TRAFFICKING AND FUNCTIONAL ANALYSIS OF YEAST DRS2

By

KE LIU

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Approved:

Professor Todd R. Graham

Professor Kendal Broadie

Professor Susan R. Wente

Professor Anne K. Kenworthy

Professor Katherine L. Friedman

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CHAPTER I

FLIPPASES AND VESICLE-MEDIATED PROTEIN TRANSPORT

The P-type ATPase Superfamily

P-type ATPases are a large, evolutionary conserved family of membrane proteins that are involved in active transport of charged substrates, such as cations, across biological membranes (Moller *et al.*, 1996; Kuhlbrandt, 2004; Paulusma and Oude Elferink, 2005). Members of the P-type ATPase superfamily are widely expressed in virtually all organisms. Based on sequence homology, this large family can be divided into five subfamilies referred to as types I –V (Axelsen and Palmgren, 1998; Palmgren and Axelsen, 1998; Kuhlbrandt, 2004). Each subfamily may be further divided into two or more subtypes, each unique for their transport substrates. Well studied examples of P-type ATPases are provided in Table 1-1 and organized by classification. Type IV ATPases, or P4-ATPases, are involved in lipid transport and will be the main focus of this thesis. These "lipid flippases" are thought to translocate phospholipids from the extracellular or luminal leaflet of the membrane to the cytosolic leaflet.

The P-type designation derives from the formation of a covalent aspartyl-phosphate catalytic intermediate, which is intimately linked with the translocation process. The general mechanism by which substrates are pumped by P-type

ATPases is described as the Post-Albers cycle. In this model, two distinct conformations of the enzyme with different affinity for ions and nucleotides are called the E1 and E2 states (Figure 1-1). In the E1 state, the ATPase contains high-affinity ion 1-binding sites that are exposed to the cytosol. Following the binding of ion 1 and Mg^{2+}-ATP, the enzyme is phosphorylated at the invariant aspartic acid residue to form a phosphorylated E1-P state. The ATPase then undergoes a change in conformation to the E2-P state, which has reduced affinity to ion 1 and releases it to the lumenal or the extracellular side of the membrane. Ion 2 binds from the lumenal or the extracellular side, and subsequently escapes to the cytosol on dephosphorylation of the aspartic acid. The ATPase is then ready to start another cycle.

Table 1-1: Members of the P-type ATPase Superfamily

SUBFAMILY	SUBTYPE	EXAMPLES
Type I	A	*E.coli* Kdp K^+-ATPase
	B	Heavy metal pumps, Cu^{2+}-ATPase
Type II	A	Sarcoplasmic-reticulum Ca^{2+}-ATPase
	B	Plasma membrane Ca^{2+}-ATPase
	C	Na^+/K^+-ATPase, Gastric H^+/K^+-ATPase
	D	Na^+-ATPase
Type III	A	H^+-ATPase
	B	Bacterial Mg^{2+}-ATPase
Type IV	/	Phospholipid translocases
Type V	/	Yeast Cod1p/Spf1p (substrate unknown)

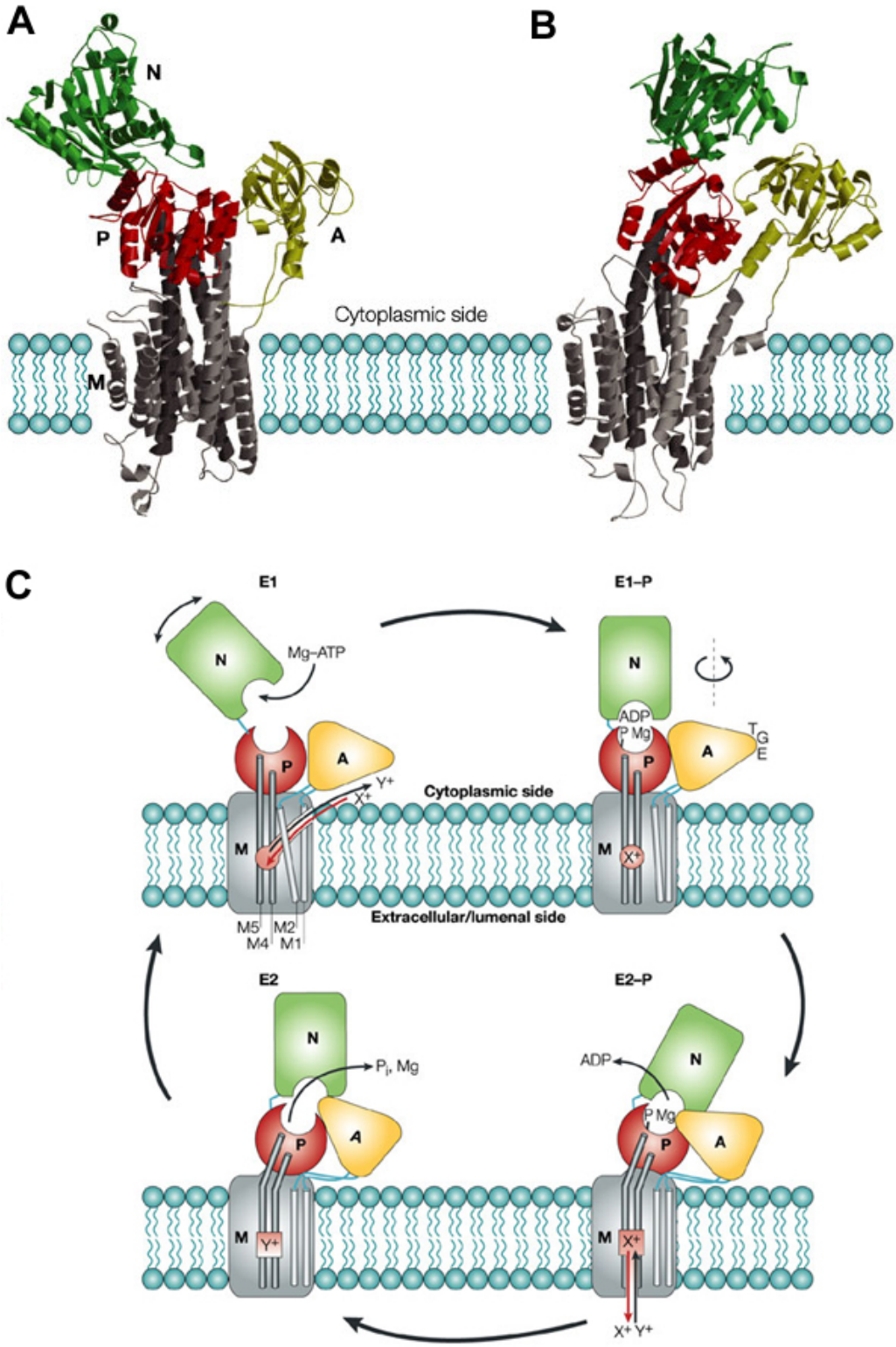

Figure 1-1: Structure and catalytic cycle of P-type ATPase

Figure 1-1: Structure and catalytic cycle of P-type ATPase.
(A) Crystal structure of the SR-Ca^{2+}–ATPase in the E1 state (From: Toyoshima *et al.*, 2000; Kuhlbrandt, 2004).
(B) Crystal structure of the SR-Ca^{2+}-ATPase in the thaipsigargin-inhibited E2 state. (From Toyoshima and Nomura, 2002; Kuhlbrandt, 2004)
(C) Schematic diagram of the catalytic cycle of P-type ATPases. In the E1 state, ion 1 (X^+) comes from the cytosol, and binds to its high-affinity binding site in the membrane (M) domain. The crucial Asp residue in the phosphorylation (P)-domain is then phosphorylated by Mg^{2+}-ATP, which is delivered by the nucleotide-binding (N)-domain, resulting in the formation of the E1-P state. In the E1-P to E2-P transition, the P domain reorientates its position, while the actuator (A)-domain rotates to bring its TGE loop into close contact with the phosphorylation site. This movement of P domain and A-domain also induces the movement of the M-domain, which shuts off the cytosolic ion-access channel and disrupts the high-affinity X^+ binding site. X^+ is then released to the extracellular or lumenal side. Ion 2 (Y^+) binds from the lumenal or the extracellular side, and subsequently escapes to the cytosol on dephosphorylation of the Asp residue. The ATPase is then ready to start another cycle. (From Kuhlbrandt, 2004)

Structural studies of P-type ATPases mainly focus on four members of this family: the Na^+/K^+-ATPase, sarcoplasmic reticulum-Ca^{2+}-ATPase (SR-Ca^{2+}-ATPase), gastric H^+/K^+-ATPase and plasma membrane H^+-ATPase. So far, crystal structures with atomic resolution are available only for the SR-Ca^{2+}-ATPase (Toyoshima *et al.*, 2000; Toyoshima and Nomura, 2002; Toyoshima and Inesi, 2004). Remarkably, the SR-Ca^{2+}-ATPase has been crystallized in four different conformations, providing a dramatic view of the conformational changes associated with the catalytic cycle. P-type ATPases share a common topology and typically have 10 transmembrane helices with both amino- and carboxyl- termini protruding into the cytosol. According to the model derived from structural studies (Figure1-1), four principal domains are defined in the SR-Ca^{2+}-ATPase. The phosphorylation (P) domain lies in the large intracellular loop between membrane-spanning helices M4 and M5. Since this domain contains the P-type signature aspartic acid which gets phosphorylated during the catalytic cycle, it is the catalytic core of the P-type ATPases. The nucleotide-binding (N) domain is a large insert in the P-domain. A conserved sequence including a lysine residue is characteristic of the nucleotide-binding site. The actuator (A) domain is formed by the loop between helices M2 and M3 and the N-terminal tail leading into M1. The A-domain rotates to make close contact with the P-domain during the catalytic cycle, causing substantial conformational changes in the transmembrane segments. The membrane (M) domain comprises the 10 transmembrane helices and the short connecting loops on the extracellular or the lumenal side of the membrane. For the SR-Ca^{2+}-ATPase, the ion-binding sites are surrounded by

transmembrane helices M4, M5, M6 and M8, which provide polar and acidic side chains to coordinate the two ions. During the ion-pumping process, the M-domain rearranges dramatically, leading to a drastic affinity change for the ion-binding sites, and the opening and closing of pathways leading to the cytosol or extracytosolic compartments.

P4-type ATPases are different from other subfamilies in that they are thought to translocate phospholipids rather than cations across the membrane bilayer. Because of the substrate specificity of the P4-ATPases, their activity appears to establish an asymmetric distribution of phospholipid species across the bilayer. Recently, studies in yeast have demonstrated a surprising requirement for these ATPases in vesicle-mediated protein trafficking. The scope of this chapter is to highlight the role of P4-ATPases in membrane asymmetry and protein trafficking, and to relate their cellular functions to their proposed phospholipid translocase activity.

P4-ATPases and Membrane Asymmetry

Membrane Asymmetry and Its Regulation

Biological membranes are lipid bilayers that are mainly formed from glycerophospholipids, sphingolipids and sterols along with the embedded integral membrane proteins. Some lipids such as diacylglycerol, cholesterol or phosphatidylglycerol can move rapidly from one leaflet to the other in seconds or minutes in artificial membranes. This transbilayer movement is called lipid “flip-flop”.

On the contrary, phospholipids that bear polar head groups, such as phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE), take hours to days to equilibrate between the two leaflets of artificial bilayers (Kornberg and McConnell, 1971) (reviewed in (Zachowski, 1993)). Consequently, these slow-flipping lipid species can be non-randomly distributed between the two leaflets of the membrane bilayer. For example, the plasma membrane of most eukaryotic cells exhibits an asymmetric lipid arrangement with sphingomyelin (SM) and PC located preferentially on the extracellular leaflet, while PS, PE and phosphatidylinositol (PI) are restricted to the cytosolic leaflet (Zachowski, 1993; Daleke, 2003).

