

P4-ATPase Requirement for AP-1/Clathrin Function in Protein Transport from the *trans*-Golgi Network and Early Endosomes

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Drs2p is a resident type 4 P-type ATPase (P4-ATPase) and potential phospholipid translocase of the *trans*-Golgi network (TGN) where it has been implicated in clathrin function. However, precise protein transport pathways requiring Drs2p and how it contributes to clathrin-coated vesicle budding remain unclear. Here we show a functional codependence between Drs2p and the AP-1 clathrin adaptor in protein sorting at the TGN and early endosomes of *Saccharomyces cerevisiae*. Genetic criteria indicate that Drs2p and AP-1 operate in the same pathway and that AP-1 requires Drs2p for function. In addition, we show that loss of AP-1 markedly increases Drs2p trafficking to the plasma membrane, but does not perturb retrieval of Drs2p from the early endosome back to the TGN. Thus AP-1 is required at the TGN to sort Drs2p out of the exocytic pathway, presumably for delivery to the early endosome. Moreover, a conditional allele that inactivates Drs2p phospholipid translocase (flippase) activity disrupts its own transport in this AP-1 pathway. Drs2p physically interacts with AP-1; however, AP-1 and clathrin are both recruited normally to the TGN in *drs2Δ* cells. These results imply that Drs2p acts independently of coat recruitment to facilitate AP-1/clathrin-coated vesicle budding from the TGN.

INTRODUCTION

Clathrin mediates protein transport between the plasma membrane, endosomes, and TGN through association with pathway-specific adaptors that link integral membrane cargo proteins to the clathrin lattice during vesicle formation. In mammalian cells, the ubiquitously expressed heterotetrameric AP-1A clathrin adaptor, composed of γ , β 1, μ 1A, and σ 1-adaptins, appears to mediate both anterograde transport of proteins from the TGN to early endosomes, as well as the retrograde trip back to the TGN (Hanners and Tooze, 2003; Robinson, 2004). For its role in anterograde transport of lysosomal enzymes, AP-1A collaborates with monomeric adaptors called GGAs (Golgi localized, γ -ear-containing, Arf-binding proteins) to recruit the mannose-6-phosphate receptor into clathrin-coated vesicles (CCVs; Doray *et al.*, 2002). The AP-1B complex, which differs from AP-1A by an alternate μ 1B subunit, has a functionally distinct role in sorting cargo from the TGN to the basolateral membrane of polarized epithelial cells (Folsch *et al.*, 1999). In

budding yeast, there is evidence that AP-1 mediates early endosome to TGN retrograde transport (Valdivia *et al.*, 2002; Foote and Nothwehr, 2006), but no anterograde role for AP-1 has yet been described. The yeast GGAs appear to function independently of AP-1 to mediate delivery of cargo from the TGN to the late endosome (Black and Pelham, 2000). Deletion of genes encoding AP-1 subunits or GGAs has little consequence to growth of yeast. However, the combined deletion of AP-1 and GGA causes a severe growth defect, suggesting that these two adaptors mediate parallel transport pathways and that yeast cannot tolerate loss of both pathways (Costaguta *et al.*, 2001; Hirst *et al.*, 2001).

The mechanism of AP-1/clathrin-coated vesicle (AP-1/CCV) formation is incompletely understood. A commonly held view is that ARF-dependent recruitment of AP-1 and clathrin to membranes induces localized assembly of clathrin triskelions into a lattice, and the intrinsic curvature of the growing clathrin lattice provides sufficient energy for the membrane deformation needed for vesicle budding. Consistent with this possibility is the observation that clathrin and AP-1 in cytosolic fractions can induce vesicle formation from liposomes *in vitro*, suggesting that AP-1/CCVs can be produced without assistance from integral membrane proteins (Zhu *et al.*, 1999). However, it is not clear if CCVs bud *in vitro* from liposomes at a rate that could sustain protein trafficking pathways *in vivo*. Therefore, whether or not integral membrane proteins contribute to AP-1/CCV biogenesis *in vivo* remains an important unanswered question. In addition, it is possible that clathrin self-assembly alone does not provide enough energy to drive bending of biological membranes without assistance from accessory proteins (Nossal, 2001). The epsins, for example, are thought to facilitate membrane bending to assist clathrin in forming vesicles (Ford *et al.*, 2002).

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Abbreviations used: ARF, ADP-ribosylation factor; CCV, clathrin-coated vesicles; CW, calcofluor white; GEF, guanine nucleotide exchange factor; GGA, Golgi-localized, γ -ear-containing, ARF-binding protein; P4-ATPase, type IV P-type ATPase; PE, phosphatidylethanolamine; PS, phosphatidylserine; PVC, prevacuolar compartment.

Phospholipid translocases, or flippases, have substantial potential to bend membranes by coupling a source of energy (ATP) to the unidirectional translocation of phospholipid across the bilayer. Flippase action can create an imbalance in phospholipid number between the two leaflets and cause bending of the membrane in the direction of lipid translocation (Graham, 2004). A large body of evidence indicates that P4-ATPases, which include the yeast Drs2, Dnf1, Dnf2, Dnf3, and Neo1 proteins, catalyze unidirectional phospholipid translocation from the extracellular leaflet of the plasma membrane, or the topologically equivalent luminal leaflet of the Golgi and endosomes, to the cytosolic leaflet of these membranes (Tang *et al.*, 1996; Gomes *et al.*, 2000; Ujhazy *et al.*, 2001; Pomorski *et al.*, 2003; Natarajan *et al.*, 2004; Saito *et al.*, 2004; Alder-Baerens *et al.*, 2006). Although a flippase activity has not been reconstituted with a purified P4-ATPase, we have shown that inactivation of a temperature-conditional mutant form of Drs2p (Drs2-ts) present in purified TGN membranes immediately disrupts phospholipid translocase activity measured with a fluorescent phospholipid derivative (Natarajan *et al.*, 2004), supporting the view that Drs2p is a flippase. In these assays, Drs2p has a preferred substrate preference for phosphatidylserine (PS), but also appears to flip phosphatidylethanolamine (PE; Natarajan *et al.*, 2004; Alder-Baerens *et al.*, 2006). This Drs2-dependent flippase activity also appears to contribute to the asymmetric distribution of endogenous PS and PE to the cytosolic leaflet as *drs2Δ* mutants exhibit a loss of PS and PE asymmetry (Pomorski *et al.*, 2003; Chen *et al.*, 2006).

P4-ATPases also play essential roles in protein transport in the secretory and endocytic pathways (Chen *et al.*, 1999; Hua *et al.*, 2002; Hua and Graham, 2003; Pomorski *et al.*, 2003; Sakane *et al.*, 2006; Furuta *et al.*, 2007). For example, inactivation of Drs2-ts in vivo rapidly blocks formation of a clathrin-dependent class of post-Golgi vesicles carrying exocytic cargo (Gall *et al.*, 2002). These post-Golgi vesicles appear to be equivalent to one of the two classes of exocytic vesicles (the "higher density" class) first described by Harsay and Bretscher (1995). Formation of these exocytic vesicles is only mildly perturbed by deletion of AP-1 subunits (Gall *et al.*, 2002); therefore, they likely differ from AP-1/CCVs proposed to mediate transport between the TGN and endosomes. Other data linking Drs2p to clathrin function include their colocalization to the TGN, a strong synthetic lethal relationship between *drs2*, *arf1*, and *chc1* (clathrin heavy chain) alleles, and *drs2* mutant phenotypes such as the accumulation of enlarged Golgi cisternae, a deficiency of CCVs, and the mislocalization of TGN resident proteins. These phenotypes are similar to what is observed in clathrin mutants (Chen *et al.*, 1999).

