



# The PQ-loop protein Any1 segregates Drs2 and Neo1 functions required for viability and plasma membrane phospholipid asymmetry<sup>S</sup>

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**Abstract** Membrane asymmetry is a key organizational feature of the plasma membrane. Type IV P-type ATPases (P4-ATPases) are phospholipid flippases that establish membrane asymmetry by translocating phospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine, from the exofacial leaflet to the cytosolic leaflet. *Saccharomyces cerevisiae* expresses five P4-ATPases: Drs2, Neo1, Dnf1, Dnf2, and Dnf3. The inactivation of Neo1 is lethal, suggesting Neo1 mediates an essential function not exerted by the other P4-ATPases. However, the disruption of *ANY1*, which encodes a PQ-loop membrane protein, allows the growth of *neo1Δ* and reveals functional redundancy between Golgi-localized Neo1 and Drs2. Here we show Drs2 PS flippase activity is required to support *neo1Δ any1Δ* viability. Additionally, a Dnf1 variant with enhanced PS flipping ability can replace Drs2 and Neo1 function in *any1Δ* cells. *any1Δ* also suppresses *drs2Δ* growth defects but not the loss of membrane asymmetry. Any1 overexpression perturbs the growth of cells but does not disrupt membrane asymmetry. Any1 coimmunoprecipitates with Neo1, an association prevented by the Any1-inactivating mutation D84G. These results indicate a critical role for PS flippase activity in Golgi membranes to sustain viability and suggests Any1 regulates Golgi membrane remodeling through protein-protein interactions rather than a previously proposed scramblase activity.—Takar, M., Y. Huang, and T. R. Graham. The PQ-loop protein Any1 segregates Drs2 and Neo1 functions required for viability and plasma membrane phospholipid asymmetry. *J. Lipid Res.* 2019. 60: 1032–1042.

**Supplementary key words** phosphatidylserine • phosphatidylethanolamine • phosphatidylcholine • trafficking • Golgi apparatus • transport

Phospholipid asymmetry is a fundamental property of the eukaryotic cell plasma membrane. For instance, phosphatidylcholine (PC) and sphingolipids are enriched in the exofacial leaflet, and aminophospholipids such as

phosphatidylserine (PS) and phosphatidylethanolamine (PE) are confined to the cytofacial leaflet (1–3). Membrane asymmetry is regulated through the transbilayer movement of amphipathic phospholipids by the concerted activities of three types of transporters: floppases, flippases, and scramblases (4). Floppases belong to the ABC transporter family and translocate phospholipid substrates to the exofacial leaflet of membranes (5). Flippases in the type IV P-type ATPase (P4-ATPase) family transport primarily aminophospholipids (PS and PE) to the cytofacial leaflet of membranes in an ATP-dependent manner (6–8), although some members transport either PC, lyso-PC, or glucosylceramide (9–12). Membrane asymmetry is thought to be produced from the net effect of the relatively slow and nonspecific outward transport of phospholipids by ABC transporters, coupled with a rapid inward transport of aminophospholipids (13). Scramblases in the TMEM16F and Xkr8 protein families are phospholipid channels that open in response to a Ca<sup>2+</sup> influx (TMEM16F) or caspase cleavage (Xkr8) to dissipate the phospholipid gradient (14–17). These scramblases play important roles in blood clotting and the removal of apoptotic cell corpses, respectively. In mammals, the loss of P4-ATPase activity is associated with liver disease (18), diabetes (19–21), hearing loss (22), increased risk of myocardial infarction (23, 24), hemolytic anemia (25), neurodegeneration (26), and intellectual disability (27). Thus, perturbations in membrane asymmetry likely contribute to these pathologies.

Membrane remodeling events within the Golgi complex play an important role in establishing plasma membrane asymmetry. Sphingolipids and glycosphingolipids are synthesized in the luminal (exofacial) leaflet of the Golgi and are exported by vesicular transport to the exofacial leaflet of the plasma membrane (28, 29). Aminophospholipids

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Abbreviations: PapA, papuamide A; PC, phosphatidylcholine; PE, phosphatidylethanolamine; P<sub>GAL</sub>, GAL promoter; PS, phosphatidylserine; P4-ATPase, type IV P-type ATPase; 5-FOA, 5-fluoroorotic acid.

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are flipped by P4-ATPases from the luminal leaflet of the Golgi to the cytosolic leaflet and subsequently move to the cytosolic leaflet of the plasma membrane by either vesicular or nonvesicular routes. These events are also coupled to sterol loading in late compartments of the Golgi complex to transition the bilayer from an ER-like composition and organization at the *cis*-face to a plasma membrane-like composition and organization at the *trans*-face (30, 31). P4-ATPases also localize to the plasma membrane and endosomal compartments to ensure asymmetry is maintained as membrane fluxes through the endocytic pathway and recycles back to the plasma membrane (32).

While differences in subcellular localization may confer unique functions, it remains unclear why a single-celled organism such as *Saccharomyces cerevisiae* expresses five different P4-ATPases. Dnf1, Dnf2, Dnf3, and Drs2 form an essential group with both overlapping and nonoverlapping functions. For example, cells harboring *drs2Δ* grow well at 30°C but fail to grow at 20°C or below because the Dnf P4-ATPases can partially compensate for the loss of Drs2 at higher temperatures but not at lower temperatures. Even at permissive growth temperatures, *drs2Δ* cells display protein-trafficking defects between Golgi and endosomal membranes. Drs2 localizes to the *trans*-Golgi network, but Neo1, Dnf1, and Dnf3 also localize significantly to the *trans*-Golgi network and yet fail to support these trafficking pathways in the absence of Drs2 (33–35). Likewise, *neo1Δ* cells cannot grow at any temperature, suggesting that this essential P4-ATPase has a unique function that none of the other P4-ATPases can perform (35, 36). Consistently, Drs2 and Neo1 regulate different vesicular transport pathways: early-endocytic/late-secretory and early-secretory pathways, respectively (35, 37–39).

The phospholipid substrate specificity of budding yeast P4-ATPases appears to be the major determinant of their specific roles in the cell. Drs2 and its mammalian orthologues (ATP8A1 and ATP8A2) are primarily PS flippases, although they are also capable of flipping PE (7, 8, 40). *drs2Δ* cells, accordingly, display a loss of plasma membrane PS and PE asymmetry (41). Substrates of Dnf1 and Dnf2 include lyso-PC and lyso-PE, phospholipids lacking one of the two fatty acyl chains, and glucosylceramide, which is not endogenously produced (10, 12). Thus, these P4-ATPases may function in nutrient scavenging and membrane repair (10). We previously mapped residues that conferred substrate specificity differences between Dnf1 and Drs2 and identified mutations that alter their specificity (12, 42–45). Separation-of-function mutations in Drs2 were isolated that abrogate PS recognition without measurably perturbing PE recognition (Drs2<sup>PS-</sup>). Conversely, gain-of-function mutations were isolated in Dnf1 that allow it to flip diacyl-PS without perturbing the recognition of its normal substrates (Dnf1<sup>PS+</sup>) (42–44). Remarkably, *drs2Δ* cells expressing Drs2<sup>PS-</sup> display trafficking defects, but normal trafficking and growth at low temperatures are restored in *drs2Δ* cells expressing Dnf1<sup>PS+</sup> (but not Dnf1) (30, 38). These observations imply that Drs2 is the primary PS flippase in the cell, and the other P4-ATPases lack sufficient PS flippase activity to compensate for the loss of Drs2.