Maintenance of phospholipid asymmetry is an important process associated with many physiological and pathological events. For example, exposure of PS and/or PE to the extracellular leaflet of the plasma membrane is important for platelet activation and clearance of apoptotic cells by phagocytes (Williamson and Schlegel, 2002; Balasubramanian and Schroit, 2003). Furthermore, lipid asymmetry between the bilayer leaflets can affect membrane morphology as suggested by the bilayer-couple hypothesis of Sheetz and Singer (Sheetz and Singer, 1974; Sheetz *et al.*, 1976). Based on the effect of various membrane intercalating compounds on the shape of red blood cells, this hypothesis postulates that the two leaflets are physically coupled together and an increase in the surface area of one leaflet relative to the other would spontaneously increase membrane curvature (Sheetz and Singer, 1974; Sheetz *et al.*, 1976). Amphipathic drugs that preferentially incorporated in to the PC and sphingomyelin enriched outer leaflet

bend the membrane outward and crenate the erythrocytes. In contrast, compounds that intercalate into the aminophospholipid-rich cytosolic leaflet cause an inward bending to generate stomatocytes.

Lipid asymmetry is tightly regulated by the synergistic action of three classes of proteins that function to translocate lipids across the membrane: flippases, floppases and scramblases (Daleke, 2003). Scramblases dissipate lipid asymmetry by mediating energy-independent, bi-directional lipid movement with little specificity. In red blood cells and platelets, scramblase activity is stimulated by an influx of calcium, leading to PS exposure and stimulation of blood clotting. The ER appears to have a similar, although calcium-independent activity that allows uniform growth of both leaflets during membrane biogenesis. In contrast to scramblases, the activities of both flippases and floppases are energy-dependent and are required to establish and maintain lipid asymmetry. Flippases catalyze the inward movement of phospholipids from either the extracellular or lumenal leaflet to the cytosolic leaflet, whereas floppases mediate a reverse outward reaction. The protein responsible for scramblase activity is unknown, while flippase and floppase activities are apparently catalyzed by P4-ATPases and ATP binding cassette (ABC) transporters, respectively.

The Aminophospholipid Translocase (APLT)

In 1984, Seigneuret and Devaux first discovered a PS and PE-specific ATP-dependent flippase, which they named aminophospholipid translocase (APLT), by

measuring the translocation of spin-labeled phospholipid derivatives across the plasma membrane of erythrocytes (Seigneuret and Devaux, 1984). This and later studies also demonstrated that the addition of different exogenous phospholipids have unique impacts on the shape of erythrocytes, depending on whether or not the phospholipid is a substrate for the APLT (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985; Daleke and Huestis, 1989). On addition of spin-labeled PC, PS or PE, erythrocytes immediately become crenated as the lipid intercalates into the outer leaflet. With further incubation, erythrocytes with the PC analogue remain crenated because this lipid is not a substrate of the APLT. In contrast, erythrocytes with PS or PE analogues display shape changes associated with translocation of the lipid to the inner leaflet, which bends the plasma membrane inward, transforming the cells to a discoid shape and eventually an uniconcave disc-like shape (Figure 1-2) (Seigneuret and Devaux, 1984). These results are consistent with the existence of a protein in erythrocytes that specifically translocates aminophospholipids to the inner leaflet of the plasma membrane.

The biochemical properties of the APLT have been well characterized. Transport activity is dependent on ATP and Mg^{2+}, but inhibited by N-ethylmaleimide, vanadate and Ca^{2+} (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985; Bitbol *et al.*, 1987; Daleke and Huestis, 1989). The stoichiometry of translocation is approximately one lipid per ATP consumed (Beleznay *et al.*, 1993). The flippase prefers PS over other lipids and the selectivity for PS is determined by multiple elements of lipid structure. The amino group is essential and monomethylation of PS is tolerable. The carboxyl group is not

absolutely required, since PE is also transported, although at a much lower rate. While the stereochemistry of serine headgroup is unimportant for recognition, the stereochemistry of the glycerol backbone is absolutely required: the *sn*-2,3-glycerol analog of the naturally-occurring *sn*-1,2-glycerol-lipid can not stimulate ATPase activity, nor can it be translated across the plasma membrane of erythrocytes (Daleke, 1995; Daleke, 2003). The transport of PS or PE seems to be independent of the length and composition of acyl chains. Thus, spin and fluorescent labeled lipids, attached to a short (e.g. C6) acyl chain in the sn-2 position, are widely used to study the APLT activity (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985; Colleau *et al.*, 1991; Devaux *et al.*, 2002).

In 1989, an APLT activity was identified in the membrane of chromaffin granules (Zachowski *et al.*, 1989). Since the biochemical properties of this APLT are similar to ATPase II purified from chromaffin granules (Moriyama and Nelson, 1988), it was suggested that ATPase II was the indeed the APLT. In 1996, the gene that encodes ATPase II was cloned and found to share a high degree of sequence similarity to Drs2p from budding yeast (Tang *et al.*, 1996). The presence of multiple P-type ATPase motifs indicated that ATPase II and Drs2p belonged to the P-type ATPase family. In fact, they are the founding members of the P4-ATPases, which are widely expressed in various eukaryotes including fungi, protozoa, insects, plants and animals (Table 1-2), but do not appear to be present in eubacteria and archaebacteria.

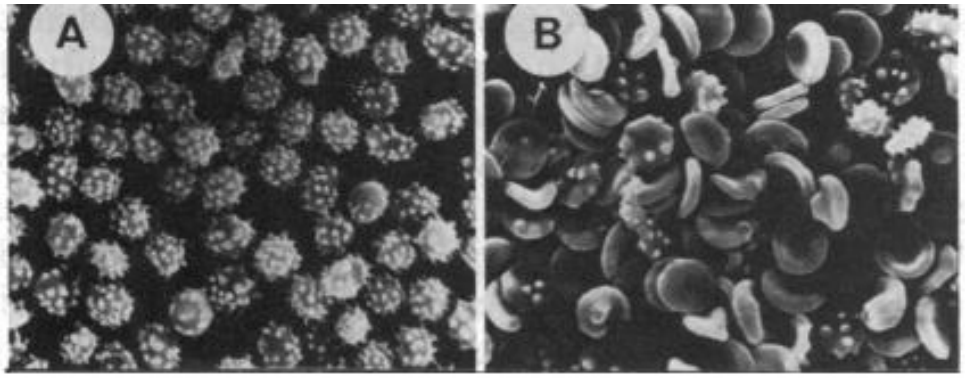

Figure 1-2: Scanning electron micrograghs of erythrocytes incubated for 3 min (A) or 6.5 hr (B) at 5°C after labeling with exogenous PS (1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylserine). (From Seigneuret and Devaux, 1984)

Table 1-2: P4-ATPases in Different Organisms

SPECIES	SUBUNIT	# OF MEMBERS	REFERENCES	REPRESENTATIVE GENES
Saccharomyces cerevisiae	α	5	(Catty *et al.*, 1997)	*NEO1* *DRS2/SWA3* *DNF1* *DNF2* *DNF3*
	β	3		*CDC50/SWA4* *LEM3/ROS3* *CRF1*
Saccharomyces Pombe	α	5	(Okorokova-Facanha *et al.*, 2003)	
Magnaporthe Grisea	α	4		*MgPDE1* *MgAPT2*
Leishmania donovani	α	/		*LdMT*
	β	/		*LdRos3*
Caenorhabditis elegans	α	6	(Halleck *et al.*, 1998; Okamura *et al.*, 2003)	
Drosophila melanogaster	α	6	(Okamura *et al.*, 2003)	
Arabidopsis thaliana	α	12	(Axelsen and Palmgren, 2001; Baxter *et al.*, 2003)	*ALA1*
	β	5		
Oryza sativa	α	10	(Baxter *et al.*, 2003)	
mammalian	α	14	(Halleck *et al.*, 1998; Halleck *et al.*, 1999; Williamson and Schlegel, 2002; Paulusma and Oude Elferink, 2005)	*ATP8A1*(ATPase II) *ATP8A2* *ATP8B1/FIC1* *ATP8B3* *ATP10A/ATP10C* *ATP10D* *ATP11A* *ATP11C*
	β	3	(Katoh and Katoh, 2004; Osada *et al.*, 2006)	*TMEM30A* *TMEM30B* *TMEM30C*

P4-ATPases Are Potential Phospholipid Translocases

1. Mammalian P4-ATPases

Database searches and phylogenetic analysis identified 14 P4-ATPases in mammals (Table 1-2). An expression profile of a panel of P4-ATPases revealed that these proteins are expressed in a development- and tissue-specific manner, suggesting that they are not simply redundant. In addition, they may also differ in subcellular localization and substrate specificity (Halleck *et al.*, 1998; Halleck *et al.*, 1999; Soupene and Kuypers, 2006; Sobocki *et al.*, 2007). Our knowledge of the biochemical properties of P4-ATPases is primarily based on characterization of ATPase II (ATP8A1), which strongly resembles the previously characterized APLT in the plasma membrane of erythrocytes (Moriyama and Nelson, 1988; Paterson *et al.*, 2006). Xie's group was the first to express and purify recombinant Atp8a1 from insect cells (Ding *et al.*, 2000). In their studies, the phosphoenzyme intermediate could form in the absence of PS, but required PS for dephosphorylation, consistent with PS being a substrate pumped towards the cytosol during the E2->E1 transition. Recently, Daleke and colleagues monitored the activity of recombinant Atp8a1 in detergent micelles containing various lipids (Paterson *et al.*, 2006). Their data strongly correlate the potential substrates that stimulate Atp8a1 activity with substrates transported across the erythrocyte plasma membrane by the APLT. However, experiments in which purified enzyme is reconstituted are still needed to directly demonstrate the transport activity of Atp8a1. In addition, the identity of the erythrocyte APLT remains unknown. So whether the APLT is Atp8a1, one of the 13 other P4-ATPase

or a different ATPase altogether is not clear.

Up to now, only one P4-ATPase gene, *FIC1* (*ATP8B1*), has been unambiguously linked to human disease (Paulusma and Oude Elferink, 2005). Mutations in *FIC1* cause inherited progressive familial intrahepatic cholestasis type 1 (PFIC1) and benign recurrent intrahepatic cholestasis (BRIC) (Bull *et al.*, 1998; Bull *et al.*, 1999; Tygstrup *et al.*, 1999; Klomp *et al.*, 2000; Eppens *et al.*, 2001; Ujhazy *et al.*, 2001; Klomp *et al.*, 2004). PFIC1/BRIC patients are characterized by impaired bile salt secretion, high levels of serum bile salts and bilirubin, but normal levels of serum cholesterol and γ-glutamyltranspeptidase, an ectoenzyme whose serum activity is high in most cholestasis disorders. Obviously, dysfunction of Atp8b1 abrogates bile salt homeostasis and bile formation, but the molecular mechanism underlying the cholestatic phenotype is still not clear. Atp8b1 localizes to the apical membrane of epithelial cells in many tissues, including hepatocytes and bile duct epithelial cells. Expression of Atp8b1 in a mutant CHO-K1 cell line, which is defective in the non-endocytic uptake of NBD-PS, increases translocation of NBD-PS across the plasma membrane (Ujhazy *et al.*, 2001). These data are consistent with a proposed role for Atp8b1 as an APTL, but do not rule out an indirect impact of Atp8b1 on NBD-PS uptake. For example, Atp8b1 may affect expression or trafficking of other proteins which are directly responsible for the APTL activity. Recently, Oude Elferink's group observed that after intravenous infusion of a hydrophobic bile salt, Atp8b1 deficient mice displayed enhanced levels of PS, cholesterol and ectoenzymes in the bile (Paulusma *et al.*, 2006). Moreover, disruption of Atp8b1

abrogates the transport of hydrophobic bile salts into bile in isolated mouse livers. Based on these observations, this group proposes that loss of membrane asymmetry caused by Atp8b1 deficiency renders the canalicular membrane more sensitive to extraction of PS and cholesterol by bile salts, which in turn perturbs ABCB11 activity, the major bile salt export pump, and causes cholestasis.