These observations are consistent with a role for Drs2p in budding CCVs. However, because the relationship of Drs2p to well-known AP-1 and GGA clathrin adaptors has not been characterized, the precise role of Drs2p in the function of clathrin and its adaptors in the TGN–endosomal system is unclear. Clathrin mediates multiple transport pathways and so this information is essential to test if Drs2p contributes to vesicle biogenesis by helping recruit specific clathrin adaptor components to membranes or through a novel mechanism. Here we demonstrate a unique functional codependence between Drs2p and AP-1. Drs2p requires AP-1 for its normal trafficking itinerary, and the enzymatic activity of this integral membrane protein is essential for AP-1/clathrin function. This work provides the first example a clathrin accessory factor essential for AP-1 function that acts independently of coat recruitment, and also suggests that an

ATP-powered pump provides a critical source of energy to drive AP-1/CCV formation from biological membranes.

MATERIALS AND METHODS

Media and Strains

Yeast strains were grown in standard rich medium (YPD) or synthetic defined (SD) minimal media. Calcoflour white (CW) sensitivity was tested on YPD 2% agar plates containing 100 μg/ml CW (Sigma-Aldrich, St. Louis, MO). Yeast strains used in this study were generated by standard genetic crosses, gene disruption, or integration methods as previously described and are listed in Supplementary Table 1. The yeast knockout collection and GFP collection were purchased from Invitrogen (Carlsbad, CA). To prevent accumulation in the endoplasmic reticulum, pGFP-DRS2 was cotransformed into yeast strains with a multicopy vector carrying CDC50 (pRS425-CDC50), encoding a chaperone for Drs2p. Strains carrying pChs3-GFP also carried a plasmid overexpressing the CHS7 chaperone (pCRP11). pGFP-*drs2-12* (encoding GFP-Drs2-ts) was constructed by replacing the AgeI-ClaI fragment of pGFP-DRS2 with the AgeI-ClaI fragment from pRS313-*drs2-12*. To create pGFP-TLG1, genomic DNA was amplified with primers Tlg1-F (GTCAGTCCGACAACAACAGTGAAGATCCGTTTC) and Tlg1-R (ACTGGTCGACTCAAGCAATGAATGCCAAACTA), and the product was digested with Sall and subcloned into the Sall site of pRS416-GFP. All plasmids used in this study are listed in Supplementary Table S2.

Microscopy

To visualize green fluorescent protein (GFP) or DsRed-tagged proteins, cells were grown to early to mid-logarithmic phase, harvested, and resuspended in imaging buffer (10 mM Tris-HCl, pH 7.4, 2% glucose). Cells were mounted on glass slides and observed immediately at room temperature as previously described. Chitin staining was performed with cells incubated in 1 mg/ml CW in water for 5 min and washed three times before imaging. To inhibit endocytosis, midlog phase cells were collected and resuspended in SD medium containing 200 μM latrunculin A (Lat A). Brefeldin A (Sigma-Aldrich) was dissolved in ethanol at 10 mg/ml and used to treat cells at 100 μg/ml in imaging buffer.

Coimmunoprecipitation of AP-1 and Drs2p

For chemical cross-linking experiments, 50 OD₆₀₀ units of each strain were incubated in 5 ml softening buffer (100 mM Tris-HCl, pH 9.4, 10 mM DTT) at 30°C for 10 min and then converted to spheroplasts by incubating in 1 ml spheroplast buffer (1 M sorbitol, 0.5% glucose, 10 mM KH₂PO₄, pH 7.0, 200 μg/ml Zymolase) at 30°C for 30 min. Spheroplasts were washed three times with cross-linking buffer (1 M sorbitol, 0.15 M NaCl, 0.1 M KH₂PO₄, pH 7.2), resuspended in cross-linking buffer supplemented with either 1 or 2 μM dithiobis(succinimidyl)propionate (DSP, 20 mg/ml stock in dimethylsulphoxide; Pierce, Rockford, IL) and incubated at room temperature for 30 min. Cells were then pelleted, resuspended in 1 ml ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% CHAPS, 1× complete protease inhibitor cocktail lacking EDTA; Roche Diagnostics, Basel, Switzerland, and 1 mg/ml BSA), and incubated on ice for 15 min. The cell lysis was cleared by incubation with 30 μl protein G Sepharose (50% suspension, Amersham Biosciences, Piscataway, NJ) at 4°C for 30 min and centrifugation at 20,000 × g for 20 min. The supernatant was incubated with 9 μg monoclonal anti-hemagglutinin (HA-7, Sigma-Aldrich) or anti-Myc (9E10, Sigma-Aldrich) antibodies for 1.5 h at 4°C and subsequently with 30 μl protein G Sepharose for 1.5 h at 4°C. The protein G Sepharose beads were washed three times with washing buffer I (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 350 mM NaCl, 1% Tween-20, 1× complete protease inhibitor cocktail lacking EDTA) and twice with washing buffer II (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1× complete protease inhibitor cocktail lacking EDTA). Bound material was eluted with SDS/urea sample buffer, separated by SDS-PAGE, and subjected to immunoblotting as previously described (Chen *et al.*, 1999).

RESULTS

Drs2p is Required for AP-1 Function in TGN-early Endosome Pathways

Prior studies implicated Drs2p in protein transport between the TGN and early endosome, suggesting a primary role for Drs2 in AP-1/clathrin function rather than the late endosome pathway mediated by GGAs (Chen *et al.*, 1999). In budding yeast, simultaneous inactivation of both AP-1 and GGA function causes a severe synthetic growth defect (Costaguta *et al.*, 2001; Hirst *et al.*, 2001). If Drs2p is required for AP-1 function, a *drs2* null allele (*drs2Δ*) should cause a strong synthetic growth defect when combined with null

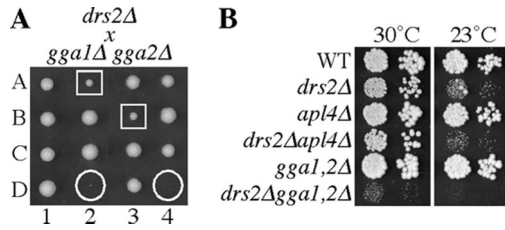


Figure 1. Synthetic growth defect between *drs2Δ* and *gga* alleles suggests that Drs2 acts in AP-1 pathways. (A) Tetrads derived from a *drs2Δ* cross with *gga1Δ gga2Δ*. Circles indicate *drs2Δ gga1Δ gga2Δ* triple mutants, and boxes are *drs2Δ gga2Δ* double mutants (tetrads, 1–4; spores, A–D). The unmarked WT and single mutant progeny grow equally well. (B) Growth of *drs2Δ apl4Δ* (γ -adaptin) and *drs2Δ gga1Δ gga2Δ* strains. Tenfold serial dilutions of strains BY4742 (WT), ZHY615M2D (*drs2Δ*), BY4742 YPR029C (*apl4Δ*), KLY691 (*drs2Δ apl4Δ*), KLY751 (*gga1Δ gga2Δ*), and KLY741 (*drs2Δ gga1Δ gga2Δ*) were spotted onto YPD plates and grown at 30 and 23°C.