However, both Drs2 and Neo1 (orthologues of mammalian ATP9A and ATP9B) are involved in regulating PS/PE plasma membrane asymmetry, and these P4-ATPases become functionally redundant when *ANY1* is deleted (39, 46, 47). The inactivation of Neo1<sup>ts</sup> more substantially perturbs PE asymmetry, but these cells also aberrantly expose PS (39). By contrast, the inactivation of the *Caenorhabditis elegans* Neo1 orthologue *Tat-5* causes exposure of PE without the loss of PS asymmetry (48). The possibility that Neo1 and Drs2 have similar substrate preferences is supported by the observation that the PQ-loop membrane protein Any1 enforces separate functions for Drs2 and Neo1 in the Golgi/endosomal membranes (47, 49). In the absence of Any1, PS and PE membrane asymmetry is restored in *neo1<sup>ts</sup> (neo1<sup>ts</sup> any1Δ)* to WT levels. Moreover, Drs2 can bypass the essential requirement for Neo1, as *neo1Δ any1Δ* viability depends on a WT copy of *DRS2* (47). Any1 was proposed to function as a scramblase in the Golgi, acting to dissipate the PS/PE gradients formed by Drs2 and Neo1 to segregate their functions (47). However, the mechanism by which Any1 antagonizes Neo1 has not been determined.

In this study, we provide evidence that the essential role of Drs2 and Neo1 in Golgi membranes lacking Any1 is to flip PS. These *any1Δ* strains also allowed us to individually compare the influence of Drs2, Neo1, or Dnf1<sup>PS+</sup> variants on membrane asymmetry, and these studies suggest a comparable ability to flip PS but that Neo1 has the greatest impact on PE asymmetry. The overexpression of Any1 is toxic to cells deficient in P4-ATPase activity, but excess Any1 does not disrupt membrane asymmetry. This result suggests that Any1 is not a scramblase and might antagonize Neo1 by a different mechanism. We found that Any1 interacts with Neo1, suggesting that it might inhibit Neo1 activity through this interaction. Thus, this study supports the proposed PE/PS flippase activity of Neo1 reported earlier and defines a protein-interaction network that facilitates membrane remodeling events in the secretory pathway.

## MATERIALS AND METHODS

### Reagents

Papuaamide A (PapA) was purchased from the University of British Columbia Depository (Vancouver, Canada). Duramycin, quinacrine dihydrochloride, calcofluor white, neomycin sulfate, monoclonal FLAG antibody, and EzView FLAG beads were purchased from Sigma-Aldrich (St. Louis, MO). 5-Fluoroorotic acid (5-FOA) was purchased from US Biologicals (Salem, MA). Monoclonal GST (clone D1 and F5) antibody was purchased from Vanderbilt Antibody and Protein Resource (Nashville, TN), and polyclonal GST (clone D1 and F5) antibody was purchased from Torrey Pines Biolabs (Secaucus, NJ). Anti-Arfl, anti-Mnn1, and anti-Drs2 antibodies have been previously described (34). IRDye 800CW goat anti-rabbit IgG (H+L) and Starbrite Blue goat anti-rabbit IgG (H+L) secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE) and Bio-Rad (Hercules, CA), respectively.

### Strains and plasmid construction

The strains and plasmids used are listed in supplemental Table 1. Yeast strains were grown and transformed using standard media

and transformation techniques (50–52). For growth and plasmid-shuffling assays, 0.1 OD<sub>600</sub> cells and 10-fold serial dilutions were spotted onto synthetic media or synthetic media containing 5-FOA and other toxic agents. Yeast knockout and knock-in strains were generated as previously described (53). For coimmunoprecipitation experiments, a *GST*-tag cassette was amplified from *pFA6a-GST-His3MX6* and integrated at the 3' end of *ANY1* in *pRS313-ANY1* or *pRS313-Any1[D84G]* plasmids using Gibson assembly (54). *pRS315-NEO1-9XGLY-5XFLAG* was generated by the Gibson assembly-mediated insertion of the *9XGLY-5XFLAG* tag into *pRS315-P<sub>NEO1</sub>-NEO1*. *pRS315-NEO1-9XGLY-5XFLAG* or *pRS315-NEO1* along with *pRS313-ANY1-GST* or *pRS313-Any1[D84G]GST* were transformed into *MTY10S (neo1Δ any1Δ)*. Any1 or Neo1 mutants were generated using 3-fragment and 2-fragment Gibson assembly according to the manufacturer's instructions.

### Toxin-sensitivity assays

For toxin-sensitivity assays, 0.1 OD<sub>600</sub> mid-log cells were distributed to each well of a 96-well plate with or without the toxin using four independent biological replicates. Plates were incubated at 30°C for 20 h. Concentrations of the cells were measured in OD<sub>600</sub>/ml with a Multimode Plate Reader Synergy HT (BioTek, Winooski, VT). Sigmodial curve-fitting modality from GraphPad Prism7 was used to fit the data point from all samples when *R*<sup>2</sup> values were equal to or greater than 0.8 for all samples.

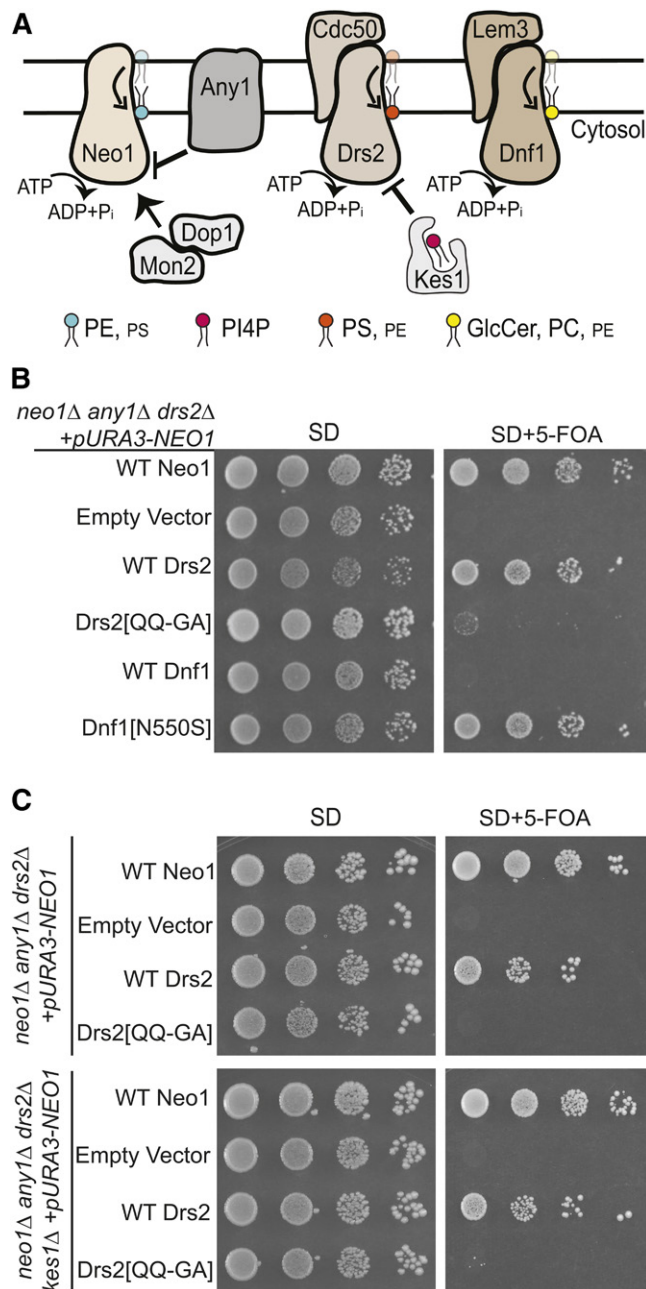
### Immunoprecipitation and Western blot analysis

Mid-log-phase cells were lysed and subjected to Western blot analysis as described previously (39). For expression analysis, mouse mono-clonal GST (1:1000) and mono-clonal FLAG (1:5000) antibodies were used. Total protein concentrations were assessed by the Bio-Rad stain-free gel system. Both Neo1 and Any1 tend to form SDS-resistant aggregates and migrate as high-molecular-weight smears by SDS-PAGE. To mitigate this problem, samples were heated at 50°C for 10 min in SDS/urea sample buffer. In addition, freezing and thawing samples seem to exacerbate the gel-migration artifact; thus, samples were stored at 4°C prior to SDS-PAGE. Total lysates from yeast spheroplasts under native conditions were prepared as described previously (34). Protein concentrations of total lysates used for immunoprecipitations were quantified using a BCA assay. Immunoprecipitations of FLAG-tagged bait were performed from 10 mg of total input (native cell lysate) using 50 μl EzView FLAG beads for 2 h at 4°C. The beads were washed three times with washing buffer I and two times with washing buffer II (34) prior to elution with SDS/urea sample buffer and SDS-PAGE.