Besides *FIC1*, a few other diseases have been tentatively linked to P4-ATPase loci. The *ATP8A2* locus is frequently deleted in tumorigenic malignancies (Sun *et al.*, 1999). X-linked hypoparathyroidism maps to a chromosomal region, Xq27, containing the 3'UTR of *ATP11C* (Andrew Nesbit *et al.*, 2004). Moreover, a role of P4-ATPases in drug resistance was suggested by a recent report, showing that elevated levels of *ATP11A* protect the lymphoblastic leukemia cells against several small molecule signal transduction inhibitors (Zhang *et al.*, 2005).

P4-ATPases have been implicated in several different physiological processes in the mouse system. Heterozygous mice with a maternally-inherited chromosomal deletion impinging on *ATP10A* are predisposed to develop obesity and type 2 diabetes when fed a high-fat diet (Dhar *et* al., 2000; Dhar *et al.*, 2002; Dhar *et al.*, 2004a; Dhar *et al.*, 2004b; Dhar *et al.*, 2006). A similar, albeit less severe phenotype is exhibited by the inbred mouse strain C57BL/6J, which is linked to a premature stop codon in the *ATP10D* gene (Flamant *et al.*, 2003). Thus, both *ATP10A* and *ATP10D* seem to be involved in the regulation of lipid metabolism. The mouse ortholog of *ATP8B3* is exclusively expressed in the acrosomal region of spermatozoa. Loss of *ATP8B3* compromises the ability of the

spermatozoa to bind and penetrate the zona pellucida and reduces the acrosome reaction (Wang *et al.*, 2004). At this moment, how these P4-ATPases contribute to disease phenotypes is still poorly understood. However, studies in yeast have shed light on cellular and molecular functions of P4-ATPases, which may illuminate physiological processes perturbed by the diseases described above.

2. Yeast P4-ATPases

Five members of the P4-ATPase family are expressed in the yeast *S. cerevisiae*, including Neo1p, Drs2p, Dnf1p, Dnf2p, and Dnf3p. While deletion of *NEO1* alone is lethal, *DRS2*, *DNF1*, *DNF2* and *DNF3* are individually nonessential genes. However, deletion of all four of these genes is lethal and so they constitute an essential group (Hua *et al.*, 2002). The fact that any one of these four proteins can support cell viability implies that they share a common biochemical function. The *drs2Δ* strain grows as well as wild-type at 30°C, but fails to grow at 23°C or below. *DNF1* and *DNF2* are the closest related genes in this family, sharing 69% sequence identity and 83% similarity. In some strain backgrounds, *dnf1Δdnf2Δ* also causes a cold-sensitive growth defect (Pomorski *et al.*, 2003). Different localization patterns are observed for individual yeast P4-ATPase members. While Drs2p and Dnf3p primarily reside in the *trans*-Golgi network (TGN), Dnf1p and Dnf2p localize to the plasma membrane and internal compartments, concentrating at the sites of polarized growth (i.e. emerging bud sites, small buds and mother-daughter neck of dividing cells) (Hua *et al.*, 2002; Pomorski *et al.*, 2003). Neo1p

appears to populate both endosomal membranes and the Golgi. All five P4-ATPases members have been implicated in protein trafficking, but act at different pathways (See below; Figure 1-3). As we will discuss later, the functional redundancy between these family members and roles they play in protein transport make it difficult to define the flippase activity of individual proteins by analyzing yeast strains carrying knockouts of P4 ATPase genes.

As in mammalian cells, the plasma membrane of yeast is asymmetric with PS and PE restricted to the inner leaflet. However, unlike most mammalian cells, yeast can efficiently translocate NBD-PC cross the plasma membrane along with NBD-PS and NBD-PE, suggesting a broader substrate specificity for yeast translocases (Kean *et al.*, 1993; Grant *et al.*, 2001; Hanson and Nichols, 2001). How active NBD-PC translocation relates to plasma membrane asymmetry remains unknown, although it is possible that most of the PC is in the inner leaflet and the outer leaflet is primarily glycosphingolipid.

Among the five yeast P4-ATPases, Dnf1p and Dnf2p are the only two present on the plasma membrane, so they are most likely to be responsible for the flippase activity at the plasma membrane. Loss of Dnf1p and Dnf2p abolishes the ATP-dependent, non-endocytic uptake of NBD-labeled PE, PC and, to a lesser extent PS (Pomorski *et al.*, 2003). The *dnf1Δdnf2Δ* double mutant also exposes elevated levels of endogenous PE and PS on the cell surface (Pomorski *et al.*, 2003; Chen *et al.*, 2006). Specifically, wild-type cells expose a small amount of PE on the extracellular leaflet, and only at sites of polarized growth. In contrast, cell surface PE is also observed at the enlarged bud

cortex of *dnf1Δdnf2Δ* cells, suggesting Dnf1p and Dnf2p are involved in retrieval of PE at the bud cortex (Iwamoto *et al.*, 2004). Uptake of three PC analogues with different Bodipy fluorophores was also tested and compared with that of NBD-PC in strains harboring deletions in *DNF1* and *DNF2* (Elvington *et al.*, 2005). Loss of *DNF1* and *DNF2* significantly reduces the translocation of NBD-PC and Bodipy FL-PC across the plasma membrane, but does not perturb translocation of Bodipy 581-PC and Bodipy 530-PC. ATP depletion and culture density also have a different impact on uptake of Bodipy 581-PC and Bodipy 530-PC. These observations suggest that in yeast there are at least three different translocation pathways which are selective for structure of the fluorophore attached to the acyl chain of PC molecules. *dnf1Δdnf2Δ* was shown to also perturb the endocytic pathway, so the data obtained from this mutant can not determine whether Dnf1p and Dnf2p directly translocate phospholipids, or whether other proteins that are depleted from the plasma membrane by the trafficking defects are more directly responsible for the phospholipid translocation.

Whether or not disruption of Drs2 significantly decreases NBD-PS translocation across the plasma membrane and perturbs PS asymmetry was controversial in initial studies (Tang *et al.*, 1996; Siegmund *et al.*, 1998; Marx *et al.*, 1999; Gomes *et al.*, 2000). Using a more sensitive probe and peptides that specifically target PS and PE on the outer leaflet, recent studies were able to show that PS and PE asymmetry on the plasma membrane is impaired in *drs2Δ* cells (Chen *et al.*, 2006). Deletion of *DRS2* from *dnf1Δdnf2Δ* cells exacerbates the PE and PS exposure on the cell surface (Pomorski *et al.*,

2003; Chen *et al.*, 2006). Since Drs2p primarily localize to the TGN, it does not seem to contribute directly to the flippase activity on the plasma membrane. Instead, its activity may affect plasma membrane asymmetry in a couple of indirect ways. First, loss of Drs2 may perturb exocytic vesicle formation at the TGN and accumulate cell-surface targeted proteins, including Dnf1p, in internal membranes (Saito *et al.*, 2004). Moreover, membrane asymmetry may be established at the TGN by Drs2p before the membrane flows to the plasma membrane by exocytosis. Although Drs2p primarily localizes to the TGN, it does travel to the plasma membrane occasionally (Saito *et al.*, 2004; Liu *et al.*, 2007). It remains under dispute whether Drs2p is active or not when present on the plasma membrane (Marx *et al.*, 1999; Saito *et al.*, 2004).

Recently, a translocase activity that flips NBD-PS, NBD-PE and NBD-PC to the cytosolic leaflet was detected on a specific class of post-Golgi secretory vesicles containing Drs2p (Alder-Baerens *et al.*, 2006). While vesicles purified from cells lacking Dnf1p and Dnf2p retained these activities, vesicles from cells deficient for Drs2p display impaired flippase activity for NBD-PS and, to a lesser extent, NBD-PE. In addition, translocation of all three lipid analogues is abolished on vesicles from cells deleted for both Drs2 and Dnf3. These data suggest that Dnf3 preferentially translocates NBD-PE and NBD-PC.

At first glance, all the defects observed in the null mutants support the proposed flippase activity for Drs2p family of proteins. However, caution should be taken in interpreting these data, because the P4-ATPase null mutants may indirectly perturb the

expression, localization or activity of other proteins that directly translocate the NBD-phospholipids. For example, *drs2Δ* was recently shown to perturb cholesterol uptake across the plasma membrane (Reiner *et al.*, 2006). Rather than directly transporting cholesterol, *drs2Δ* disrupts trafficking of the Aus1p and Pdr11p ABC transporters that appear to be the steroid transporters. The TGN membrane contains an energy-dependent flippase activity, which translocates NBD-labeled PS and PE from the luminal to the cytosolic leaflet (Natarajan *et al.*, 2004). TGN membranes from *drs2Δ* cells exhibited a substantial decrease in this flippase activity (Our unpublished data). However, it was impossible to conclude that Drs2p was directly responsible for this flippase activity, because deletion of *DRS2* causes mislocalization of several TGN resident enzymes and the TGN membranes purified from wild-type and *drs2Δ* cells have different protein compositions (Natarajan *et al.*, 2004). In addition, other mutants with disrupted protein trafficking pathways, such as clathrin and endocytosis null mutants, also exhibit a loss of membrane asymmetry that is comparable to *drs2Δ* or *dnf1Δdnf2Δ* cells (Chen *et al.*, 2006). More importantly, acute inactivation of *drs2-ts* or *chc-ts*, results in defects in protein transport but fails to induce a measurable loss of PS asymmetry. Thus, PS exposure in the *drs2-ts* or *chc-ts* cells seems to be a secondary consequence of a chronic disruption in protein and membrane trafficking to and from the plasma membrane,

Ideally, to determine if Drs2p is directly responsible for a flippase activity, one would like to compare membrane samples that differ only in the presence or absence of Drs2p activity. To achieve this goal, we applied two strategies with an in vitro flippase

assay using purified TGN membranes (Natarajan *et al.*, 2004). First, all Dnf proteins were deleted from the cell to eliminate possible contribution of these proteins to the TGN flippase activity. Second, to avoid the chronic defects associated with loss of Drs2p, cells harboring either WT or temperature-sensitive alleles of Drs2 were grown at permissive temperature. By inactivating Drs2p-ts after membrane isolation, we could compare the flippase activity in the same membranes before and after inactivation of this single protein. These data demonstrated that Drs2p was required for ATP-dependent translocation of NBD-PS across TGN membranes, whereas no active translocation of NBP-PE or NBD-PC was detected with the TGN membranes isolated from the *DRS2 dnf1,2,3Δ* cells. So far, these studies provide the most convincing evidence that Drs2p is indeed an APLT. Drs2p also appears to weakly translocate NBD-PE, an activity that can be detected when the protein is overexpressed.

3. The Cdc50-Lem3 Family

In yeast, an evolutionarily conserved family of proteins was found to associate with P4-ATPases as a potential noncatalytic subunit. Cdc50p, Lem3p and Crf1p are glycosylated proteins with two putative transmembrane domains. Cdc50p was originally identified in a screen for cold-sensitive, cell-division-cycle mutants, although it is still not clear why disruption of *CDC50* causes a cold-sensitive block in the cell cycle (Moir *et al.*, 1982). *CDC50* was later found to be allelic to *SWA4* (described in more detail below) (Chen *et al.*, 2006), a gene recovered in a genetic screen that also identified *DRS2* (Chen

et al., 1999). Similar to *drs2Δ*, *cdc50Δ* mutants are impaired in protein transport, cell polarity and PS asymmetry of the plasma membrane (Saito *et al.*, 2004; Chen *et al.*, 2006). Cdc50p physically interacts with Drs2p and the formation of this complex is required for ER exit of both proteins (Saito *et al.*, 2004; Chen *et al.*, 2006). Thus, the defects shown in *cdc50Δ* cells may be caused by loss of Drs2p function. Like Drs2p, Cdc50p localizes to the TGN and early endosome system, and accumulates on the plasma membrane when endocytosis is blocked, suggesting that the Drs2p-Cdc50p complex is maintained in post-ER compartments (Saito *et al.*, 2004).