alleles of the two *GGA* genes (*gga1Δ* and *gga2Δ*), comparable to the poor growth of an *apl4Δ* (γ -adaptin) *gga1Δ gga2Δ* strain. In addition, deletion of an AP-1 subunit should have no additional consequence to a *drs2Δ* strain if AP-1 function is already lost in this strain. To test these predictions, we crossed *drs2Δ* with *gga1Δ gga2Δ* and *apl4Δ* and performed tetrad analyses. The *drs2Δ gga1Δ gga2Δ* triple mutant progeny were either inviable or grew extremely slowly. In addition, the *drs2Δ gga2Δ* double mutants grew slower than the single mutant or WT progeny (Figure 1, A and B). By contrast, the *drs2Δ apl4Δ* mutant exhibited a growth rate identical to the *drs2Δ* single mutant, even at 23°C, a semipermissive temperature for growth of cold-sensitive *drs2Δ* strains (Figure 1B). These results suggest that Drs2p acts in parallel with GGAs and functions in the same pathway as AP-1.

A well-defined role for AP-1 in yeast is in the trafficking of chitin synthase III (Chs3p) between the early endosomes and TGN (Valdivia *et al.*, 2002), and so we tested if Drs2p is required in this pathway. Chs3p deposits a ring of chitin around emerging buds that remains as a scar after the bud is released. Most Chs3p is retained intracellularly by AP-1-dependent transport between the TGN and early endosome, but a portion is diverted to the plasma membrane in a cell cycle-regulated process that requires the Chs5p and Chs6p coat complex (Trautwein *et al.*, 2006; Wang *et al.*, 2006). Thus, *chs6Δ* cells retain nearly all Chs3p intracellularly and have reduced chitin incorporation at the bud site. This makes *chs6Δ* cells resistant to the toxic effects of CW, a chitin-binding fluorescent compound (Figure 2A). In the *chs6Δ* background, deletion of any AP-1 subunit disrupts intracellular Chs3p retention and allows cell surface transport of Chs3p in constitutive exocytic vesicles, restoring the appearance of chitin rings and CW sensitivity to an AP-1 *chs6Δ* double mutant (Valdivia *et al.*, 2002). Like the *chs6Δ apl4Δ* strain, the *chs6Δ drs2Δ* double mutant failed to grow on the medium containing CW (Figure 2A) and formed chitin rings indistinguishable from those in the WT and *drs2Δ* cells (Figure 2B).

As a cargo of the AP-1-mediated early endosome to TGN retrograde pathway, Chs3p normally avoids transport to the late endosome, but transits this compartment frequently in AP-1 mutants (Valdivia *et al.*, 2002). This perturbation is revealed in a class E *vps* AP-1 double mutant (*apl4Δ vps27Δ*), where Chs3p is trapped in enlarged endosomal compartments adjacent to the vacuole (class E compartment) that also accumulate the endocytic tracer FM4-64. As in *apl4Δ vps27Δ* cells, Chs3p accumulates in the class E compartment

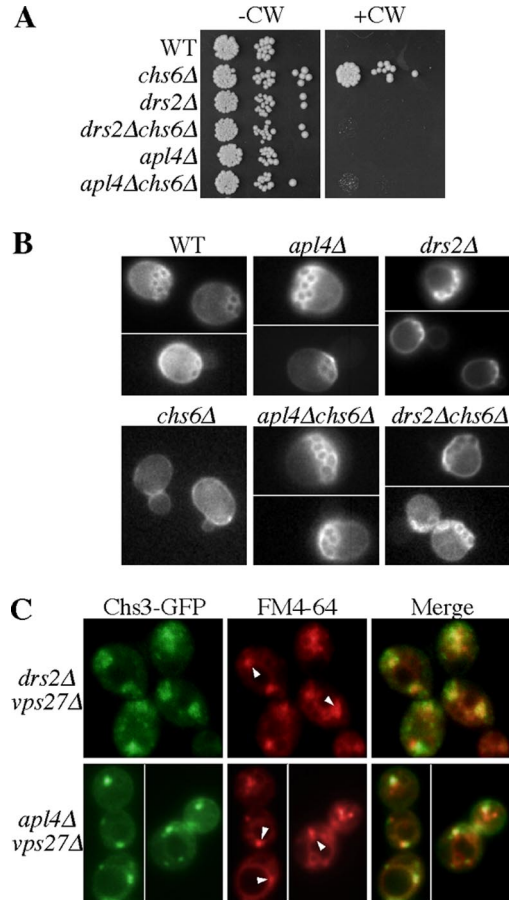


Figure 2. Drs2p is required for AP-1-dependent retrograde transport of Chs3p. (A) Loss of Drs2p or AP-1 restores CW sensitivity to *chs6Δ* cells. Tenfold serial dilutions of strains BY4742 (WT), ZHY615M2D (*drs2Δ*), BY4742 YPR029C (*apl4Δ*), BY4742 YJL099W (*chs6Δ*), KLY022 (*drs2Δ chs6Δ*), and KLY492 (*apl4Δ chs6Δ*) were spotted onto rich medium containing 100 μ g/ml CW and incubated at 30°C. (B) Loss of Drs2p or AP-1 restores chitin-rich bud scars to *chs6Δ* cells. Cells (same as in A) were stained with CW and visualized by fluorescence microscopy. (C) Loss of Drs2p or AP-1 causes missorting of Chs3-GFP to the prevacuolar compartment (arrowheads) of *vps27Δ* cells. Cells (KLY782 and KLY711) expressing Chs3-GFP were stained with FM4-64 to mark the prevacuolar compartment as previously described (Liu *et al.*, 2007).

of *drs2Δ vps27Δ* cells (Figure 2C). The class E compartment in *drs2Δ vps27Δ* cells has a larger and more diffuse morphology than in *vps27Δ* or *apl4Δ vps27Δ* cells. However, the *drs2Δ vps27Δ* and *vps27Δ* cells exhibit a similar defect in the kinetics of FM4-64 delivery to the vacuole, and so the *drs2Δ* mutation, although altering the class E compartment morphology, does not suppress the *vps27Δ* FM4-64 trafficking defect. In summary, the data shown in Figure 2 indicates that loss of Drs2p disrupts Chs3p trafficking in a manner similar to loss of AP-1.