## RESULTS

### PS flippase activity is required in *any1*-deficient Golgi membranes

Neo1, Drs2, and Dnf1 have nonredundant functions in the Golgi complex despite their similar effects on plasma membrane asymmetry (39) (see Fig. 1A and supplemental Table 2 for an overview of the proteins analyzed in this work). Removing Any1 reveals a redundancy between Neo1 and Drs2, although it was not clear whether a PS or PE flippase activity, or both, were required to support viability in a *neo1Δ any1Δ drs2Δ* strain. To test this, we expressed WT Neo1, WT Drs2, Drs2<sup>PS-</sup> (Drs2[QQ→GA]), WT Dnf1, or Dnf1<sup>PS+</sup> (Dnf1[N550S]) in *neo1Δ drs2Δ any1Δ* cells expressing



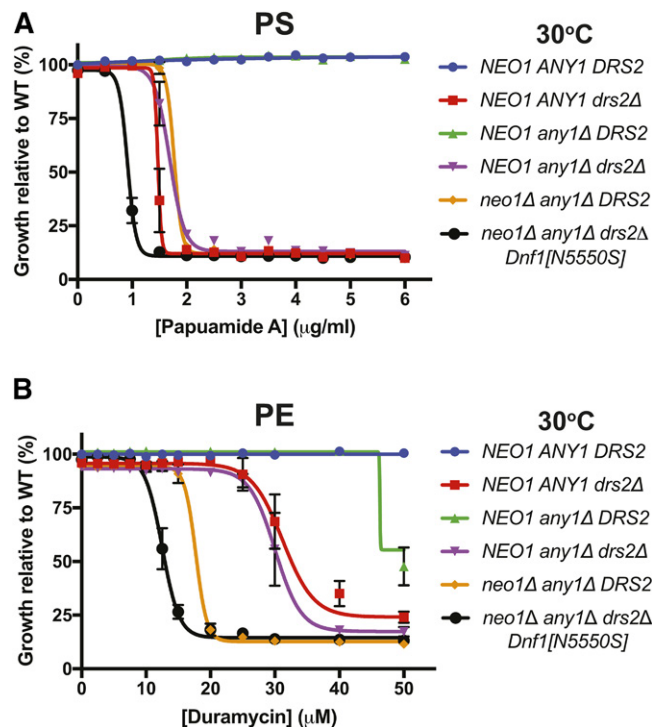
**Fig. 1.** PS translocation in Golgi membranes is required for viability. A: Interacting network of proteins important for membrane organization whose function in membrane asymmetry and cell viability is explored in this study. B: Neo1, Drs2, and Dnf1 [N550S] can support the growth of *neo1Δ drs2Δ any1Δ* cells. *neo1Δ drs2Δ any1Δ* + pRS416-*NEO1* (MTY10-615M2DS) expressing single-copy (*cen*) *NEO1* (pRS313-*NEO1*), empty vector (pRS313), *cen* *DRS2* (pRS313-*DRS2*), *cen* *Drs2*[QQ→GA] (pRS313-*Drs2*[QQ→GA]), *cen* *DNF1* (pRS313-*DNF1*), or *cen* *Dnf1*[N550S] (pRS313-*Dnf1*[N550S]), respectively, were spotted onto minimal media plates, and the pRS416-*NEO1* plasmid was counter-selected on 5-FOA. C: Loss of Kes1 failed to suppress the synthetic lethality of *neo1Δ drs2Δ any1Δ*. *neo1Δ drs2Δ any1Δ kes1Δ* + pRS416-*NEO1* (MTY10-615M2DS) expressing single-copy (*cen*) *NEO1* (pRS313-*NEO1*), empty vector (pRS313), *cen* *DRS2* (pRS313-*DRS2*), and *cen* *Drs2*[QQ→GA] (pRS313-*Drs2*[QQ→GA]), respectively, were spotted onto minimal media plates, and the pRS416-*NEO1* plasmid was counter-selected on 5-FOA. These images are representative of four independent growth assays.

WT *NEO1* on a *URA3*-marked plasmid. We counter-selected *pURA3-NEO1* on 5-FOA plates that maintained selection for the *HIS3*-marked plasmids bearing the P4-ATPase variants to be tested and incubated the plates at 30°C (Fig. 1B). As previously reported (47), WT Neo1 or Drs2 was sufficient to complement *neo1Δ drs2Δ any1Δ* synthetic lethality and support growth, but the empty vector and extra copy of WT Dnf1 failed to suppress this synthetic lethality. Importantly, while *Drs2<sup>PS-</sup>* failed to complement the growth defect, *Dnf1<sup>PS+</sup>* fully suppressed the *neo1Δ drs2Δ any1Δ* synthetic lethality (Fig. 1A). Thus, bypassing *neo1Δ* in cells lacking Any1 requires a PS flippase activity that can be provided by either Drs2 or Dnf1.

The cold-sensitive growth defect of *drs2Δ* can be fully suppressed by deleting *KES1/OSH4*, which encodes an ergosterol/phosphatidylinositol-4-phosphate exchange protein involved in Golgi membrane remodeling (Fig. 1A) (31, 55). Therefore, we tested whether the synthetic lethality between *neo1Δ* and *drs2Δ* alleles could be suppressed by *kes1Δ*. The same plasmid-shuffling strategy was used to express WT Neo1, WT Drs2, *Drs2<sup>PS-</sup>*, and empty vector control in *neo1Δ any1Δ drs2Δ* and *neo1Δ any1Δ drs2Δ kes1Δ* cells (Fig. 1B). In this experiment, *kes1Δ* failed to suppress the *neo1Δ any1Δ drs2Δ* synthetic lethality (empty vector and *Drs2*[*QQ→GA*]), and only WT Neo1 or Drs2 were able to support the growth of *neo1Δ drs2Δ any1Δ kes1Δ* cells (Fig. 1C).

#### Loss of Any1 does not suppress membrane asymmetry defects of P4-ATPase null mutants

We previously found that *neo1<sup>fs</sup>* mutants expose both PS and PE in the exofacial leaflet at semipermissive growth temperatures. The loss of asymmetry can be probed by hypersensitivity to pore-forming toxins that specifically target PS (PapA) or PE (Duramycin) exposed on the exofacial leaflet of the plasma membrane (56, 57). The loss of *neo1<sup>fs</sup>* plasma membrane asymmetry is completely suppressed in *neo1<sup>fs</sup> any1Δ* cells (47). Here we examined membrane asymmetry in strains carrying *neo1Δ*, *any1Δ*, and *drs2Δ* null alleles. WT and *any1Δ* cells were resistant to both PS-binding (PapA) and PE-binding (Duramycin) toxins. Although *any1Δ* cells were partially sensitive to Duramycin at the highest concentration tested (Fig. 2A, B). The *neo1Δ drs2Δ any1Δ* cells expressing Neo1, Drs2, or *Dnf1<sup>PS+</sup>* were hypersensitive to PapA and showed a similar level of sensitivity with *Dnf1*[N550S], displaying a slightly lower capacity to establish PS asymmetry relative to Drs2 or Neo1 (Fig. 2A). Thus, none of these P4-ATPases could prevent PS exposure in the plasma membrane outer leaflet independently in this strain background. A comparison of *NEO1 ANY1 drs2Δ* to *NEO1 any1Δ drs2Δ* seemed to show that *any1Δ* weakly suppressed *drs2Δ* PS exposure, but these data were not significantly different (Fig. 2A). Note that we cannot analyze the *neo1Δ ANY1 DRS2* strain because it is inviable. *Dnf1*[N550S], Drs2, and Neo1 conferred increasing resistance to Duramycin, with Neo1 restoring PE asymmetry to a significantly greater extent than Drs2 or *Dnf1*[N550S] in *neo1Δ drs2Δ any1Δ* cells. Again, none of the P4-ATPases were able to restore PE asymmetry to WT levels on their own, and *any1Δ* did not suppress PE exposure caused by