LEM3 (also known as *ROS3*) was independently discovered in two screens for mutants that are either sensitive to an antifungal peptide that specifically binds to PE exposed on the outer leaflet of the plasma membrane, or are defective in the uptake of a toxic PC analogue (Kato *et al.*, 2002; Hanson *et al.*, 2003). Lem3p associates with and chaperones ER exit of Dnf1p and Dnf2p (Saito *et al.*, 2004; Furuta *et al.*, 2007). Thus, *lem3Δ* cells exhibit a deficiency of both Dnf1p and Dnf2p at the plasma membrane and consequently are impaired in translocation of NBD-PE and NBD-PC, but not NBD-PS across the plasma membrane (Kato *et al.*, 2002; Hanson *et al.*, 2003). Recently, a *lem3* mutant was isolated that exhibits normal association with Dnf1p and Dnf2p, an apparent normal localization of Dnf1p and nearly normal uptake of NBD-PE and NBD-PC, but displays a synthetic growth defect with *cdc50Δ* (Noji *et al.*, 2006). This result suggests that Lem3p may regulate Dnf1p or Dnf2p function in more ways than just chaperoning their ER exit, or that Lem3p has some functions totally independent of Dnf1p and Dnf2p.

Like Cdc50p and Lem3p, Crf1p is a potential noncatalytic subunit that associates with Dnf3p (Furuta *et al.*, 2007). Members of Cdc50p family proteins have also been identified in mammalian cells, although their function has not yet been explored (Katoh and Katoh, 2004; Osada *et al.*, 2006). Many interesting questions remain about the Cdc50-Lem3 family of proteins: Are they dedicated subunits with P4-ATPases, or do they chaperone other proteins as well? Is their only function to escort the P4-ATPases from the ER or are they playing an essential role in lipid translocation? Are they required for proper membrane insertion, folding or association with COP II for ER exit? More experiments need to be done to resolve these issues.

4. P4-ATPases in Other Organisms

The cold sensitive growth of yeast *drs2* mutants prompted the investigation of P4-ATPases in Arabidopsis, to determine if they are involved in chilling tolerance of plants (Gomes *et al.*, 2000). Among the 11 P4-ATPases in Arabidopsis, *ALA1* is the closest relative to *DRS2*, and is able to complement the cold sensitivity of the *drs2* yeast mutants. Downregulation of ALA1 in *Arabidopsis* greatly impairs cold tolerance of the plant. The actual mechanism by which P4-ATPases participate in cold tolerance is not known, but is likely to be related to membrane integrity or fluidity.

Two P4-ATPases from the rice blast fungus *Magnaporthe grisea*, *MgAPT2* and *MgPDE1,* are required for fungal pathogenicity. Whereas MgPde1 is required for appressoria formation, an outgrowth of the plasma membrane used to penetrate the plant

cuticle (Balhadere and Talbot, 2001), MgApt2 plays a role in exocytosis of virulence-associated proteins and plant tissue colonization (Gilbert *et al.*, 2006).

A P4-ATPase called LdMT from *Leishmania donovani*, a parasite that causes visceral leishmaniasis, is associated with drug resistance. Parasites defective in LdMT are resistant to miltefosine, an alkylphosphocholine drug used to treat leishmaniasis. In addition, overexpression of LdMT increases the uptake of miltefosine by the parasites (Perez-Victoria *et al.*, 2003). In fact, it was demonstrated earlier that yeast *lem3Δ* mutants are resistant to miltefosine (Hanson *et al.*, 2003). A functional homolog of *LEM3/ROS3* was recently discovered in *Leishmania donovani*, and given the name *LdRos3* (Perez-Victoria *et al.*, 2006).

Yeast P4-ATPases in Protein Transport

The Proposed Role of Flippases in Vesicle Biogenesis

It has long been proposed that flippases, whose primary function is to establish and maintain membrane asymmetry, may play a role in vesicle biogenesis. Translocation of phospholipids by flippases increases the surface area of the cytosolic leaflet and coincidently decreases that of the extracellular leaflet. As suggested by the bilayer couple hypothesis (Sheetz and Singer, 1974), this imbalance of phospholipid number would induce membrane curvature towards the cytosol and hence facilitate vesicle formation. In fact, induced bilayer asymmetry can trigger shape changes of spherical liposomes into

tubular and interconnected vesicular structures (Farge and Devaux, 1992). Sphingomyelinase treatment, which changes the composition of the plasma membrane outer leaflet, induces an ATP-independent budding of functional endocytic vesicles (Zha *et al.*, 1998). Moreover, exogenous PS or PE, when incorporated in the outer leaflet of the plasma membrane and then pumped to the inner leaflet, significantly enhances endocytosis (Farge *et al.*, 1999). Conversely, addition of lyso–α-PS or a cholesterol derivative, which cannot be translocated across the plasma membrane and remains in the outer layer, inhibits endocytosis. Although these data described above are in agreement with the role of flippases in vesicle budding, they all deal with artificial membranes or perturbed cell systems. As we will discuss later, the requirements for yeast P4-ATPases in protein trafficking provide the first line of evidence that cells use a bilayer-couple mechanism under normal physiological conditions to generate vesicles.

The role of generating the tight membrane curvature required in vesicle formation has traditionally been assigned to vesicle coat proteins like clathrin. Structural studies have revealed an intrinsic curvature in clathrin triskelia (Smith *et al.*, 1998; Musacchio *et al.*, 1999) and clathrin can self-assemble into polyhedral baskets in the absence of lipids. In an in vitro system, clathrin-coated buds and even clathrin-coated vesicles can form on appropriate protein-free liposomes, with a minimal requirement for ARF, GTP, adaptor proteins and clathrin (Takei *et al.*, 1998; Zhu *et al.*, 1999). However, recent theoretical studies estimated that the rigidity of clathrin-adaptor protein complexes is of the same order of magnitude as the resistance of lipid membranes to bending (Nossal, 2001). Thus,

clathrin assembly is unlikely to provide sufficient energy to drive membrane curvature in vivo, and instead, it may serve to stabilize an already curved membrane and prevent the membrane from collapsing back into a planar form. In the "Brownian ratchet" model, membranes spontaneously fluctuate, resulting in transient membrane invaginations, which could be captured by the clathrin lattice to form vesicles (Shraiman, 1997). Consistent with this model, additional accessory proteins with the ability to deform the membrane have been identified that participate in CCV formation, including BAR-domain containing proteins, the epsins (Itoh and De Camilli, 2006; Ren *et al.*, 2006) as well as flippases.

Requirements for P4-ATPases in Vesicle-mediated Protein Transport

Drs2p was first implicated in vesicle-mediated protein transport by a genetic screen for mutants defective in *ARF*-dependent vesicle biogenesis (Chen *et al.*, 1999). ARF (ADP-ribosylation factor) is a small GTP-binding protein that regulates coat protein assembly on transport vesicles (Randazzo *et al.*, 2000; D'Souza-Schorey and Chavrier, 2006). ARF acts as a molecular switch by cycling between active GTP-bound and inactive GDP-bound forms. The GDP-bound form is largely soluble, whereas the GTP-bound form exposes its myristoylated N-terminus and associates with the membrane via both the myristoyl group and several hydrophobic and basic residues from the N-terminal α-helix. Binding and hydrolysis of GTP by ARF require catalytic assistance from two classes of proteins. Guanine nucleotide exchange factors (GEFs) promotes the

exchange of GDP for GTP, while GTPase-activating proteins (GAPs) stimulate the hydrolysis of GTP by ARF to form GDP.

In yeast *Saccharomyces cerevisiae*, ARF is encoded by two genes, *ARF1* and *ARF2*, which are 96% identical in sequence and redundant in function. Single deletion of either gene has little effect on cell growth, but strains with the double deletion are inviable (Stearns *et al.*, 1990a). In wild type cells, Arf2p is only expressed at 10% of the level of Arf1p, and *arf2Δ* shows a wild-type phenotype. However, *arf1Δ* exhibits partial defects in protein secretion and Golgi-specific glycosylation, and the morphology of Golgi and endosomes is substantially changed in *arf1Δ* mutants (Stearns *et al.*, 1990b; Gaynor *et al.*, 1998).

The critical role of ARF in protein trafficking is to directly bind and recruit vesicle coats, which then capture cargos on the donor membrane and drive the formation of transport vesicles. ARF-dependent coats include the COPI coatomer complex, clathrin/adaptor protein (AP)-1, clathrin/GGA (Golgi associated, γ-ear homologous, ARF interacting) proteins, AP-3 and AP-4 complexes. COPI vesicles participate in the intra-Golgi transport and the retrograde transport from the Golgi complex to the endoplasmic reticulum (ER), whereas clathrin coated vesicles (CCV) mediate protein traffic from the plasma membrane to endosomes and between the *trans*-Golgi network (TGN) and endosomes. The polyhedral clathrin coats are assembled from triskelions, which are three-legged structures consisting of three clathrin heavy chains and three light chains (Brodsky *et al.*, 2001). The interaction between the clathrin basket and the

membrane surface of the vesicle is mediated by adaptor proteins, which also facilitate cargo selection and bind additional accessory proteins (Robinson, 2004). A number of different adaptor proteins have been discovered to date, among which AP-1 and GGA are two major classes of adaptors that function in clathrin-mediated transport between the TGN and endosomes. While GGA proteins are monomeric adaptors, AP-1 is composed of two large subunits (β1 and γ), one medium subunit (μ1), and a small subunit (σ1). AP-3 and AP-4 are heterotetrameric complexes with homology to AP-1, but they appear to be able to function independently of clathrin. AP-3 is involved in protein transport from endosomes and the TGN to lysosomes. AP-4, absent in yeast, has been implicated in sorting of cargos destined for lysosomes and the basolateral membrane in different cell types.

The fact that a single ARF protein is involved in multiple protein transport pathways suggests that other regulators exist to coordinate its function. To better understand the function of ARF in vivo, the Graham lab performed a genetic screen for mutant alleles that displayed synthetic lethality with *arf1Δ*. This genetic interaction often implies that the two gene products involved play roles in the same pathway or in parallel pathways. Seven complementation groups were discovered in the screen and named *SWA1-7* for synthetic lethality with *arf1Δ*. *SWA5* was identified as clathrin heavy chain gene (*CHC1*), providing the first genetic evidence for a functional interaction in vivo between ARF and clathrin (Chen and Graham, 1998). *SWA2* encodes a DnaJ protein and is the yeast ortholog of auxilin, a clathrin binding protein that recruits Hsc70p via its

DnaJ domain to uncoat CCVs (Gall *et al.*, 2000). *SWA7* is allelic to *NMT1*, which encodes the yeast N-myristoyltransferase (Our unpublished data). Myristoylation of the N-terminus of ARF is critical for its function. Unexpectedly, *SWA3* and *SWA4* were found to be allelic to *DRS2* and *CDC50* respectively (Chen *et al.*, 1999; Chen *et al.*, 2006). Drs2p and Cdc50p were later shown to be required for clathrin function in the TGN-early endosome system. Thus, most of the genes identified so far from this genetic screen are involved in regulation of clathrin dynamics, demonstrating that the screen is biased towards clathrin function.

