PS translocation at the TGN seems to be more relevant than vesicle formation to the generation of plasma membrane PS asymmetry.

mRFP-Lact-C2 did not localize to either the ER or cis-/medial-Golgi membranes, suggesting that PS flipping was suppressed in these membranes. This could be accomplished by specific localization or/and activation of a putative phospholipid translocase at the TGN. In a previous study, the results of immuno-electron microscopy suggested that PS is present in the luminal leaflet of the ER in mammalian cells (Fairn et al. 2011b). Because PS is synthesized at the cytosolic face of the ER in yeast
(Carman and Han 2011), it must be translocated to the luminal leaflet by an unknown mechanism, and then subsequently transported to the TGN and the plasma membrane via the secretory pathway. Recently, Maeda et al. (2013) reported another PS transport route to the plasma membrane, in which oxysterol-binding proteins Osh6/7p transport PS from the ER to the plasma membrane via a nonvesicular pathway. Thus, PS is efficiently removed from the cytosolic leaflet of the ER by two different mechanisms and transported to the plasma membrane.

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**Conflict of Interest**

None declared.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Cell viability of flippase mutants containing sec6-4 after flippase depletion and temperature upshift. Cells were incubated in YPGA or YPDA medium at 30°C for 8.5 h (Neo1p depletion) or 4 h (Cdc50p depletion), and then further incubated at 30°C or 37°C for 2 h. Cell viability was determined by PI staining and flow-cytometric analysis. (A) Viability of *PGAL1-3HA-NEO1 sec6-4* cells. The strains used were *PGAL1-3HA-NEO1* (YKT1660) and *sec6-4 PGAL1-3HA-NEO1* (YKT1894). (B) Viability of *sec6-4 PGAL1-3HA-CDC50 lem3Δ crf1Δ* cells. The strains used were *PGAL1-3HA-CDC50 lem3Δ crf1Δ* (YKT1860) and *sec6-4 PGAL1-3HA-CDC50 lem3Δ crf1Δ* (YKT1855). (C) Viability of *sec6-4 PGAL1-3HA-CDC50 lem3Δ crf1Δ* cells in SD medium. Viability was determined as in (B), except that cells were grown in SD medium instead of YPDA at 37°C.