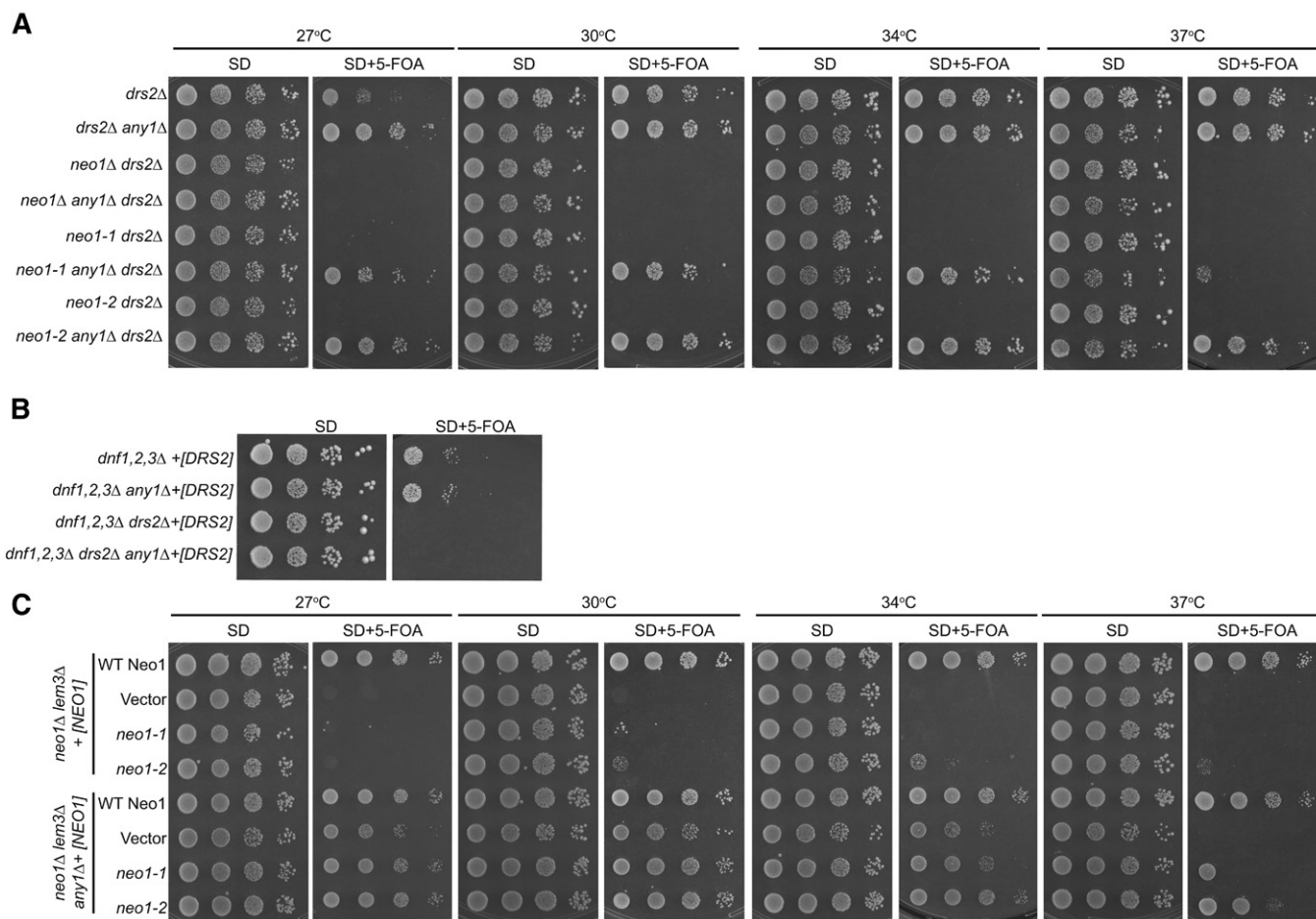


**Fig. 2.** None of the Golgi P4-ATPases can individually restore membrane asymmetry when expressed in *neo1Δ any1Δ drs2Δ* cells. The strains indicated were grown with increasing concentrations of PapA (A) or Duramycin (B) to assess the exposure of PS or PE, respectively. Growth ( $OD_{600}$ ) after 20 h at 30°C is plotted relative to untreated WT cells ( $n \geq 4$ ; error bars indicate  $\pm$  SEM).

*drs2Δ* (Fig. 2B). These findings indicate that Drs2, Neo1, and *Dnf1<sup>PS+</sup>* have a near-equivalent ability to support the growth of *neo1Δ any1Δ drs2Δ* cells and a comparable ability to support PS asymmetry, whereas Neo1 has a more substantial impact on PE asymmetry than Drs2 or *Dnf1<sup>PS+</sup>*.

To further explore these genetic relationships, we tested whether *any1Δ* could suppress growth phenotypes in strains deficient for several different P4-ATPases. Even though *any1Δ* did not measurably suppress the loss of membrane asymmetry in *drs2Δ*, it was able to partially suppress the cold-sensitive growth defect of *drs2Δ* (Fig. 3A). Hypomorphic *neo1-1* and *neo1-2* alleles are synthetically lethal with *drs2Δ* (37) (Fig. 3A), but *any1Δ* effectively suppressed this synthetic lethality. The more stringent *neo1-1* allele still displayed a temperature-sensitive growth phenotype in the *neo1-1 any1Δ drs2Δ* background, while *neo1-2* was suppressed across the full growth temperature range (Fig. 3A). However, *any1Δ* could not suppress the lethality caused by *drs2Δ dnf1,2,3Δ* (Fig. 3B). *Dnf1* and *Dnf2* form heterodimers with *Lem3* (Fig. 1A, supplemental Table 2), and *lem3Δ* cells grow well but are deficient for both *Dnf1* and *Dnf2* activities. Here we show that *lem3Δ* is synthetically lethal with *neo1-1* and *neo1-2* (Fig. 3C, top), but the growth defects of *neo1<sup>fs</sup> lem3Δ* and *neo1Δ lem3Δ* are substantially, but not completely, suppressed by *any1Δ* (Fig. 3C, bottom).

To test the influence of Any1 on the loss of membrane asymmetry caused by Drs2 and Dnf deficiency, we performed pore-forming toxin-sensitivity assays with WT, *drs2Δ*, *drs2Δ any1Δ*, *dnf1Δ dnf2Δ*, *dnf1Δ dnf2Δ any1Δ*, and *any1Δ*