Drs2p localizes to the late Golgi and early endosomal membranes, where CCVs are actively budded. *drs2Δ* is synthetically lethal with *arf1* and *chc-ts* alleles and exhibits a marked reduction in CCVs isolated from subcellular fractions (Chen *et al.*, 1999). Similar to clathrin mutants, *drs2Δ* accumulates swollen Golgi cisternae and mislocalizes the TGN resident protein Kex2p, which normally cycles between the TGN and endosomal compartments to maintain its steady-state localization. Therefore, clathrin and Drs2p seem to participate in the protein traffic between the TGN and endosomes. In yeast, protein transport to the plasma membrane is mediated by two classes of exocytic vesicles, which are distinguished by density and cargos. Specifically, both clathrin and Drs2p are required for the formation of the dense exocytic vesicles which carry invertase and acid phosphatase (Gall *et al.*, 2002). Moreover, Drs2 has genetic interactions with Gcs1, an ARF-GTPase activating protein, and physically associates with Rcy1, an F-box protein involved in recycling of cargo from the early endosome back to the TGN (Sakane

et al., 2006; Furuta *et al.*, 2007). A role for Drs2p in the early endosome to TGN pathway has been proposed but still needs further investigation. In summary, most available data imply that Drs2p functions to facilitate CCV formation from the TGN and endosomal membranes.

A *drs2* allele bearing a truncation of the carboxyl terminal cytosolic tail (C-tail) can not complement the cold-sensitive growth and trafficking defects of *drs2*Δ, suggesting that the C-tail makes an essential contribution to Drs2 function (Chantalat *et al.*, 2004; Liu *et al.*, 2007). Three motifs have been mapped within the Drs2 C-tail thus far. The Gea2p interaction motif (GIM) directly interacts with the catalytically important Sec7 domain of the ARF-GEF Gea2p (Chantalat *et al.*, 2004). Adjacent to GIM, there is a region highly conserved among all, including mammalian, Drs2p homologues. Function of this conserved motif is still unknown, although deletion of this motif abrogates function in vivo, suggesting that the conserved motif is primarily responsible for the essential function of the C-tail (Chantalat *et al.*, 2004). At the membrane distal end of the C-tail there are two $NPFX_{(1,2)}D$ motifs, which are potential endocytosis signals (Tang *et al.*, 1996; Valdivia *et al.*, 2002). How the C-tail contributes to the function of Drs2p is still not clear, but it likely mediates protein interactions and/or TGN localization.

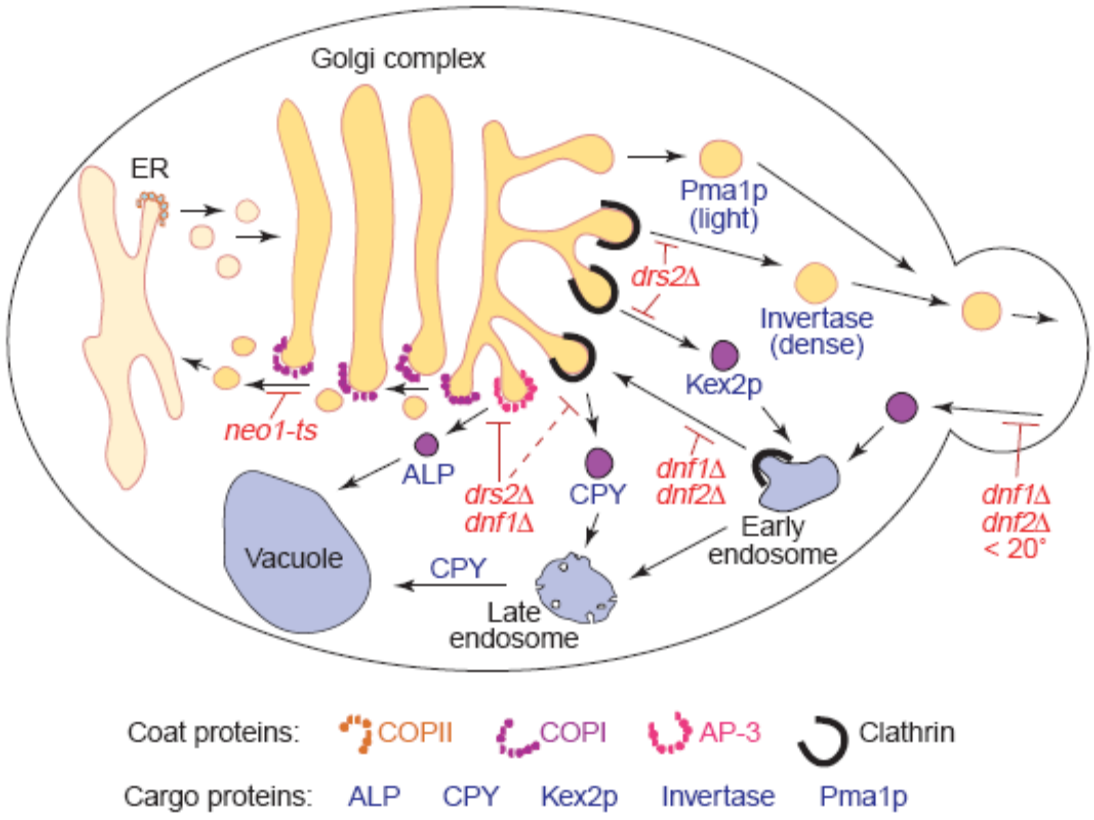

Figure 1-3: Vesicle-mediated protein-transport pathways that require Drs2p family P4-ATPases. (From Graham, 2004)

Studies have been done to determine if other members of yeast P4-ATPase family participate in protein traffic. *NEO1* is essential for viability, so conditional alleles of *NEO1* were isolated and studied (Hua and Graham, 2003; Wicky *et al.*, 2004). After shifting to nonpermissive temperatures, *neo1-ts* mutants show several defects in common with COPI mutants, including a cargo-specific defect in secretion, aberrant glycosylation of cargo in the Golgi and mislocalization of Rer1-GFP to vacuole (Hua and Graham, 2003). Rer1p is an integral membrane protein that continuously cycles between the ER and the Golgi. These data, in combination with the synthetic lethality between *neo1-ts* and COPI mutations, suggest that Neo1p is required for a retrograde transport pathway from the Golgi to the ER. In another study, *neo1-ts* mutants exhibited fragmented vacuole and defects in endocytosis (Wicky *et al.*, 2004). Deletion of *ARL1*, an ARF like protein that functions within the endosomal/Golgi system, rescues the temperature sensitivity of *neo1-ts*. In addition, Neo1p have genetic and physical interactions with Ysl2p, a potential guanine nucleotide exchange factor for Arl1p. Thus, Neo1p seems to also play a role in protein traffic within the endosomal/Golgi system.

Snc1p is an exocytic v-SNARE, which cycles in the TGN -> plasma membrane -> early endosome ->TGN pathway, but primarily localizes to the plasma membrane at steady-state. Disruption of *DNF1* and *DNF2* causes accumulation of Snc1p in internal membranes but has no affect on the trafficking of Ste2p, the a-factor receptor, which travels the endocytic pathway to the vacuole for degradation (Hua *et al.*, 2002). Therefore, Dnf1p and Dnf2p play overlapping roles in an early endosome to TGN pathway.

Interestingly, *dnf1Δ dnf2Δ* mutant also displays a cold-sensitive defect in the internalization step of endocytosis, which is exacerbated by additional deletion of *DRS2* (Pomorski *et al.*, 2003). Deletion of both *DNF1* and *DRS2* abrogates transport of alkaline phosphatase to the vacuole, whereas the *dnf1Δ* or *drs2Δ* single mutants transport alkaline phosphatase relatively normally (Hua *et al.*, 2002). Thus, Dnf1p and Drs2p have redundant functions in this AP-3 mediated TGN to vacuole pathway. Remarkably, members of Drs2p family participate in all the protein transport pathways, in which ARF seems to be involved.

Objectives of This Study

The above evidence suggested that P4-ATPases function as a phospholipid translocases, and are important for establishing membrane asymmetry. These ATPases also play a critical role in vesicle mediated protein trafficking. The mechanism for coupling a specific P4-ATPase to specific transport pathways requires the appropriate localization and regulated local interactions. However, the precise mechanism that P4-ATPases use in facilitating vesicle biogenesis is unclear.

In Chapter II of this thesis, the trafficking itineraries of Drs2p and Dnf1p are determined by studying the location of these proteins in various mutants defective for different trafficking pathways. Multiple endocytosis signals were defined in Drs2p, including two NPFXDs near the C-terminus and PEST-like sequences near the N-terminus for ubiquitin (Ub)-dependent endocytosis. Surprisingly, in a mutant that is

constitutively defective for Ub-dependent endocytosis but not for NPFXD-dependent endocytosis, Drs2p must be internalized through the NPFXD/Sla1p pathway to sustain viability. Thus, Drs2p seems to be an essential endocytic cargo for the NPFXD/Sla1p system.

In Chapter III, we examined which clathrin adaptors Drs2p preferentially acts in conjunction with, and found that Drs2p and AP-1/clathrin function together in a TGN-early endosome pathway distinct from the TGN to late endosome pathway mediated by GGA/clathrin. A physical interaction was observed between Drs2p and AP-1. However, deletion of *DRS2* does not seem to affect recruitment of AP-1 to the TGN membrane, suggesting that Drs2p is required at late stages in clathrin-coated vesicle formation. Interestingly, disruption of AP-1 alters Drs2p trafficking but not its steady-state localization to the TGN, demonstrating that Drs2p is a cargo of AP-1 specifically for the anterograde TGN to early endosome pathway.

Based on these observations, we propose that at the TGN, Drs2p facilitates formation of vesicles coated with clathrin and AP-1 by generating positive membrane curvature. Drs2p embarks within these AP-1/clathrin-coated vesicles for delivery to the early endosome, but uses an AP-1 independent pathway for retrieval back to the TGN.

CHAPTER II

YEAST P4-ATPASES DRS2p AND DNF1p ARE ESSENTIAL CARGOS OF THE NPFXD/SLA1p ENDOCYTIC PATHWAY

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Introduction

Drs2p is a resident P-type ATPase of the yeast *trans*-Golgi network (TGN) that is required for vesicle-mediated protein transport from this organelle. Most well characterized P-type ATPases are cation pumps that control the concentration of ions in both intracellular and extracellular spaces (for example, the Na^+/K^+ ATPase, Ca^{++} ATPase, and H^+/K^+ ATPase) (Kuhlbrandt, 2004). Drs2p, in contrast, is the founding member of a large P-type ATPase subfamily, called P4-ATPases (Catty *et al.*, 1997), that are proposed to translocate phospholipid rather than ions. This flippase activity is responsible for translocating specific phospholipid molecules from the exoplasmic leaflet to the cytosolic leaflet to establish asymmetry of the membrane bilayer (Pomorski *et al.*, 2003; Graham, 2004; Pomorski *et al.*, 2004; Paulusma and Oude Elferink, 2005; Devaux *et al.*, 2006). For Drs2p, ATPase activity and presumably phospholipid translocation are essential, because mutation of the aspartic acid that forms an aspartyl-phosphate intermediate during catalysis (D560N) renders Drs2p nonfunctional *in vivo* (Chen *et al.*, 1999). In

addition, after shifting to the nonpermissive temperature, a *drs2* temperature-sensitive (ts) allele causes a rapid loss of exocytic vesicle formation *in vivo* (Gall *et al.*, 2002) and the loss of an ATP-dependent phosphatidylserine (PS) flippase activity in purified Golgi membranes containing Drs2-ts (Natarajan *et al.*, 2004). Mammalian homologues of Drs2p include the chromaffin granule ATPase II (now called ATP8A1) (Tang *et al.*, 1996), which is likely responsible for a PS translocase activity observed with these exocytic vesicles (Zachowski *et al.*, 1989), and FIC1 (ATP8B1), for which mutations in humans cause an impairment of bile flow through the liver (cholestasis) (Bull *et al.*, 1998; Klomp *et al.*, 2004). In addition, deletions removing the mouse *Atp10c* gene cause diet-induced obesity and type 2 diabetes phenotypes (Dhar *et al.*, 2004b). P4-ATPases are also agriculturally important, as they are required for pathogenesis of the rice blast fungus *Magneporthe griseus* (Balhadere and Talbot, 2001; Gilbert *et al.*, 2006) and growth of plants at cold temperatures (Gomes *et al.*, 2000).