Missorting of Drs2p to the Plasma Membrane of AP-1 Mutants

Our previous work suggested that Drs2p primarily traffics between the TGN and early endosome in order to maintain its TGN localization (Liu *et al.*, 2007). To test if Drs2p localization requires AP-1, we first examined the distribution of GFP-Drs2p relative to the TGN marker Sec7-RFP in WT and

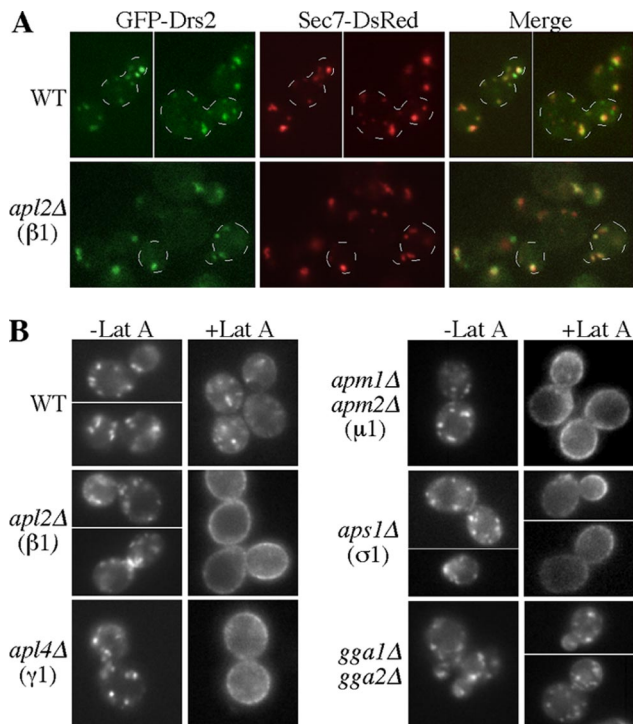


Figure 3. Loss of AP-1 perturbs Drs2p trafficking but not its steady-state TGN localization. (A) Colocalization of GFP-Drs2 and Sec7-DsRed at the TGN of WT (KLY1101) and AP-1-deficient (*apl2Δ*) cells (KLY1351). Some of the cells are outlined for clarity. (B) Disruption of AP-1 subunit genes causes missorting of GFP-Drs2p to the plasma membrane, where accumulation was induced by blocking endocytosis with latrunculin A (Lat A) at room temperature for 30 min. WT (BY4741) and AP-1 mutant cells (BY4741 derivatives) were treated with Lat A and imaged as previously described (Liu *et al.*, 2007).

AP-1-deficient cells (Figure 3A). GFP-Drs2p localized to several discrete puncta in WT cells, and this pattern was not altered in cells lacking AP-1 (*apl2Δ*). Furthermore, 81% of Drs2p-GFP puncta were coincident with Sec7-DsRed puncta in WT cells, whereas 82% of Drs2p-GFP puncta colocalized with Sec7-DsRed puncta in *apl2Δ* cells. Therefore, the steady-state localization of GFP-Drs2p to the TGN is not noticeably perturbed by AP-1 disruption.

We then addressed whether deletion of AP-1 has an effect on trafficking of GFP-Drs2p. Acute inhibition of endocytosis by treating cells with Lat A, an inhibitor of actin assembly that blocks endocytosis in yeast, causes a slow accumulation of GFP-Drs2p on the plasma membrane over the course of about 3 h. This indicates a relatively rare incorporation of Drs2p into transport vesicles targeted to the plasma membrane. In addition, Drs2p contains multiple endocytosis signals, and so any Drs2p molecules that are transported to the plasma membrane are rapidly retrieved through endocytosis (Liu *et al.*, 2007). If GFP-Drs2p requires AP-1 for TGN to early endosome transport, we would expect an increased frequency of Drs2p transport to the plasma membrane in an AP-1 mutant. To test this, we treated WT and AP-1 mutant strains expressing GFP-Drs2p with Lat A to inhibit endocytosis and imaged the cells over time (Figure 3B). In WT cells, GFP-Drs2p was primarily retained intracellularly in small puncta after 30 min of Lat A treatment. In stark contrast, deletion of the AP-1 β subunit (*apl2Δ*), γ subunit (*apl4Δ*), or σ subunit (*aps1Δ*) caused accumulation of nearly all GFP-

Drs2p on the plasma membrane within 30 min of blocking endocytosis with Lat A.

Cell surface accumulation of GFP-Drs2p was less pronounced in *apm1Δ* and nearly absent in *apm2Δ* cells treated with Lat A. These mutants harbor deletions of the partially redundant AP-1 μ 1 subunits (unpublished observations). However, deletion of both *APM1* and *APM2* leads to plasma membrane accumulation comparable to deletion of other AP-1 subunits (*apm1Δ apm2Δ*; Figure 3B). These data indicate that Drs2p trafficking primarily relies on Apm1p, although Apm2p can partially contribute. The *gga1Δ gga2Δ* strain was also treated with Lat A, and these cells slowly accumulated GFP-Drs2p on the plasma membrane, similarly to WT cells (Figure 3B). We conclude that AP-1 is required, and GGAs dispensable, for efficient exclusion of Drs2p from exocytic vesicles targeted to the plasma membrane. This likely reflects a role for AP-1 in transporting Drs2p from the TGN to early endosomes; however, see *Discussion* for an alternative model.

Missorting of Drs2p to the plasma membrane of AP-1 null cells could be an indirect consequence of chronically perturbing protein transport between the early endosomes and TGN. To distinguish chronic versus acute effects of AP-1 inactivation on Drs2p trafficking, an *apl2* temperature-sensitive (*ts*) mutant was shifted to the nonpermissive temperature (37°C) to inactivate AP-1 just before Lat A addition. The *apl2-ts* strain accumulated GFP-Drs2p on the plasma membrane within 30 min at 37°C, whereas Lat A-treated cells maintained at the permissive temperature (24°C) or WT cells shifted to 37°C retained significant internal punctate fluorescence (Figure 4A). For other potential anterograde AP-1 cargos tested, a minor fraction of Chs3-GFP but no Kex2-GFP or GFP-Tlg1 accumulated on the plasma membrane of Lat A-treated *apl4Δ* cells (Figure 4B and unpublished observations). Thus, missorting of Drs2p to the plasma membrane is a specific and immediate consequence of AP-1 inactivation.

To rule out the possibility that missorting of GFP-Drs2p to the plasma membrane is a secondary consequence of inactivating AP-1 retrograde function, we examined the trafficking of GFP-Drs2p in two other mutants that display a defect in the retrograde pathway. The Snx4-Snx41-Snx42 sorting nexin complex and the F-box protein Rcy1p function in early endosome to TGN retrograde transport (Wiederkehr *et al.*, 2000; Hettema *et al.*, 2003). However, disruption of *RCY1* only slightly increased GFP-Drs2p accumulation on the plasma membrane upon addition of Lat A, whereas disruption of *SNX4* has no apparent impact on Drs2p trafficking (Figure 4C). Therefore, wholesale missorting of GFP-Drs2p to the plasma membrane in AP-1-deficient cells is not a secondary consequence of disrupting early endosome to TGN retrograde transport.

The observation that AP-1 disruption rerouted Drs2p to the plasma membrane without substantially perturbing its steady-state localization to the TGN, suggested that endocytosis and early endosome to TGN transport of GFP-Drs2p was unaffected. To test if AP-1 disruption perturbs retrograde transport of Drs2p similarly to Chs3p, localization of GFP-Drs2p was monitored in *apl4Δ vps27Δ* cells. In contrast to Chs3-GFP (shown in Figure 2), GFP-Drs2p did not accumulate in the class E compartment marked with FM4-64 (Figure 5A). In addition, GFP-Drs2p was efficiently endocytosed from the plasma membrane and transported to the TGN in AP-1-deficient cells. To show this, the *apl2Δ* strain expressing GFP-Drs2p and Sec7-DsRed was treated with Lat A to accumulate GFP-Drs2p on the plasma membrane. After washing out the Lat A to lift the endocytosis block, GFP-