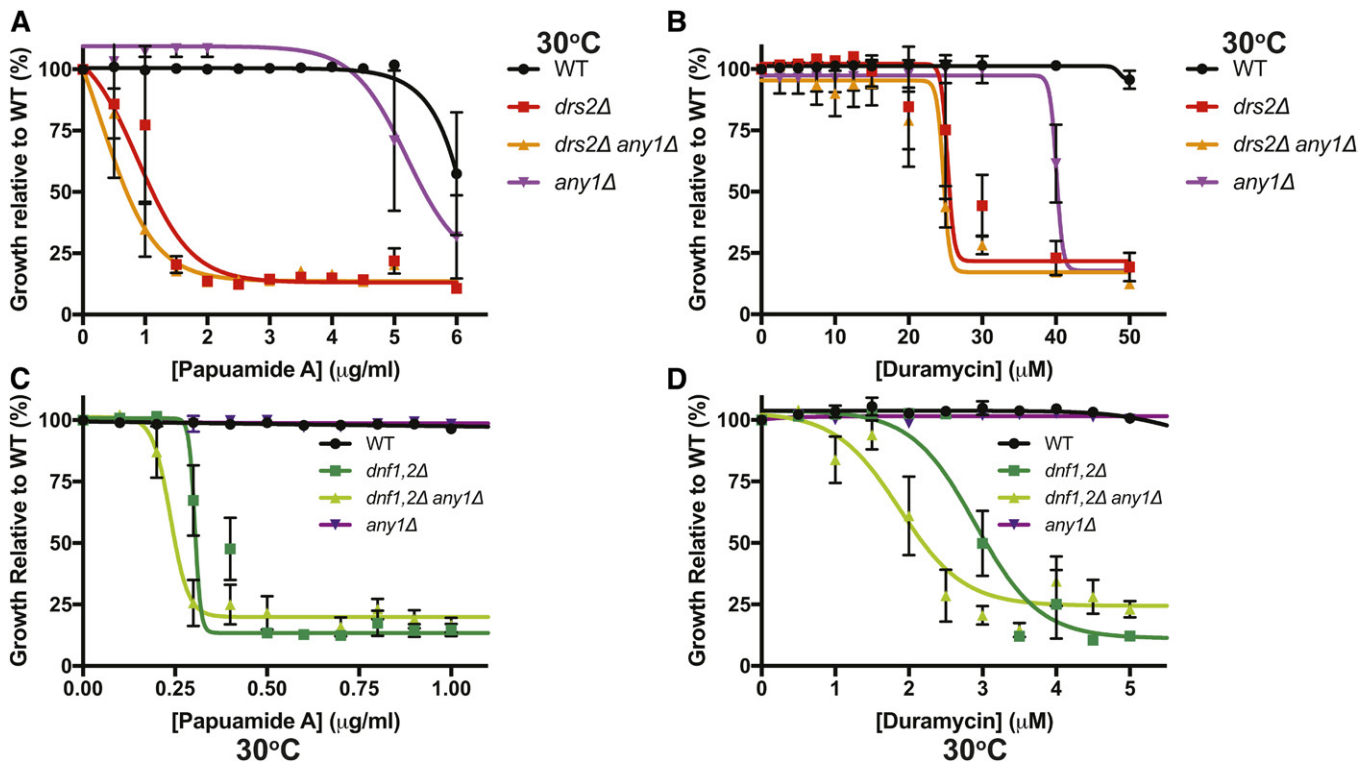
**Fig. 3.** *any1Δ* genetic interactions with P4-ATPase mutants. A: *any1Δ* suppresses the cold-sensitive growth of *drs2Δ*, high-temperature growth defects of *neo1-1* and *neo1-2*, and the synthetic lethality between *drs2* and *neo1<sup>ts</sup>* alleles. Both *neo1-1<sup>ts</sup>* and *neo1-2<sup>ts</sup>* can support the viability of *any1Δ drs2Δ* cells at all temperatures tested except for 37°C. B: Loss of Any1 failed to suppress the synthetic lethality of *dnf1,2,3Δ drs2Δ*. C: Loss of Any1 can suppress the synthetic lethality of *neo1Δ lem3Δ* or *neo1<sup>ts</sup> lem3Δ* at all temperatures tested except for 37°C. These images are representative of four independent growth assays.

cells. *drs2Δ* and *drs2Δ any1Δ* were equally sensitive to PapA and equally sensitive to Duramycin (Fig. 4A, B). We further examined membrane asymmetry in WT, *dnf1Δ dnf2Δ*, and *dnf1Δ dnf2Δ any1Δ* cells. Again, no significant difference in sensitivity to the toxins was observed with or without Any1 (Fig. 4C, D). In summary, *any1Δ* suppresses growth and membrane asymmetry defects caused by hypomorphic *neo1* alleles and bypasses the essential function of Neo1 in a Drs2-dependent manner. *any1Δ* modestly suppresses the cold-sensitive growth defect of *drs2Δ* but does not enhance the ability of the remaining P4-ATPases to restore PS/PE asymmetry in these cells. Likewise, *any1Δ* does not enhance the ability of remaining P4-ATPases to restore membrane asymmetry defects caused by *dnf1,2Δ*.

#### Any1 overexpression is toxic to P4-ATPase mutants

We also tested whether the overexpression of Any1 would perturb cell growth and/or alter membrane organization, as would be expected if it inhibits Neo1 and/or is a scramblase. *ANY1* was placed under transcriptional control of the *GAL* promoter (*P<sub>GAL</sub>*), which is repressed by glucose and strongly induced by galactose. This *P<sub>GAL</sub>-ANY1* construct was ex-

pressed in WT and *neo1-2 any1Δ* strains, and the growth on galactose or glucose media was examined over a range of temperatures (Fig. 5A). Any1 overexpression caused a mild growth defect in WT cells relative to the empty vector control. In contrast, *neo1-2* was very sensitive to Any1 overexpression and failed to grow at any temperature on the galactose plates. The robust growth of *neo1-2 any1Δ* on glucose plates as well as galactose plates with empty vector at high temperatures shows the strong suppression conferred by the absence of Any1. The deletion of *ANY1* also suppresses growth defects caused by mutations in the Neo1-interacting proteins Dop1 and Mon2 (Figs. 1A, 5A). *dop1-1* is temperature-sensitive for growth, but the *dop1-1 any1Δ* double mutant (empty vector) grows well at 37°C (47) (Fig. 5A). Similarly, *mon2Δ* mutants grow slowly, but this phenotype is suppressed in *mon2Δ any1Δ* strains (47). Like *neo1-2*, the growth of *dop1-1* and *mon2Δ* is severely inhibited by Any1 overexpression (Fig. 5A). We also found that the growth of *drs2Δ* and *dnf1,2Δ* strains is inhibited by Any1 overexpression (Fig. 5B), but not as potently as with *neo1*, *dop1*, and *mon2* mutants. Thus, cells deficient for Neo1/Dop1/Mon2 function are most sensitive to changes in Any1 levels within the cell.

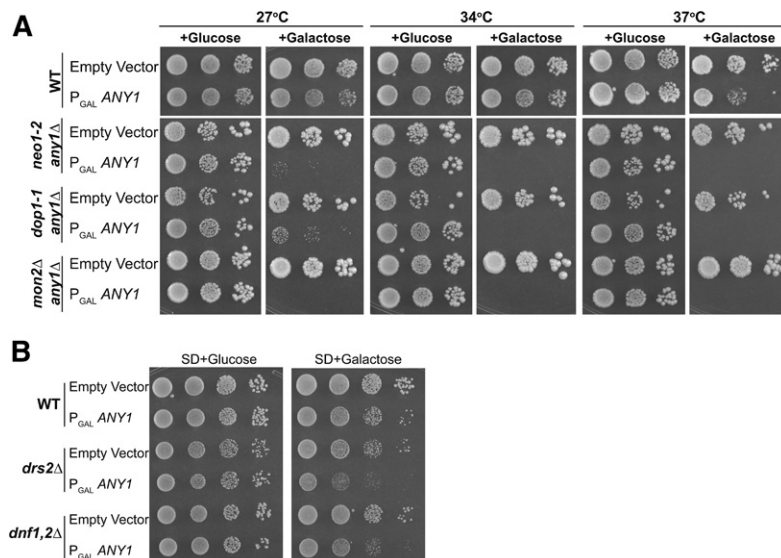


**Fig. 4.** Deletion of *Any1* failed to suppress the loss of PS and PE asymmetry in *drs2Δ* or *dnf1,2Δ* mutants, respectively. The strains indicated were grown with increasing concentrations of Papa A (A, C) or Duramycin (B, D) to assess the exposure of PS or PE, respectively. Growth (OD<sub>600</sub>) after 20 h at 30°C is plotted relative to untreated WT cells ( $n \geq 4$ ; error bars indicate  $\pm$  SEM).