The yeast Drs2p family of P4-ATPases, including Neo1p, Dnf1p, Dnf2p and Dnf3p, are all involved in protein transport in the secretory and endocytic pathways, but at different stages (Graham, 2004). Drs2p and the Dnf proteins form an essential group and at least one of these proteins must be present to sustain yeast viability. The Drs2/Dnf P4-ATPases have both overlapping and non-overlapping functions in protein transport (Hua *et al.*, 2002). Strains carrying a deletion of *DRS2* (*drs2*Δ) are viable, but strongly cold-sensitive for growth, and exhibit defects in forming one of the two classes of exocytic vesicles (high density) targeted to the plasma membrane (Gall *et al.*, 2002).

The *drs2*Δ mutant also exhibits defects in protein trafficking between the TGN and early endosome that are comparable to clathrin mutant phenotypes (Chen *et al.*, 1999; Hua *et al.*, 2002). Thus, the Dnf ATPases cannot compensate for loss of Drs2p in these pathways and moreover, deletion of all three *DNF* genes does not perturb these Drs2p-dependent pathways. Dnf1p and Dnf2p are 69% identical in amino acid sequence, localize to the plasma membrane and internal membranes (TGN, early endosomes and transport vesicles), and have redundant functions in the internalization step of endocytosis (at cold temperatures) and an early endosome to TGN transport pathway traveled by the Snc1p soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) (Hua *et al.*, 2002; Pomorski *et al.*, 2003). Drs2p is also required for Snc1p recycling suggesting that Drs2p and Dnf1,2p are partially redundant in the this pathway. In addition, Drs2p and Dnf1p have redundant functions in the AP-3-dependent transport of alkaline phosphatase from the TGN directly to the vacuole. The individual *drs2*Δ or *dnf1*Δ mutants show little to no defect in the AP-3 pathway while this pathway is blocked in the *drs2*Δ *dnf1*Δ double mutant (Hua *et al.*, 2002). The mechanism for coupling a specific P4-ATPase to a specific protein transport pathway is unclear, but likely involves translocation substrate specificity, unique protein interactions and appropriate localization.

Localization of Drs2p to the Golgi requires interaction with the Cdc50p chaperone subunit in order for the Drs2p/Cdc50p complex to exit the ER (Saito *et al.*, 2004). In *cdc50*Δ, Drs2p is retained in the ER and these cells show protein transport

defects at the TGN comparable to *drs2*Δ (Chen *et al.*, 2006). Similarly, the Cdc50p homolog Lem3p (also called Ros3p) is required for transport of Dnf1p and presumably Dnf2p to the plasma membrane and so *lem3*Δ phenocopies *dnf1*Δ *dnf2*Δ (Saito *et al.*, 2004). The Drs2p carboxyl-terminal cytosolic tail (C-tail) makes an essential contribution to its function, apparently by mediating protein interactions and/or TGN localization. Drs2p is linked to the vesicle budding machinery by a direct interaction between the ARF-GEF Gea2p and a short motif in the C-tail (called GIM, for Gea2p interaction Motif) (Chantalat *et al.*, 2004). Adjacent to GIM, there is a region highly conserved among all, including mammalian, Drs2p homologues. Function of this conserved motif (CM) is still unknown, although a mutational analysis suggested that the CM is primarily responsible for the essential function of the C-tail (Chantalat *et al.*, 2004). At the membrane distal end of the C-tail there are two $NPFX_{(1,2)}D$ motifs (hereafter referred to as NPFXD), which are potential endocytosis signals (Tang *et al.*, 1996; Howard *et al.*, 2002).

In yeast, two types of endocytosis signals have been characterized that recruit membrane proteins into a clathrin/actin-based endocytic pathway for internalization from the plasma membrane: sequences that mediates phosphorylation and ubiquitination of cargo, such as PEST-like sequences, and the NPFXD motif (Tang *et al.*, 1996; Howard *et al.*, 2002; Wang *et al.*, 2003). The NPFXD signal is recognized by the Sla1p subunit of an endocytic coat complex consisting of clathrin, Pan1p, End3p, Sla2p/End4p (related to mammalian Hip1R) and Sla1p (related to mammalian CIN85 and intersectin) (Tang *et*

al., 1996; Howard *et al.*, 2002; Newpher *et al.*, 2005; Kaksonen *et al.*, 2006). Pan1p, a member of the Eps15 family of modular scaffolding proteins, interacts with the clathrin binding proteins AP180 and epsin, and also binds to and stimulates the ARP2/3 complex (Wendland and Emr, 1998; Duncan *et al.*, 2001; Aguilar *et al.*, 2003). Therefore, Pan1p has the capacity to link adaptor-bound cargo proteins to clathrin-coated pits and sites of actin assembly. Pan1p, End3p and actin assembly are required for both ubiquitin (Ub)-dependent and NPFXD-dependent endocytosis, although Sla1p is only required for endocytosis of cargo bearing the NPFXD signal (Howard *et al.*, 2002; Miliaras *et al.*, 2004). Not only does Drs2p have the potential to physically interact with the Sla1p/Pan1p/End3p complex, it is also functionally linked to this complex as *drs2*Δ is synthetically lethal with the temperature-conditional *pan1-20* allele (Chen *et al.*, 1999). However, the nature of these relationships between Drs2p and this endocytic complex is unclear.

Drs2p exhibits a steady-state localization to the TGN, although recent reports showed accumulation of Drs2p on the plasma membrane of a verprolin (*vrp1*) mutant and the presence of Drs2p in exocytic vesicles, suggesting that Drs2p transits the plasma membrane as part of its trafficking itinerary (Saito *et al.*, 2004; Alder-Baerens *et al.*, 2006). Dnf1p also appears to cycle between the exocytic and endocytic pathways (Hua *et al.*, 2002; Saito *et al.*, 2004). In this work, we further examined the trafficking itinerary of Drs2p and Dnf1p and tested whether the NPFXD motifs contribute to the function and localization of these P4-ATPases.

Materials and Methods

Media and Strains

Yeast were grown in standard rich medium (YPD) or synthetic defined (SD) minimal media containing the required nutritional supplements (Sherman, 1991). Yeast transformations were performed using the lithium acetate method. *Escherichia coli* strains DH5α and XL1-Blue were used for plasmid construction and amplification.

Yeast strains used in this study are summarized in Table 2-1. The yeast knockout strain collection was originally purchased from Research Genetics (Huntsville, AL), which is now Resgen, Invitrogen Corporation (Carlsbad, CA). Strains carrying multiple disruptions were generated by standard genetic crosses and tetrad dissection. The genotype of each spore was determined by a PCR method as described by the Saccharomyces genome deletion project (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html). Strains expressing Myc and HA tagged Dnf1p were generated by PCR-based targeting into BY4741 and BY4741 *sla1*Δ using pPF6a-13Myc-HisMX6 or pPF6a-3HA-HisMX6 as the PCR template (Longtine *et al.*, 1998). Transformants were selected on SD plates without histidine and the integrated tags were confirmed by PCR.

Plasmid Construction

Plasmids used in this study are listed in Table 2-2. To generate pGBT9-Drs2CT

used in the two-hybrid test, a BamHI fragment from pDHS279 (Chantalat *et al.*, 2004) containing the Drs2p C-tail (amino acids 1230-1355) was cloned into the pGBT9 BamHI site, and the orientation was confirmed by PCR. PCR products used to generate *DRS2* C-tail truncation plasmids pRS315-Drs2-ΔCT, pRS315-Drs2-ΔEnd, pRS315-Drs2-ΔNPF2, pRS315-Drs2-ΔNPF and pRS315-Drs2-Δ1274 were produced using a forward primer (CAGCTGATATAGCTCTTGG) that anneals 5' of an endogenous NcoI site in *DRS2* and reverse primers with a stop codon and MluI site added to the 3' end. PCR products were then used to replace the NcoI/MluI region of pRS315-*DRS2*. The *DRS2* internal deletion mutant pRS315-Drs2-ΔCM was generated from the truncation plasmid pRS315-Drs2-Δ1274 with the C-terminal sequence added as a MluI/SalI PCR fragment. A megaprimer PCR method (Barik and Galinski, 1991) was used to introduce point mutations into the MscI/SalI fragment of the *DRS2* gene to produce plasmids pRS315-Drs2-NPW1 and pRS315-Drs2-NPW2. Using similar methods, pRS315-Drs2-ΔGIM-NPW1,2 was generated from pSC33 (pRS315-Drs2-ΔGIM). Sequencing of the resulting plasmids indicated that the specific mutations were introduced with no additional mutations. All other clones generated from the PCR fragments described below were also sequenced for confirmation.

The full-length *DNF1* gene was cloned by PCR amplification using primers JN01F (CTATGTAATCACCTACTTCCC) and GR02R (CTGGAGTGCTACATGAGCC) and subcloned into pRS416 after treating both the vector and PCR product with SpeI and HindIII. The SpeI/XhoI fragment of pRS416-*DNF1* was inserted into SpeI/XhoI site of

pRS313 to produce pRS313-*DNF1*. Site-directed mutagenesis of *DNF1* to produce pRS313-Dnf1-NAI was carried out in plasmid pRS313-*DNF1* using the QuickChange protocol (Stratagene, La Jolla, CA).

For construction of GFP-DRS2, a 1.3kb SpeI/ClaI fragment from pGOGFP (Cowles *et al.*, 1997) consisting of the *PRC1* promoter and GFP(S65T) was inserted into pRS416 to generate pRS416-GFP. The plasmid pRS315-*DRS2* was used as a PCR template with primers SalI-Drs2-F (ACGTAGTCGACAATGACGACAGAGAAACCCC C) and Drs2-CT-R (CCCCTCGAGGTCGACGGTA) to generate a 3.7kb fragment that placed SalI sites at both the start and end of the *DRS2* coding region. This fragment was subcloned into SalI site of pRS416-GFP, creating the plasmid pGFP-*DRS2*. To eliminate mutations produced by PCR, most of the *DRS2* coding sequence in pGFP-*DRS2* was further replaced by an AgeI/ClaI fragment from pRS315-*DRS2*. This form of GFP-DRS2 fully complemented the cell growth defect of *drs2*Δ at 20°C. To generate C-terminal tail mutated GFP-DRS2 (pGFP-Drs2-ΔNPF2, pGFP-Drs2-ΔNPF or pGFP-Drs2-NPW1,2), PCR amplifications of different regions of DRS2 C terminus were used to replace the NheI/ClaI region of pGFP-*DRS2*. To generate N-terminal truncated GFP-DRS2 (pGFP-Drs2-ΔN2 or pGFP-Drs2-ΔN3), primers Drs2ΔN2F (GATGAGATCTCATGAAAATCTATTTATGAGCAAT) or Drs2ΔN3F (GACTGAGATCTCGAGCAGTCAAGCCTCCC) were used with Drs2NR (GAACCACAGTTGGGGTATCAG) to produce fragments to replace the BglII region of pGFP-*DRS2*. To generate pGFP-Drs2-ΔN3-NPW1,2, the 1.44kb NheI/ClaI fragment

from pGFP-Drs2-NPW1,2 was used to replace the corresponding sequence in pGFP-Drs2-ΔN3. The *PRC1* promoter is stronger than the *DRS2* promoter, and so to avoid accumulation of GFP-Drs2p in the ER (Saito *et al.*, 2004),we co-transformed yeast strains with a multicopy vector carrying *CDC50* (pRS425-*CDC50*).