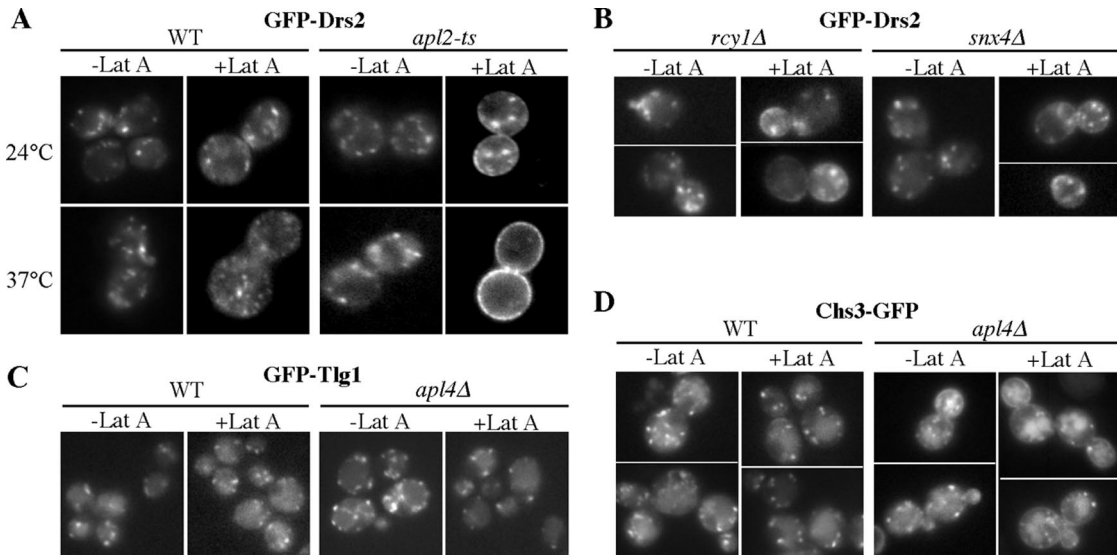


Figure 4. GFP-Drs2p missorting to the plasma membrane is an immediate and specific consequence of AP-1 deficiency. (A) GFP-Drs2p is missorted to the plasma membrane of an *apl2-ts* (β 1-adaptin) strain preincubated 30 min at the nonpermissive temperature (37°C) before Lat A addition. Partial plasma membrane accumulation of GFP-Drs2p was also observed at the permissive temperature suggesting a partial loss of AP-1 function at 24°C. Lat A incubations were for 30 min at the indicated temperature. (B) Localization of GFP-Drs2 in *rcy1Δ* and *snx4Δ* cells treated with Lat A for 30 min at 30°C. Localization of GFP-Tlg1 (C) and Chs3-GFP (D) in WT and AP-1-deficient (*apl4Δ*) cells treated with Lat A as in B.

Drs2 efficiently returned to Sec7-DsRed-marked TGN cisternae (Figure 5B). In summary, these data indicate that Drs2p requires AP-1 at the TGN for exclusion from exocytic vesicles. However, Drs2p can be retrieved from early endosomes and transported to the TGN in the absence of AP-1.

Drs2p Activity Drives Its Own Transport in the AP-1 Pathway

Drs2p is the only cargo thus far characterized in yeast that depends solely on AP-1 in what appears to be a TGN-to-endosome anterograde pathway. To determine if Drs2p ac-

tivity is required for the function of AP-1 in this pathway, we examined the trafficking of a GFP-tagged Drs2 temperature-sensitive protein (Drs2-ts) at the permissive (24°C) and nonpermissive (38°C) temperatures. Because Drs2-ts is the only source of Drs2p in these cells, we could determine if inactivation of Drs2p ATPase and flippase activities at high temperature disrupts its own trafficking in the AP-1-dependent anterograde direction. GFP-Drs2 and GFP-Drs2-ts were expressed in a strain deficient for multiple P4-ATPases (*drs2Δ dnf1Δ dnf2Δ dnf3Δ*), where Drs2p is required to support viability (Hua *et al.*, 2002), and we confirmed that the temperature-sensitive growth phenotype caused by the *drs2-ts* allele was not perturbed by the GFP moiety (unpublished observation). WT GFP-Drs2 maintained a punctate distribution at both temperatures in the presence or absence of Lat A (Figure 6). This indicates that the Dnf1, Dnf2, and

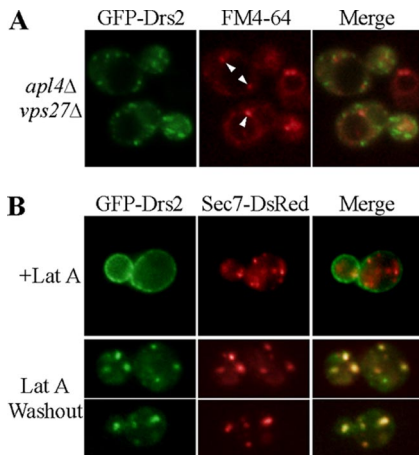


Figure 5. Drs2p does not require AP-1 for early endosome-to-TGN retrograde transport. (A) An *apl4Δ vps27Δ* strain (KLY711) expressing GFP-Drs2 was stained with FM4-64 to mark the class E compartment and imaged. Arrowheads are within the vacuole region and point to the class E compartments. (B) An *apl2Δ* strain (KLY351) expressing GFP-Drs2 and Sec7-DsRed was treated with Lat A for 1 h to completely accumulate GFP-Drs2p on the plasma membrane. The cells were washed twice, resuspended in fresh medium without Lat A (Lat A washout) and incubated for 1 h at 30°C before imaging.

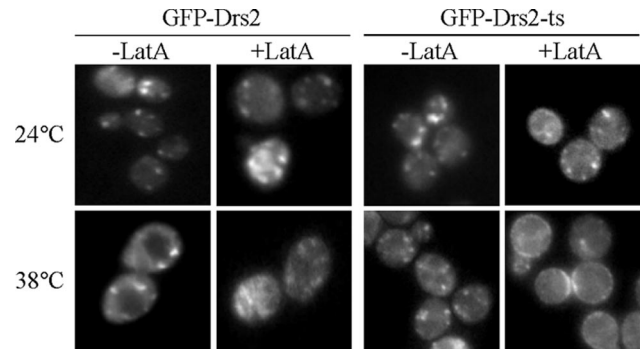


Figure 6. Acute inactivation of Drs2p enzymatic activity causes its missorting to the plasma membrane. Strains KLY921 and KLY931, deficient for Dnf P4-ATPases and expressing GFP-Drs2 or GFP-Drs2-ts (pGFP-*drs2-12*) as the sole source of Drs2p, were incubated at the permissive (24°C) or nonpermissive (38°C) temperature for 1 h before a 30-min incubation with or without Lat A at the same temperature. Plasma membrane fluorescence is only observed with Lat A-treated cells expressing GFP-Drs2-ts at 38°C.