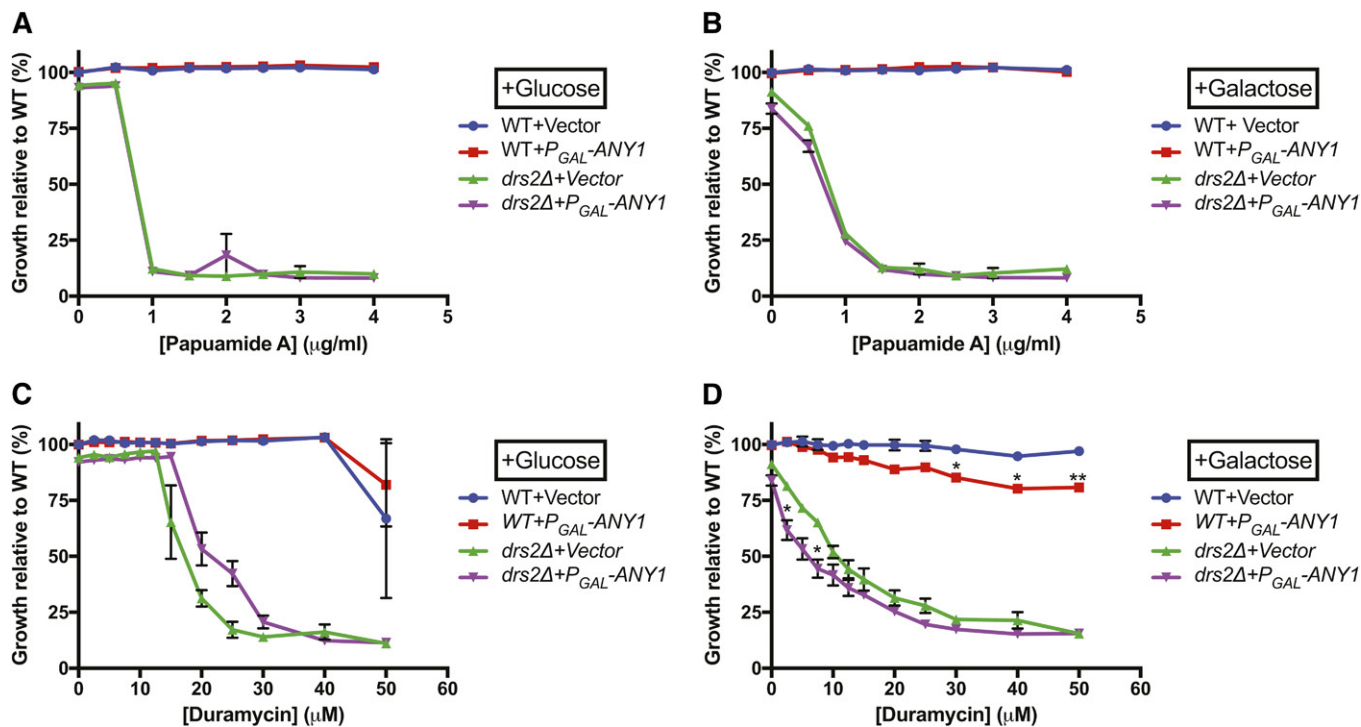
If *Any1* were functioning as a scramblase, we might expect that overexpression would cause a loss of membrane asymmetry. However, WT and *drs2Δ* showed no difference in papuamide B sensitivity between cells harboring *P<sub>GAL</sub>-ANY1* and empty vector grown on galactose (Fig. 6A, B). WT cells harboring *P<sub>GAL</sub>-ANY1* displayed a small increase in sensitivity to Duramycin, although no significant difference was observed for *drs2Δ* cells with or without *ANY1* overexpression (Fig. 6C, D). These experiments suggest that if *Any1* is a scramblase or phospholipid transporter of some type, it may be specific for PE.

#### Interaction between *Neo1* and *Any1*

Two spontaneous point mutations, G80R and D84G, were identified in *Any1* that were capable of suppressing *mon2Δ* growth defects (47). Interestingly, we found that *any1-G80R* did not suppress *neo1Δ*, but *any1-D84G* suppressed *neo1Δ* as effectively as deleting the *ANY1* gene (Fig. 7A). We also overexpressed these *Any1* mutants in WT, *drs2Δ*, and *dnf1,2Δ* cells using the strong *GAL1* promoter. The *P<sub>GAL</sub>-ANY1* construct used in this experiment was on a different vector backbone than the construct used in Fig. 5 and caused a more substantial growth defect in WT cells



**Fig. 5.** *ANY1* overexpression partially inhibits the growth of *drs2Δ* and *dnf1,2Δ* mutants but is most detrimental to *neo1<sup>ts</sup>*, *dop1<sup>ts</sup>*, and *mon2Δ* mutants. A: *ANY1* overexpression completely inhibits the growth of *neo1<sup>ts</sup> any1Δ*, *dop1<sup>ts</sup> any1Δ*, and *mon2Δ any1Δ* mutants. Growth assays were performed with WT, *neo1<sup>ts</sup> any1Δ*, *dop1<sup>ts</sup> any1Δ*, and *mon2Δ any1Δ* mutants expressing empty vector or *pBY011-ANY1* (*P<sub>GAL</sub>-ANY1*) at 27, 34, and 37°C. B: *ANY1* overexpression partially inhibits the growth of *drs2Δ* and *dnf1,2Δ* mutants to a greater extent than WT cells. WT, *drs2Δ*, and *dnf1,2Δ* expressing empty vector or *pBY011-ANY1* (*P<sub>GAL</sub>-ANY1*) were spotted onto the minimal media plates containing glucose or galactose at 30°C. These images are representative of four independent growth assays.

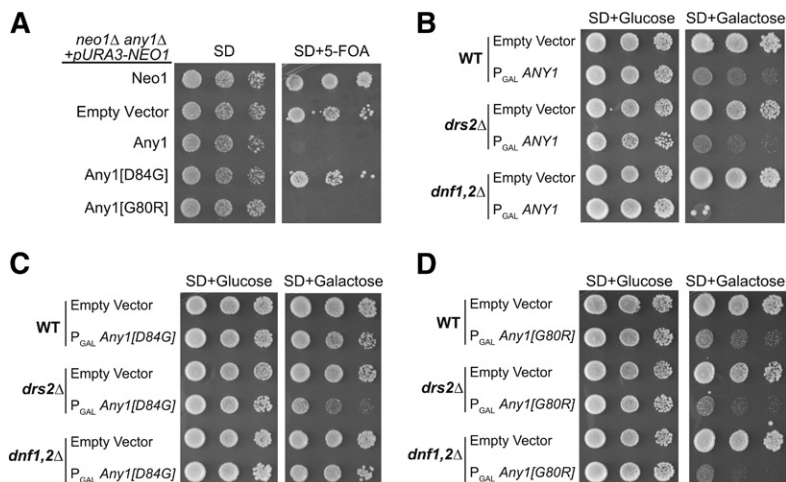


**Fig. 6.** *ANY1* overexpression induces a modest loss of PE asymmetry but does not increase PS exposure in WT or *drs2Δ* cells. A, B: *ANY1* overexpression does not induce a loss of PS asymmetry in WT and *drs2Δ* cells. C, D: *ANY1* overexpression induces a partial but significant loss of PE asymmetry in WT and *drs2Δ* cells. WT and *drs2Δ* cells expressing empty vector (*pRS416*) or *pRS416-P<sub>GAL</sub>-ANY1* were grown to mid-log phase in the minimal media containing glucose. Cells (0.2 OD<sub>600</sub>) were shifted to minimal medium containing galactose or glucose (control) in the presence of pore-forming toxins PapA or Duramycin. Growth relative to the vehicle control was plotted. Student's *t*-test was performed for each concentration tested between WT and *drs2Δ* cells ( $n \geq 2$ ).