To generate pGFP-*CDC50*, pRS315-*CDC50* was used as template with primers CDC50KpnIF (CGGTACCGTTTCATTGTTCAAAAGAGGTA) and CDC50KpnIR (CGGTACCCACAAATACCTACAGGCACTA) to produce a 1.2 kb fragment with KpnI sites at both ends of the *CDC50* coding region. The fragment was subcloned into the KpnI site of pRS416-GFP.

Microscopy

Cells were observed using an Axioplan microscope (Carl Zeiss, Thornwood, NY). Fluorescent images were captured with a charge-coupled-device camera and processed with Metamorph 4.5 software (Molecular Devices, Sunnyvale, CA). To visualize GFP-tagged proteins, cells were grown to early-mid logarithmic phase, harvested and resuspended in imaging buffer (10 mM Tris-HCl, pH 7.4, and 2% glucose). Cells were mounted on glass slides and observed immediately using a GFP (green) bandpass filter set.

To study the kinetics of GFP-Drs2p transport to the plasma membrane, mid-log phase cells were collected and resuspended in SD medium containing 200 μM latrunculin A. Samples of cells were harvested at different time points and imaged. To label

endosomes, we incubated cells in ice-cold SD medium containing 10 μg/ml FM4-64 (Invitrogen) for 20 min. Cells were washed twice with cold medium without FM4-64 and then incubated for 30 min at 30°C before microscopic examination.

Subcellular Fractionation and Immunological Methods

For subcellular fractionation experiments, ~25 OD_{600} units of each strain were grown in YPD media to an OD_{600} of 0.5-1.0. The cells were harvested and converted to spheroplasts in HB buffer (1.4 M sorbitol, 50 mM KPi, pH 7.5, 10 mM NaN_3, 10 mM NaF, 40 mM β-mercaotpethanol), by using 200 μg/ml Zymolyase 100T (MP Biomedicals, Irvine, CA) at 30°C for 30min. The spheroplasts were washed twice with HB buffer and lysed by resuspension in triethanolamine (TEA) lysis buffer (0.5 M sorbitol, 25 mM TEA, pH 8.0, 1 mM EDTA) containing 1x complete protease inhibitor cocktail (PIC) lacking EDTA (Roche Diagnostics, Basel, Switzerland). The extract was centrifuged at 400 x g for 5 min and the resulting supernatant at 9,000 x g for 15 min in a refrigerated microcentrifuge. After each centrifugation step, the supernatant was transferred to a separate tube, and the pellet was resuspended in an equal volume of TEA lysis buffer supplemented with PIC. SDS/urea buffer was added to 1X (20 mM Tris-HCl pH6.8, 4 M urea, 0.05 mM EDTA, 0.5% β-mercaptoethanol, 2.5% SDS, 0.125% bromophenol blue), and the samples were heated at 65°C for 10 min before electrophoresis.

Immunoblotting and immunofluorescence experiments were performed as described previously (Chen *et al.*, 1999). The 9E10 mouse monoclonal c-Myc antibody

(Oncogene Research Products, Darmstadt, Germany) was used at 1:2000 for Western Blot and 1:100 for immunofluorescence. Polyclonal rabbit anti-Pma1p antibody was a gift from Amy Chang (University of Michigan, Ann Arbor, MI), and was used at 1:1000 to detect Pma1p by Western Blot. Polyclonal rabbit anti-G6PDH antibody (Sigma-Aldrich, St. Louis, MO) was used at 1:10,000 dilutions. Alexa-594 goat anti-mouse IgG (Invitrogen) was used at 1:200 as secondary antibodies for immunofluorescence.

Table 2-1: Yeast strains used in chapter II

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Research Genetics
BY4742	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	Research Genetics
BY4741 YBL007C	BY4741 *sla1Δ::KanMX6*	Research Genetics
KLY011	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 sla1Δ::KanMX6*	This study
BY4742 YNL084C	BY4742 *end3Δ::KanMX6**	Research Genetics
KLY201	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 end3Δ::KanMX6*	This study
BY4742 YLR337C	BY4742 *vrp1Δ::KanMX6*	Research Genetics
BY4741 YPR173C	BY4741 *vps4Δ::KanMX6*	Research Genetics
BY4741 YNR006W	BY4741 *vps27Δ::KanMX6*	Research Genetics
ZHY615D1C	*MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 drs2Δ::KanMX6*	Hua *et al*., 2002
ZHY615M2D	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 drs2Δ::KanMX6*	Hua *et al*., 2002
ZHY2149D	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 drs2Δ::KanMX6 dnf1Δ::KanMX6*	Hua *et al*., 2002
KLY041	BY4742 *DNF1::13XMYC*	This study
KLY054	KLY011 *DNF1::13XMYC*	This study
KLY035	*MATα his3Δ1 leu2Δ0 ura3Δ0 drs2Δ::KanMX6 sla1Δ::KanMX6*	This study
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al*., 1988
SEY6211	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade suc2-Δ9*	Robinson *et al*., 1988
SEY6210 *drs2Δ*	SEY6210 *drs2Δ::TRP1*	Chen *et al*., 1999
TGY1907	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 pan1-20*	Chen *et al*., 1999
TGY1906	*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 pan1-20*	Chen *et al*., 1999
TGY1912	*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 end4-1*	Chen *et al*., 1999
ZHY823	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 pan1-20 drs2Δ::TRP1 (pRS416-DRS2)*	This study
CCY2808	*MATa leu2 ura3-52 his3 trp1-Δ901 ade2 ade3 arf1Δ::HIS3 drs2-2 (pRS416-DRS2)*	Chen & Graham, 1998
YJF1165	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James *et al*., 1996

* An extragenic suppressor mutation was found in this strain.

Table 2-2: Plasmids used in chapter II

Plasmid	Description	Reference or Source
pRS416 GFP-*SNC1*	*GFP-SNC1 URA3 CEN*	Lewis *et al*., 2000
pGO41	*GFP-ALP URA3 2*	Cowles *et al.*, 1997
pRS426-*KEX2*-GFP	*KEX2-GFP URA3 2*	Gift from Tom Vida
pSM1493	*STE6-GFP URA3 2*	Kelm *et al.*, 2004
pRS315-*DRS2*	*DRS2 LEU2 CEN*	Chen *et al.*, 1999
pDRS2(D560N)	*drs2(D560N) LEU2 CEN*	Chen *et al.*, 1999
pRS315-Drs2-ΔCT	*drs2-Δ1259-1355 LEU2 CEN*	Chantalat *et al.*, 2003
pRS315-Drs2-ΔEnd	*drs2-Δ1337-1355 LEU2 CEN*	Chantalat *et al.*, 2003
pRS315-Drs2-ΔNPF2	*drs2-Δ1315-1355 LEU2 CEN*	Chantalat *et al.*, 2003
pRS315-Drs2-ΔNPF	*drs2-Δ1310-1355 LEU2 CEN*	Chantalat *et al.*, 2003
pRS315-Drs2-ΔCM	*drs2-Δ1274-1283 LEU2 CEN*	Chantalat *et al.*, 2003
pSC33 [pRS315-Drs2- GIM]	*drs2-Δ1250-1270 LEU2 CEN*	Chantalat *et al.*, 2003
pRS315-Drs2-ΔGIM-ΔNPF	*drs2-Δ1250-1263, Δ1310-1355 LEU2 CEN*	This study
pRS315-Drs2-NPW1	*drs2(F1313W) LEU2 CEN*	This study
pRS315-Drs2-NPW2	*drs2(F1335W) LEU2 CEN*	This study
pRS315-Drs2-NPW1,2	*drs2(F1313W,F1335W) LEU2 CEN*	This study
pRS315-Drs2-ΔGIM-NPW1,2	*drs2-Δ1250-1270, F1313W, F1335W LEU2 CEN*	This study
pGBT9-Drs2-CT	*DRS2(aa 1230-1355) TRP1 2*	This study
pZH429 [pGBT9-Drs2-CTΔNPF]	*DRS2(aa 1230-1310) TRP1 2*	This study
pGBT9-Drs2-CT-NPW1,2	*DRS2(aa 1221-1355, F1313W,F1335W) TRP1 2*	This study
pGBT9-Drs2-CT-ΔGIM	*DRS2(aa 1221-1355, Δ1250-1263) TRP1 2*	This study
pPAN1.1 [Pan1-EH]	*PAN1(aa 96-713) TRP1 2*	Wendland and Emr, 1998
pGAD-SLA1-555 [Sla1-SHD1]	*SLA1(aa 471-555*) *LEU2 2*	Gift from Gregory Payne Lab
pGAD-SLA1charged [Sla1-Charged]	*SLA1(aa 511-855*) *LEU2 2*	Gift from Gregory Payne Lab
pRS313-*DNF1*	*DNF1 HIS3 CEN*	This study
pRS313-Dnf1-NAI	*DNF1(PF147,148AI) HIS3 CEN*	This study
pGOGFP	*CPY5'UTR-GFP URA3 2*	Cowles *et al.*, 1997
pRS416-GFP	*CPY5'UTR-GFP URA3 CEN*	This study
pGFP-*DRS2*	*CPY5'UTR-GFP-DRS2 URA3 CEN*	This study
pGFP-Drs2-ΔNPF2	*CPY5'UTR-GFP-drs2 Δ1333-1355 URA3 CEN*	This study
pGFP-Drs2-ΔNPF	*CPY5'UTR-GFP-drs2 Δ1310-1355 URA3 CEN*	This study
pGFP-Drs2-NPW1,2	*CPY5'UTR-GFP-drs2(F1313W,F1335W) URA3 CEN*	This study
pGFP-Drs2-ΔN2	*CPY5'UTR-GFP-drs2Δ1-72 URA3 CEN*	This study
pGFP-Drs2-ΔN3	*CPY5'UTR-GFP-drs2Δ1-103 URA3 CEN*	This study
pGFP-Drs2-ΔN3-NPW1,2	*CPY5'UTR-GFP-drs2Δ1-103(F1313W,F1335W) URA3 CEN*	This study
pRS425-*CDC50*	*CDC50 LEU2 2*	Chen *et al.*, 2006
pGFP-*CDC50*	*CPY5'UTR-GFP-CDC50 URA3 CEN*	This study

Results

The C-tail is Essential for Drs2p Function in Protein Transport

Previously, we had shown that the ATPase dead *drs2-D560N* allele and a *drs2* allele bearing a truncation of the last 96 amino acids of the C-tail (*drs2-ΔCT)* could not complement the cold-sensitive growth defect of *drs2*Δ (Chen *et al.*, 1999; Chantalat *et al.*, 2004). To test whether these alleles could complement trafficking defects of *drs2*Δ, we examined the localization of the exocytic v-SNARE Snc1p, which cycles between the plasma membrane, early endosomes and the TGN (Hettema *et al.*, 2003). In wild-type cells, although a small fraction of GFP-Snc1p localizes to punctuate structures within the cell, GFP-Snc1p primarily localizes to the plasma membrane, concentrating in bud or the regions of polarized growth. In contrast, *drs2*Δ cells carrying an empty plasmid exhibited very little GFP-Snc1p at the plasma membrane and most of this fusion protein was found in internal structures, which may be either early endosomes or the TGN (Figure 2-1, empty; (Hua *et al.*, 2002)). Introduction of a plasmid bearing wild-type *DRS2* restored normal plasma membrane localization of GFP-Snc1p, but neither the *drs2-D560N* mutant nor the *drs2-ΔCT* allele were able to restore normal localization of GFP-Snc1p (Figure1; *D560N*, Δ*CT*).