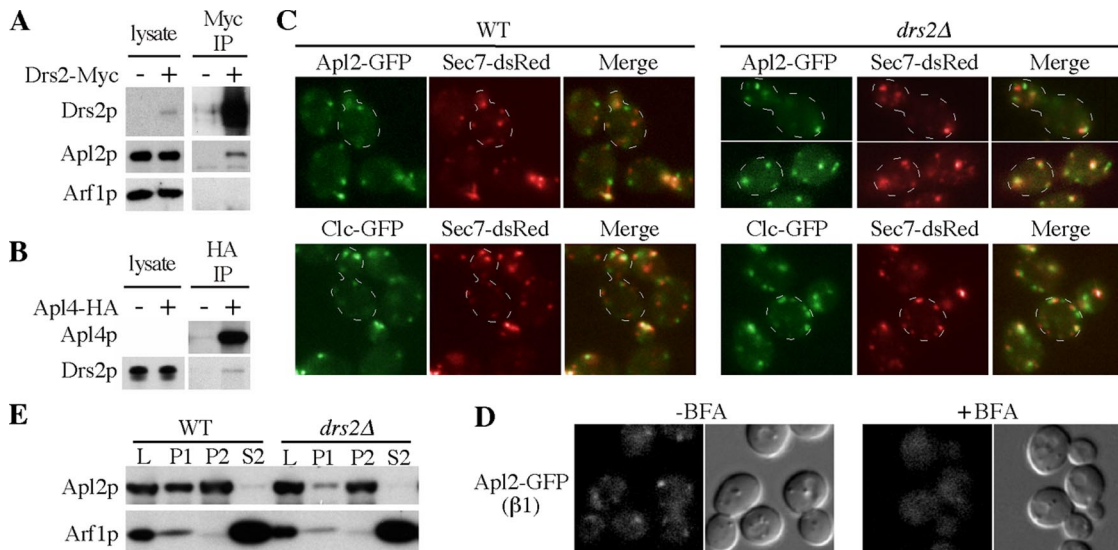


Figure 7. Drs2p interacts with AP-1 but is not required for AP-1 or clathrin recruitment to Golgi membranes. Coimmunoprecipitation of AP-1 and Drs2p. Cells expressing epitope tagged Drs2p (A), Apl4p (B), or control cells without the tag were treated with chemical cross-linker before lysis and immunoprecipitation (IP) with tag antibodies. 0.5% of the initial lysate was loaded in the “lysate” lanes, and the indicated proteins were detected by immunoblot. (C) WT (KLY791, KLY792) and *drs2Δ* (KLY361, KLY363) strains expressing the indicated fusion proteins were grown at 30°C to midlog phase, shifted to 15°C for 2 h (a nonpermissive growth temperature for *drs2Δ*), and imaged by fluorescence microscopy. The same results were obtained with cells maintained at 30°C (not shown). (D) Cells expressing GFP-tagged Apl2p (KLY551) were grown at 30°C, treated with or without brefeldin A (BFA), in imaging buffer for 30 min and imaged. (E) Lysates (L) from WT and *drs2Δ* cells grown at 30°C were prepared as previously described (Chuang and Schekman, 1996) and centrifuged sequentially at 13,000 × g for 20 min (P1 pellet) and 100,000 × g for 1 h (P2 pellet and S2 supernatant). Each fraction was then immunoblotted for Apl2p and Arf1p.

Dnf3 P4-ATPases do not significantly contribute to the AP-1-dependent trafficking of Drs2p. In contrast, Lat A treatment caused accumulation of GFP-Drs2-ts on the plasma membrane of the cells shifted to 38°C, but not the cells maintained at permissive temperature (Figure 6). Therefore, the enzymatic activity of Drs2p is required to drive its own exclusion from exocytic vesicles in a pathway that also requires AP-1.

Drs2p Associates With AP-1 but does Not Recruit AP-1/Clathrin to Membranes

The functional codependence of AP-1 and Drs2p suggested that these proteins might interact. To test this, cells expressing functionally Myc-tagged Drs2p were treated with a cross-linking agent before lysis and immunoprecipitation of Drs2p-Myc. After disruption of cross-links by reduction, the immunoprecipitates were probed by immunoblotting with antibodies to Arf1p and Apl2p, the AP-1β subunit. Apl2p, but not Arf1p, coimmunoprecipitated with Drs2p-Myc and Apl2p could not be detected in control immunoprecipitates from cells lacking the Myc tag on Drs2p (Figure 7A). In addition, cross-linking followed by immunoprecipitation of HA-tagged Apl4p, the AP-1 γ subunit, specifically isolated endogenous Drs2p (Figure 7B). However, only a small percentage (<0.5%) of Drs2p coimmunoprecipitated with AP-1 and so this complex is likely to be transient in vivo.

To determine if Drs2p is required to recruit AP-1 and clathrin onto TGN-endosomal membranes, we compared the localization of AP-1β-GFP (Apl2-GFP) to the TGN marker Sec7-RFP in WT and *drs2Δ* cells. Both strains showed a pattern of Apl2-GFP spots typical of the yeast TGN and early endosomal compartments with 51% of Apl2-GFP puncta coincident with Sec7-DsRed puncta in WT cells and 73% of Apl2-GFP puncta colocalized with Sec7-DsRed puncta in *drs2Δ* (Figure 7C). The increased association of

Apl2-GFP with Sec7-positive membranes is likely caused by normal recruitment of AP-1 to the TGN, combined with a defect in the budding of AP-1/clathrin-coated vesicles. Localization of GFP tagged clathrin light chain (Clc1-GFP), Gga2 and AP-3 were also examined in these strains, and their membrane association was unaffected (Figure 7C and data not shown). As a control, cells expressing AP-1-GFP were treated with brefeldin A, an inhibitor of ARF guanine nucleotide exchange factors (Peyroche *et al.*, 1996), and the punctate pattern was dispersed (Figure 7D). Membrane association of ARF and AP-1 was also examined in WT and *drs2Δ* mutant cells by differential centrifugation. Cell lysates (L) were sequentially centrifuged at 10,000 × g and 130,000 × g to collect P1 and P2 pellets, respectively, and 130,000 × g supernatants (S2), which were probed for AP-1 (Apl2p) and ARF. No significant loss from the membrane fractions (P1 and P2) or increase in the cytosolic (S2) fraction of AP-1 or ARF was detected for the *drs2Δ* strain relative to WT (Figure 7E). Thus, Drs2p is not required for recruitment of AP-1 or clathrin to membranes, even though AP-1/clathrin pathways are disrupted by *drs2Δ*.

DISCUSSION

The results of this work provide a number of unique observations with significant mechanistic implications for clathrin-mediated protein transport from the TGN and early endosomes. We find that an integral membrane P-type ATPase called Drs2p is essential for AP-1/clathrin function in vivo. The strong genetic interaction between *drs2* and *gga1/gga2* alleles, the complete lack of a genetic interaction between *drs2* and AP-1 alleles, and the phenotypic similarities between *drs2* and AP-1 mutants strongly suggest that AP-1 function is lost in *drs2* cells. In fact, Drs2p and AP-1 exhibit a novel functional codependency as we show that AP-1 is