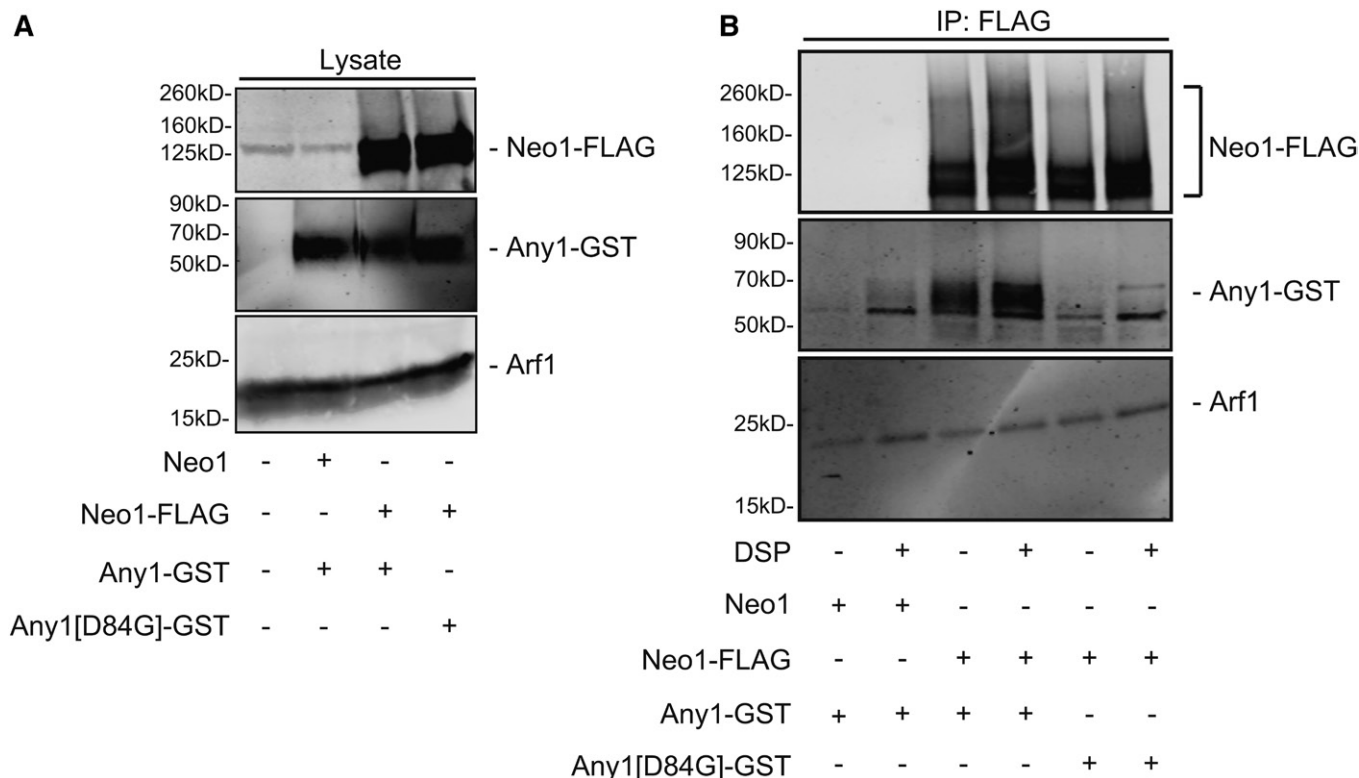
grown on galactose (compare Fig. 7B to 5B). This is likely due to slight differences in expression between the two constructs. In contrast, *P<sub>GAL</sub>-ANY1[D84G]* caused only a minor growth defect in WT and *dnf1,2Δ* cells but was still able to perturb the growth of *drs2Δ* cells. Again, the influence of *P<sub>GAL</sub>-ANY1[G80R]* expression on the growth of these strains was indistinguishable from *P<sub>GAL</sub>-ANY1*. These results indicate D84G partially inactivates Any1 function in suppressing the growth of WT or *dnf1,2Δ* cells, while the G80R variant is fully functional in these assays. We suspect that the *drs2Δ*

cells are more sensitive to Neo1 inhibition by Any1, which is why Any1[D84G] is still capable of inhibiting the growth of *drs2Δ*.

To test for a physical interaction between Any1 and Neo1, we immunoprecipitated Neo1-5XFLAG from cells expressing either Any1-GST or Any1[D84G]GST (58). The Any1-GST and Any1[D84G]GST variants were expressed at comparable levels in these cells (Fig. 8A). An interaction between Neo1 and Any1 can be clearly detected in the immunoprecipitates from Neo1-FLAG cells relative to the



**Fig. 7.** Effect of Any1 point mutations on its ability to antagonize Neo1 and inhibit the growth of P4-ATPase mutants. A: Any1[D84G] suppressed *neo1Δ* lethality, whereas Any1[G80R] inhibited growth comparably to WT *ANY1*. Plasmid shuffling assays were performed with *neo1Δ any1Δ* + *pRS416-NEO1* (MTY10) expressing single copy NEO1 (*pRS313-NEO1*), empty vector (*pRS313*), ANY1 (*pRS313-ANY1*), ANY1[D84G] (*pRS313-Any1[D84G]*) or ANY1[G80R] (*pRS313-Any1[G80R]*); each strain was then spotted onto minimal media plates (SD) and the *pRS416-NEO1* plasmid was counter selected on 5-fluorotic acid (5-FOA). B: Overexpression of *ANY1* using *pRS416-P<sub>GAL</sub>-ANY1* construct inhibits the growth of WT, *drs2Δ*, and *dnf1,2Δ* mutants. WT, *drs2Δ*, and *dnf1,2Δ* expressing *pRS416* (empty vector) or *pRS416-P<sub>GAL</sub>-ANY1* were spotted onto the minimal media plates containing glucose or galactose at 30°C. C, D: Growth inhibition due to *ANY1* overexpression is suppressed by the D84G mutation but not the G80R mutation.



**Fig. 8.** Any1 physically interacts with Neo1, and the [D84G] mutation abolishes this interaction. **A:** Expression of Neo1-FLAG, Any1-GST, and Any1[D84G]-GST in cells used for coimmunoprecipitation experiments. *neo1Δ any1Δ* (MTY10S) cells expressing *pRS315-NEO1* or *pRS315-NEO1-5XFLAG* along with *pRS313-ANY1-GST* or *pRS313-Any1[D84G]-GST* were lysed in SDS/urea and probed for Neo1-FLAG and Any1-GST expression. **B:** Any1 interacts with Neo1. *neo1Δ any1Δ* (MTY10S) cells expressing *pRS315-NEO1* or *pRS315-NEO1-5XFLAG* along with *pRS313-ANY1-GST* or *pRS313-Any1[D84G]-GST* were spheroplasted and lysed in the presence of detergent. Immunoprecipitations were performed using these total lysates in the presence or absence of the DSP cross-linker. Immunoprecipitations were also probed for Arf1 as a negative control. Immunoprecipitations were performed two independent times.

control sample from cells lacking the 5XFLAG tag (Fig. 8B). Strikingly, the inactivating D84G mutation completely abrogated the Neo1-Any1 interaction. A minor fraction of total Any1 in the cell immunoprecipitated with Neo1-FLAG, and the recovery of Any1 was only slightly enhanced by the addition of a membrane-permeable cross-linker (DSP) to the cells prior to lysis and immunoprecipitation. We also probed these Neo1-FLAG immunoprecipitates with anti-Arf1 as an additional control and found no significant difference in the background binding of Arf1 to the beads with or without Neo1-FLAG (Fig. 8B). Thus, we identify a weak but significant association between Neo1 and Any1 that is disrupted by the D84G mutation in Any1.

## DISCUSSION

Regulating membrane asymmetry requires a complex network of membrane remodeling machineries (Fig. 1A). P4-ATPases are membrane remodelers that generate a steep gradient of specific glycerophospholipids in biological membranes. Prior studies have shown that the Golgi P4-ATPases Neo1 and Drs2 execute nonredundant biological functions despite their apparently similar substrate preferences and localizations, and the PQ-loop protein Any1 somehow enforces these separate functions (39). In this

study, we found that PS flippase activity is essential in supporting the viability of *neo1Δ drs2Δ any1Δ* cells. Even a Dnf1<sup>PS+</sup> variant (Dnf1[N550S]) is capable of providing the PS flippase activity required for viability in this background, while WT Dnf1 or a Drs2<sup>PS-</sup> variant cannot. While none of the flippases can fully restore PS or PE asymmetry in *neo1Δ drs2Δ any1Δ* mutant cells, Drs2 and Neo1 establish an equivalent degree of PS asymmetry. In contrast, Neo1 displays a greater ability than Drs2 or Dnf1<sup>PS+</sup> in preventing PE exposure in the extracellular leaflet in this background.