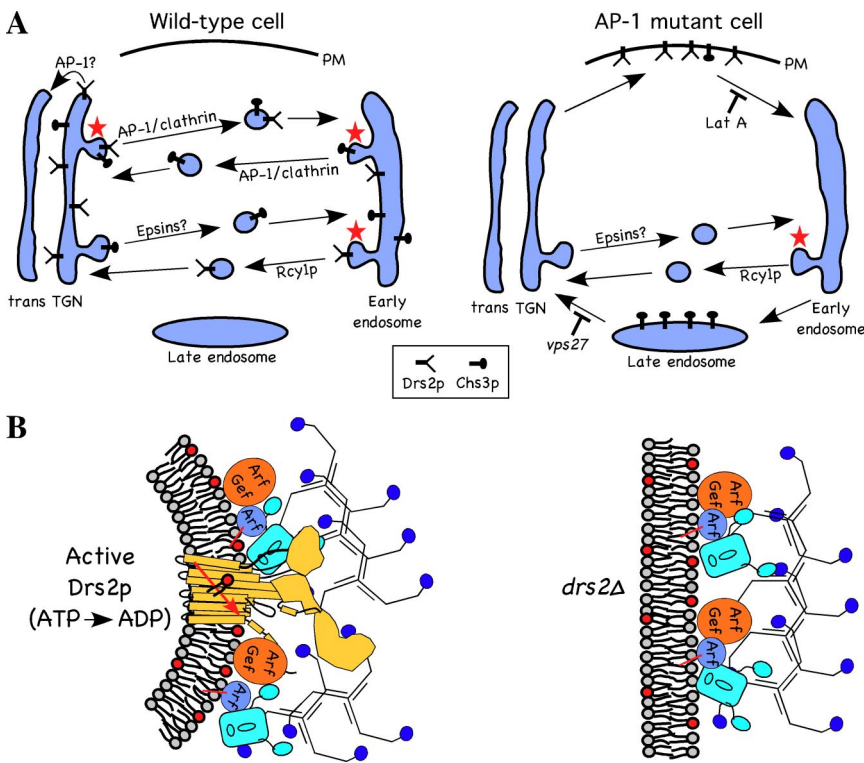


Figure 8. Models for the codependency of Drs2p and AP-1 in vesicle-mediated protein transport. (A) AP-1 and clathrin are required for anterograde transport of Drs2p from the TGN to the early endosome. Alternatively, it is possible that AP-1 mediates retrieval of Drs2p from the TGN to earlier Golgi cisternae (pathway marked AP-1?). Anterograde transport of Chs3p occurs, for the most part, independently of AP-1 and perhaps requires the Golgi epsins (Ent3p and Ent5p). AP-1 mutant cells treated with Lat A accumulate all of Drs2p and a fraction of Chs3p on the plasma membrane. Retrograde transport of Drs2p from the early endosome back to the TGN does not depend on AP-1, but appears to use the Rcy1p pathway. In contrast, Chs3p depends solely on AP-1 for early endosome to TGN retrograde transport. AP-1 mutants mis-sort most of Chs3p, but no Drs2p, to the late endosome where it can be trapped behind a vps27 block. Pathways that require Drs2p activity for vesicle formation are indicated with a red star. (B) In the absence of Drs2p (*drs2Δ*), AP-1 and clathrin are recruited to the membrane, but vesicles are not produced because the coat components cannot induce sufficient curvature in the membrane. (C) Enzymatically active Drs2p (yellow), modeled with a similar structure as SERCA1 (Toyoshima *et al.*, 2000), is required for budding AP-1/clathrin-coated vesicles. Interaction of Drs2p with Arf guanine nucleotide exchange factors (Arf Gef; Chantala-

et al., 2004) and AP-1 should concentrate activated Arf-GTP, AP-1 and clathrin at sites of lipid translocation where the clathrin coat can efficiently mold the curved membrane into a vesicle.

required at the TGN to prevent Drs2p transport to the plasma membrane. In addition, Drs2p flippase activity is required to drive its own trafficking in the AP-1 pathway that excludes Drs2p from exocytic vesicles. Importantly, we also show that Drs2p is not required for recruitment of AP-1 and clathrin to the TGN, thus providing the first example of an essential AP-1/clathrin accessory protein that acts independently of coat recruitment.

AP-1 was initially found to localize to the TGN of mammalian cells and was assumed to mediate protein transport from the TGN to the endosomal system. However, the finding that mutations of AP-1 subunits in mice and yeast appeared to cause trafficking defects in endosome to TGN retrograde transport raised questions as to whether AP-1 plays a primary role in retrograde or anterograde transport (reviewed in Hinners and Tooze, 2003). Our results show that AP-1 is required at the TGN to sort Drs2p out of the exocytic pathway. The simplest interpretation of these data are that Drs2p is packaged into AP-1/CCVs at the TGN for delivery to the early endosome (Figure 8A, WT cell). In the absence of AP-1, Drs2p is rerouted to the plasma membrane where it can be trapped by blocking endocytosis with Lat A (Figure 8A, AP-1 mutant cell). Mislocalization of Drs2p to the plasma membrane is an immediate consequence of inactivating a temperature-conditional AP-1 mutant allele (*apl2-ts*). It is also a specific consequence as other TGN resident proteins are not mislocalized to the plasma membrane in AP-1 mutants. In contrast to Chs3p, Drs2p does not depend solely on AP-1 for early endosome-to-TGN retrograde transport. Cdc50p, a Drs2p binding partner and chaperone, accumulates in the early endosomes of an *rcy1Δ* mutant (Furuta *et al.*, 2007). Therefore, Drs2p is likely retrieved back to the TGN primarily through the Rcy1p (AP-1-inde-

pendent) pathway (Figure 8A). However, these observations do not rule out a minor role for AP-1 in Drs2p-Cdc50p retrieval from the early endosome.

The yeast TGN appears to be a transient organelle with a half-life of only 1–2 min before it is consumed into many types of vesicles that are targeted to either the plasma membrane, early endosomes, late endosomes, the vacuole, or potentially earlier Golgi cisternae (Losev *et al.*, 2006; Matsuura-Tokita *et al.*, 2006). Thus, TGN residents rely on an active trafficking mechanism to maintain their TGN localization. As an alternative model that is consistent with our data, it is possible that AP-1/CCVs containing Drs2p bud from the TGN and are targeted directly to an earlier Golgi cisternae to facilitate the maturation of *trans* cisternae to TGN compartments (Figure 8A, pathway designated “AP-1?”). An intra-Golgi retrograde pathway would be a novel role for AP-1/clathrin and further work is needed to distinguish between these two models. Regardless of the vesicle destination, the current studies, combined with previously published data (Valdivia *et al.*, 2002; Foote and Nothwehr, 2006), support a direct role for AP-1 in selecting cargo for inclusion into CCVs at the TGN (Drs2p) and early endosome (Chs3p and Ste13p). Most Chs3p is rerouted to the late endosome of AP-1 null cells, where it can be trapped behind a *vps27Δ* block (Figure 8A, AP-1 mutant cell). However, Chs3p is also weakly mislocalized to the plasma membrane of AP-1 null cells, and so this cargo can likely use either AP-1 or another adaptor, such as Ent3/5p, for anterograde transport (this work and Copic *et al.*, 2007).

Several lines of evidence indicate that Drs2p plays an essential and direct role in bidirectional protein transport between the TGN and early endosome. *DRS2* mutant alleles exhibit genetic interactions with mutations in a number of

protein transport factors operating in the late secretory and early endocytic pathways. We originally linked Drs2p to clathrin-mediated transport through the recovery of several *drs2* alleles in an *arf1* synthetic lethal screen and the observation that *drs2* is also synthetically lethal with clathrin heavy-chain temperature-sensitive alleles but not with mutations in COPI or COPII subunits (Chen *et al.*, 1999). Subsequently, genetic interactions were discovered between Drs2p and ARF guanine nucleotide exchange factors (Gea1p and Gea2p), an ARF guanine nucleotide activating protein (Gcs1p), GGAs, Ypt31p (a rab protein), TRAPP II subunits, and a phosphatidylinositol 4-kinase (Pik1p; Chantalat *et al.*, 2004; Sciorra *et al.*, 2005; Sakane *et al.*, 2006). In addition, the Drs2-Cdc50 complex physically interacts with Gea2p, AP-1, and Rcy1p, providing a direct link to ARF/AP-1/clathrin pathways as well as the Rcy1-dependent early endosome-to-TGN recycling pathway (this work and Chantalat *et al.*, 2004; Furuta *et al.*, 2007).

Consistent with these genetic and physical interactions, *drs2* mutants exhibit protein transport defects specific to the TGN and early endosomal system (the red stars in Figure 8A indicate pathways that require Drs2p). As shown here, AP-1/clathrin-dependent pathways associated with the TGN and early endosome are abrogated in cells deficient for Drs2p. In addition, Drs2p is required for the early endosome to TGN retrograde transport of the SNARE protein Snc1 (Hua *et al.*, 2002; Furuta *et al.*, 2007), which is independent of AP-1 (our unpublished observations) but requires Rcy1p (Galan *et al.*, 2001). Cdc50p, presumably in a complex with Drs2p, accumulates in the early endosomes of *rcy1* mutants (Furuta *et al.*, 2007). Therefore, Drs2 activity likely drives its own retrieval back to the TGN in the Rcy1p pathway. Because the majority of Drs2p localizes to the TGN, inactivation of Drs2p activity initially causes its missorting to the plasma membrane rather than accumulation in the early endosome. The precise role of the F-box protein Rcy1 is unclear, although it functions with the Ypt31/32 rab pair, Gcs1p (Arf-GAP) and a COPI subcomplex in this recycling pathway (Chen *et al.*, 2005; Robinson *et al.*, 2006; Furuta *et al.*, 2007).

In addition to the AP-1 and Rcy1 pathways, we previously described a role for Drs2p and clathrin in the formation of one class of post-Golgi exocytic vesicles (Gall *et al.*, 2002). AP-1 mutations partially perturb formation of these exocytic vesicles, although it was not clear at the time whether the small defect observed reflected a primary or secondary consequence of AP-1 disruption. On the basis of the current studies, we suggest that AP-1 mutations influence the exocytic vesicles indirectly by altering the trafficking patterns of Drs2p (and perhaps other TGN residents as well). Drs2p is required to form the dense exocytic vesicles and AP-1 retrograde vesicles, but does not appear to be significantly incorporated into these vesicles. This is consistent with a role for Drs2p in priming the membrane of the TGN and early endosome to produce a structure that coats can more efficiently mold into a vesicle.

P4-ATPases in yeast (Drs2p, Dnf1p, Dnf2p, Dnf3p, and Neo1p) have a general function in vesicle-mediated protein transport in the Golgi-endosomal system (Chen *et al.*, 1999; Hua *et al.*, 2002; Hua and Graham, 2003). Although some vesicle classes budding from the TGN and early endosomes primarily require Drs2p (e.g., AP-1, Rcy1, and the dense exocytic vesicles), other pathways are less discriminate for their P4-ATPase requirement. For example, AP-3-dependent protein transport from the TGN to the vacuole requires either Drs2p or Dnf1p, such that a severe defect in this pathway is only observed in the *drs2Δ dnf1Δ* double mutant

(Hua *et al.*, 2002). Likewise, transport of carboxypeptidase Y from the TGN to late endosomes, perhaps carried in GGA/clathrin-coated vesicles, is kinetically delayed in the *drs2Δ dnf1Δ* double mutant (Hua *et al.*, 2002). Importantly, not all vesicles budding from the Golgi require Drs2p. The “lighter density” class of exocytic vesicles appears to form normally in *drs2Δ* cells, and cargos normally secreted in the dense vesicles, such as invertase, get rerouted into the light exocytic vesicles (Chen *et al.*, 1999; Gall *et al.*, 2002; Hua *et al.*, 2002). In fact, the rate of protein transport from the ER to the Golgi and subsequently to the cell surface in *drs2Δ* or *drs2Δ dnf1Δ*, or *dnf1,2,3Δ* cells is similar to that of WT cells (Chen *et al.*, 1999; Gall *et al.*, 2002; Hua *et al.*, 2002). These data also indicate that COPI and COPII function in the early secretory pathway is unperturbed by loss of Drs2 and Dnf P4-ATPases. However, conditional alleles of the essential P4-ATPase *NEO1* cause transport defects that are similar to COPI mutants and also perturb ARL-dependent transport from endosomes (Hua and Graham, 2003; Wicky *et al.*, 2004). The proposed phospholipid flippase activity for P4-ATPases is best supported by studies showing a temperature-conditional loss of flippase activity in purified TGN membranes carrying a temperature conditional form of Drs2p (Drs2-ts; Natarajan *et al.*, 2004). Inactivation of Drs2-ts *in vivo* causes an immediate defect in the budding of dense exocytic vesicles (Gall *et al.*, 2002), and rerouting of Drs2-ts to the plasma membrane, implying a defect in budding AP-1/CCVs from the TGN (this work). Therefore, Drs2p flippase activity appears to drive formation of vesicles from the TGN.

Two models have been proposed to explain how the phospholipid flippase activity of Drs2p contributes to vesicle biogenesis (Chen *et al.*, 1999; Gall *et al.*, 2002; Graham, 2004). One possibility is that Drs2p recruits coat proteins to the membrane through protein-protein interactions, and/or by increasing the concentration of specific phospholipids on the cytosolic leaflet. However, our results rule out this possibility by showing that membrane association of ARF, AP-1, and clathrin does not require Drs2p (Figure 8B, right), in spite of the Drs2p requirement for AP-1 function. Therefore, our data argue that membrane recruitment of ARF, AP-1, and clathrin is insufficient to bud AP-1/CCVs in the absence of Drs2p. Even though epsins have been implicated in membrane deformation and AP-1 function (Ford *et al.*, 2002; Hirst *et al.*, 2003; Mills *et al.*, 2003; Costaguta *et al.*, 2006), the yeast Golgi epsins (Ent3p and Ent5p) also fail to support AP-1/CCV function in the absence of Drs2p.

The model best supported by our data is that ATP-dependent transbilayer movement of lipids catalyzed by Drs2p imparts curvature to the membrane that is required to facilitate vesicle budding (Figure 8B, left). The bilayer-couple hypothesis predicts that an increase in surface area of one leaflet relative to the other will spontaneously induce membrane curvature (Sheetz and Singer, 1974). As little as a 1–2% difference in surface area between leaflets of a bilayer can have a substantial impact on membrane shape (Lim *et al.*, 2002). Consistent with this hypothesis, bilayer surface area asymmetry induced by insertion of additional phospholipid in the extracellular leaflet of the plasma membrane and their subsequent flip to the cytosolic leaflet, catalyzed by the aminophospholipid translocase, leads to dramatic changes in the shape of red blood cells (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985). Translocase-mediated influx of phospholipid to the cytosolic leaflet of the plasma membrane also stimulates endocytosis (Farge *et al.*, 1999). By translocating phospholipid from the luminal leaflet to the cytosolic leaflet of the TGN or early endosomes, Drs2p would increase the surface area of the cytosolic leaflet at the

expense of the luminal leaflet and bend the membrane toward the cytosol. This could provide a critical priming step to produce a membrane substrate that coat proteins can more efficiently mold into a vesicle. We propose that Drs2p imparts curvature to the membrane through a bilayer-couple mechanism that is subsequently captured and molded by AP-1/clathrin to produce vesicles.

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