Our data show that PS flipping by a P4-ATPase in the Golgi/endosomal system of budding yeast is essential for growth, yet strains lacking the sole PS synthase (*cho1Δ*) are viable. How do we explain this apparent discrepancy? We favor the idea that PS in the luminal leaflet antagonizes vesicle budding from Golgi membranes, while the translocation of this negatively charged phospholipid and its concentration in the cytosolic leaflet promotes vesicle budding. Failure to flip PS will increase luminal leaflet PS and reduce cytosolic leaflet PS, with both effects likely contributing to the strong trafficking defect observed in *drs2* and *neo1* mutants (37–41). The *cho1Δ* mutant would obviously lack the luminal leaflet PS, thus relieving negative effects, and compensatory changes in the lipidome to this perturbation might provide sufficient anionic phospholipids in the cytosolic leaflet to support vesicular transport, which



appears normal in this mutant (40). In addition, the transport of other phospholipid substrates by Drs2 and Neo1 may increase significantly in *cho1Δ* cells in the absence of competition by PS, providing another compensatory mechanism to support vesicular transport.

We have previously shown a requirement for PS flipping to support protein trafficking in the Golgi/endosomal system using the same Drs2<sup>PS-</sup> and Dnf1<sup>PS+</sup> variants described here (38). PS flipping increases the positive curvature (bending into the cytosol) and negative charge needed to recruit the ArfGAP Gcs1 via its ArfGAP lipid-packing sensor motif onto Golgi/endosomal membranes from the cytosol (38). In mammalian cells, PS flipping by ATP8A1 or ATP8A2 facilitates the recruitment of EHD1, a membrane fission factor, to the cytosolic leaflet of recycling endosomes (59). Thus, the concentration of PS in the cytosolic leaflet has positive roles in vesicle budding. A negative role for PS in the luminal leaflet is supported by the observation that endocytic recycling defects in the *C. elegans tat-1* mutant (orthologue of Drs2/ATP8A1/ATP8A2) are suppressed by the depletion of PS synthase (60). This result suggests that the accumulation of PS in the luminal leaflet of *tat-1* endosomes inhibits vesicle budding and the depletion of PS can relieve this inhibition. In budding yeast, *drs2Δ cho1Δ* cells grow very poorly and transport defects are not suppressed (40), suggesting that the loss of PS in the luminal leaflet is insufficient to overcome the deficit in the cytosolic leaflet in this case.

The loss of Any1 has been shown previously to suppress deficiencies in Neo1 and Drs2/Dnf P4-ATPases (47, 49). Yamamoto et al. (49) found that *any1Δ* (also called *cfs1Δ*) suppressed *drs2Δ* and *cdc50Δ* cold-sensitive growth and the synthetic lethality of *lem3Δ cdc50Δ crf1Δ*, a strain deficient for the β-subunits needed for the activity of Dnf1-Lem3, Dnf2-Lem3, Dnf3-Crf1, and Drs2-Cdc50 heterodimers (supplemental Table 2). On the contrary, we found that the loss of Any1 failed to suppress the *dnf1,2,3Δ drs2Δ* synthetic lethality, a mutant that should be equivalent to *lem3Δ cdc50Δ crf1Δ*. This raises the possibility that *lem3Δ cdc50Δ crf1Δ* cells retain some residual activity of P4-ATPase α-subunits to allow suppression by *any1Δ/cfs1Δ*. However, it is also possible that some minor difference in the strain background was responsible for the different results, particularly considering that *lem3Δ cdc50Δ crf1Δ any1Δ* cells grew very slowly (49). We also found that *neo1* alleles are synthetically lethal with *lem3Δ*, but *neo1Δ any1Δ lem3Δ* cells grow similarly to WT and must rely on Drs2 for growth. In total, these genetic interactions suggest that Any1 segregates the functions of multiple P4-ATPases and eliminating Any1 allows Drs2, in particular, to carry out the essential function of the P4-ATPase group.

The deletion of *KES1* or *ANY1* similarly suppresses the growth and trafficking defects of *drs2Δ/cdc50Δ* mutants (30, 31), raising the possibility that Any1 and Kes1 are performing comparable tasks. *kes1Δ* mutants display a substantial increase in the anionic phosphatidylinositol-4-phosphate in the cytosolic leaflet, which is likely the reason *kes1Δ* suppresses *drs2* and *sec14*. *any1Δ* does not suppress *sec14* and *kes1* does not suppress *neo1* (49), suggesting that the mech-

anism for suppressing P4-ATPase deficiency is distinct for *any1Δ* and *kes1Δ*.

The biochemical function of Any1 remains enigmatic, although this study provides some insight into potential roles for this protein in membrane organization. PQ-loop proteins share this simple PQ motif in a cytosolic loop and a similar membrane topology with seven or eight transmembrane segments. Beyond this, the PQ-loop proteins share little sequence homology. The best-characterized PQ-loop proteins include the KDEL receptor, the sweet and semisweet sugar transporters in plants, and basic amino-acid transporters in lysosomes/vacuoles (61). The potential transporter activity of Any1 and its ability to antagonize Neo1 function led to the proposal that Any1 is a scramblase that disrupts the phospholipid gradient formed by Neo1. However, it is also possible that Any1 antagonizes Neo1 through protein interactions. Consistent with this latter possibility, Any1 and Neo1 were found to interact based on a coimmunoprecipitation assay. The observation that an inactivating mutation in the second transmembrane domain of Any1, D84G, causes a loss of interaction with Neo1 supports the specificity and physiological relevance of this interaction. In addition, the influence of *any1Δ* on membrane asymmetry is most profound when assayed with hypomorphic *neo1<sup>ts</sup>* alleles, which could be explained by the removal of a protein interaction that negatively regulates Neo1 flippase activity. Any1 overexpression is particularly toxic to *neo1<sup>ts</sup>* mutants and slightly increases PE exposure in the outer leaflet of WT cells, a result more consistent with the inhibition of Neo1 activity by Any1.

The potential role of Any1 as a negative regulator of Neo1 activity does not fully explain the role of this PQ-loop protein, as it seems to actively inhibit growth when *NEO1* is deleted. It could be that additional unknown protein interactions or a transport activity of Any1 becomes dysregulated in the absence of Neo1. Dop1 and Mon2 interact with Neo1 and are critical for the growth of yeast, but they have no known biochemical function. Potential interactions between Any1 and Dop1 or Mon2 have not yet been explored and could help to define the full nature of this regulatory network. If Any1 can scramble phospholipids as a monomer or homo-oligomer, we would expect to see a more substantial impact on membrane organization when highly expressed from the strong *GAL* promoter or disrupted in P4-ATPase-deficient backgrounds. However, it remains possible that Any1 is a scramblase if it is part of a hetero-oligomeric complex and/or tightly regulated. For example, the majority of Any1 expressed from the Gal promoter could be inactive if another subunit is required, or Any1 scramblase activity may be turned on only as a pressure-relief valve to counterbalance excessive flippase activity.

In summary, we identified Any1 as a key regulator that enforces separate functions for Drs2 and Neo1 in Golgi membranes. When Any1 is deleted, essential Golgi function can be provided by either Neo1 or variants of Drs2 or Dnf1 that are able to flip PS. This result emphasizes the importance of PS translocation for Golgi function and supports the possibility that Neo1 can flip PS. Our studies do not support the proposed scramblase function for Any1,

and we also provide evidence that Neo1 and Any1 form a complex in cells. However, further work is needed to determine whether this interaction directly inhibits Neo1 activity or if Any1 acts upstream of Neo1 to limit substrate availability (for both Neo1 and Drs2) or downstream of Neo1 to dissipate phospholipid gradients in the membrane. The possibility that Neo1 suppresses Any1 activity will also be an interesting avenue to pursue. **■**

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