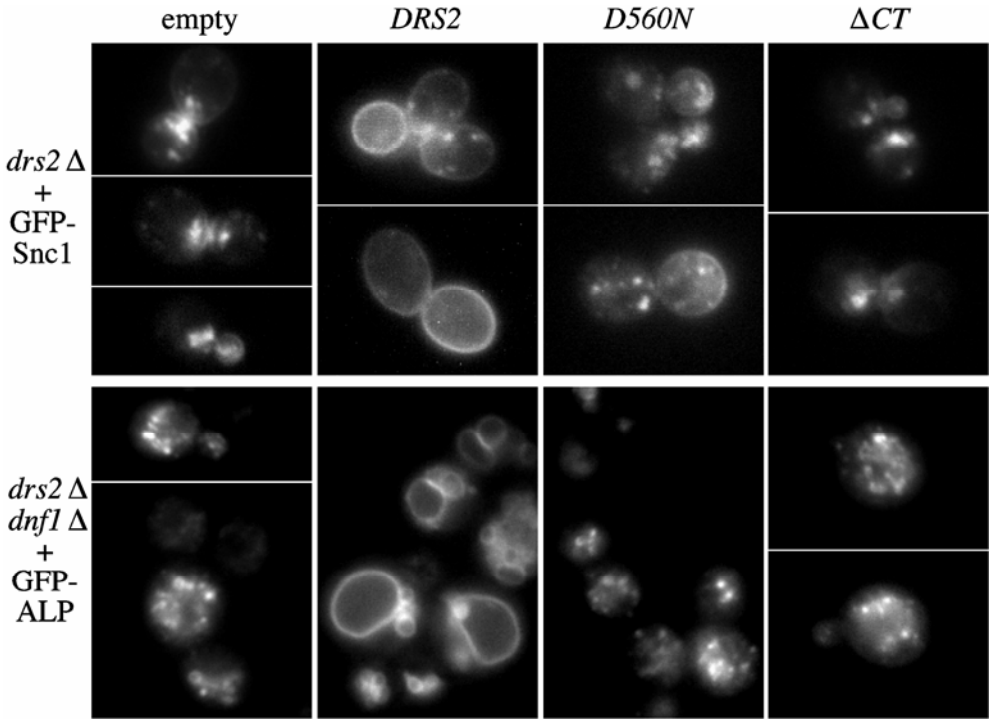

Figure 2-1: ATPase activity and the C-tail region are essential for Drs2p function in protein trafficking.

(A) Requirement for Drs2p ATPase activity and the C-tail region in Snc1p-GFP recycling. Plasmids pRS315 (empty), pRS315-*DRS2* (*DRS2*), pDRS2-D560N (*D560N*, ATPase dead) and pRS315-Drs2-ΔCT (Δ*CT*) were introduced into strain ZHY615M2D (*drs2*Δ) along with pRS416-GFP-SNC1 (GFP-Snc1). Transformants were grown at 30°C to mid-log phase and examined by fluorescence microcopy. Snc1-GFP is at the plasma membrane of *DRS2* (wild-type) cells and is trapped in internal membranes in the *drs2* mutants.

(B) Requirement for Drs2p ATPase activity and the C-tail region in alkaline ALP transport to the vacuole. The same *DRS2* plasmids as in (A) were co-transformed into strain ZHY2149D (*drs2*Δ*dnf1*Δ) with pGO41 (GFP-ALP). Cells were grown at 30°C to mid-log phase, shifted to 15°C for 2 h and then imaged. Fluorescent rings in the *DRS2* (wild-type) cells are vacuoles while GFP-ALP was mislocalized to extravacuolar puncta in *drs2dnf1* mutants.

We also tested if these two *drs2* mutant alleles could complement the alkaline phosphatase (ALP) trafficking defect shown by *drs2*Δ *dnf1*Δ cells. Drs2p and Dnf1p have redundant functions in the transport of GFP-ALP from the TGN to the vacuole (Hua *et al.*, 2002), a pathway mediated by AP-3 coated vesicles (Cowles *et al.*, 1997). In wild-type cells, GFP-ALP primarily localizes to the vacuole membrane, and one to three punctate structures outside of the vacuole in a small percentage of cells. In *drs2*Δ *dnf1*Δ cells, however, most of the GFP-ALP localizes to extravacuolar puncta (Figure 2-1, empty; (Hua *et al.*, 2002)). Wild-type *DRS2* complemented this phenotype, but *drs2-*Δ*CT* and *drs2-D560N* failed to restore the normal vacuolar GFP-ALP localization pattern (Figure 2-1; *D560N*, *ΔCT*). These results indicate that both the ATPase activity and the C-tail are critical for Drs2p function in protein trafficking from the TGN.

Functional Requirement for the Drs2p NPFXD Motifs

Three motifs have been mapped within the Drs2 C-tail thus far (Figure 2-2A), the Gea2p interaction motif (GIM), a highly conserved motif (CM) and the two NPFXD motifs, which could potentially interact with the Sla1p homology domain 1 (SHD1) of Sla1p (Howard *et al.*, 2002). A two-hybrid analysis was done to test for an interaction between the Drs2p C-tail and the Sla1p SHD1 domain. The Eps15 homology (EH) domain of Pan1p interacts with NPF motifs (Wendland and Emr, 1998) and so the Pan1-EH domain was also tested for interaction with the Drs2p C-tail. The Drs2p C-tail gave a positive two-hybrid interaction with the Sla1p SHD1 domain but did not interact

with the Pan1p EH domain (Figure 2-2B). The Drs2p C-tail also failed to interact with a fragment of Sla1p containing the SHD2 domain and a number of charged amino acids (Fig 2-2B, Sla1-charged). Deletion of the C-terminal region containing the two NPFXD motifs abolished the two-hybrid interaction as did mutating both NPFXDs to NPWXD (Drs2-CTΔNPF and Drs2-CT-NPW). The F to W mutation was previously shown to disrupt the two-hybrid interaction of Sla1p with the NPFXD motif of Kex2p, and to disrupt the ability of this motif to serve as an endocytosis signal (Howard *et al.*, 2002). In contrast, deletion of the C-tail GIM sequence had no effect on the interaction (Drs2-CTΔGIM). These data indicate that the interaction between Drs2p and Sla1p is mediated by the NPFXD motifs.

As shown previously, deletion of the conserved motif (Δ*CM*) partially abrogated the ability of this *drs2* allele to complement the cold-sensitive growth defect of *drs2*Δ, while deletion of both GIM and CM abolished Drs2p function. In contrast, deletion of C-terminal sequences containing the NPFXD motifs (Δ*NPF*) did not appear to perturb Drs2p function or exacerbate the defect caused by Δ*CM* (Chantalat *et al.*, 2004). These data initially suggested that the NPFXD motifs did not contribute to the essential function of the Drs2p C-tail. However, while neither deletion of the NPFXD motifs *(*Δ*NPF*) nor deletion of GIM (Δ*GIM*) perturbed complementation of the *drs2*Δ cold-sensitive growth defect, the Δ*GIM*-Δ*NPF* double mutant failed to complement (Figure 2-2C). These results indicate that the interaction with ARF-GEF and the C-terminal 44 residues bearing the two NPFXD motifs make important contributions to Drs2p function *in vivo*.

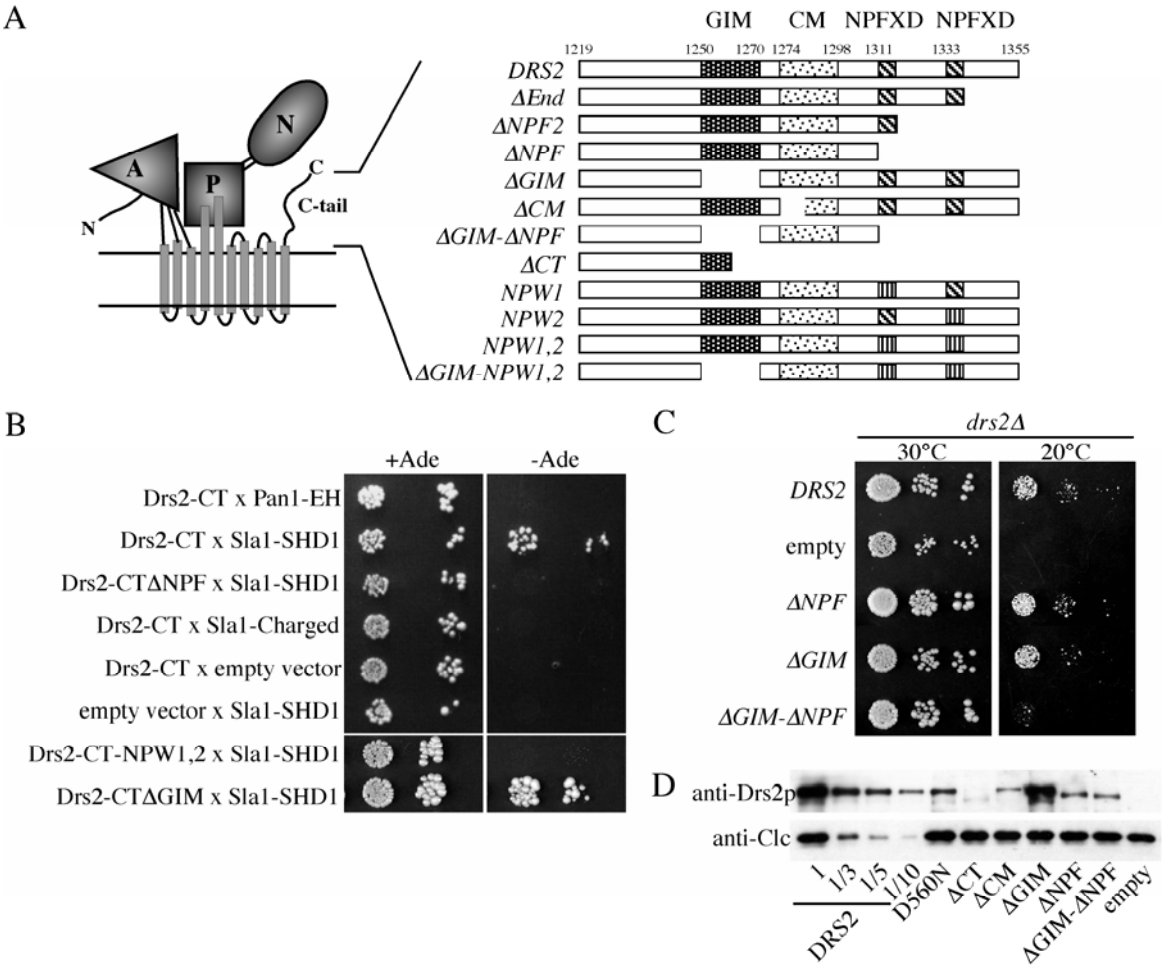

Figure 2-2: C-tail sequences containing the NPFXD motifs bind Sla1p and contribute to Drs2p function.

Figure 2-2: C-tail sequences containing the NPFXD motifs bind Sla1p and contribute to Drs2p function.

(A) Predicted topology and domain structure of Drs2p based on the crystal structure of the related sarcoplasmic reticulum Ca^{2+} ATPase ((A) actuator, (P) phosphorylation, (N) nucleotide binding) (Toyoshima and Inesi, 2004). Schematic diagram of motifs in the Drs2p C-tail and constructs used in this study ((GIM) Gea2p interaction motif, (CM) conserved motif).

(B) Two-hybrid test for interaction between Drs2p C-tail and Sla1p SHD1 domain. Bait and prey plasmids used are described in Table 2-2. Serial dilutions of the cells were spotted on minimal medium with or without adenine (Ade). Growth in the absence of adenine indicates a two-hybrid interaction.

(C) Synergistic defect caused by deleting both the GIM and C-terminal 44 amino acids bearing the two NPFXD motifs. Serial dilutions of *drs2*Δ strains (ZHY615M2D) expressing the indicated constructs were tested for their ability to grow at 20°C. *DRS2* is the wild-type gene and "empty" received the vector without an insert, thus showing the *drs2*Δ growth phenotype.

(D) Western blot of cell lysates probed for Drs2p and clathrin light chain (Clc1p). Lysates were normalized for cell equivalents and compared to a dilution series from cells expressing wild-type *DRS2*.

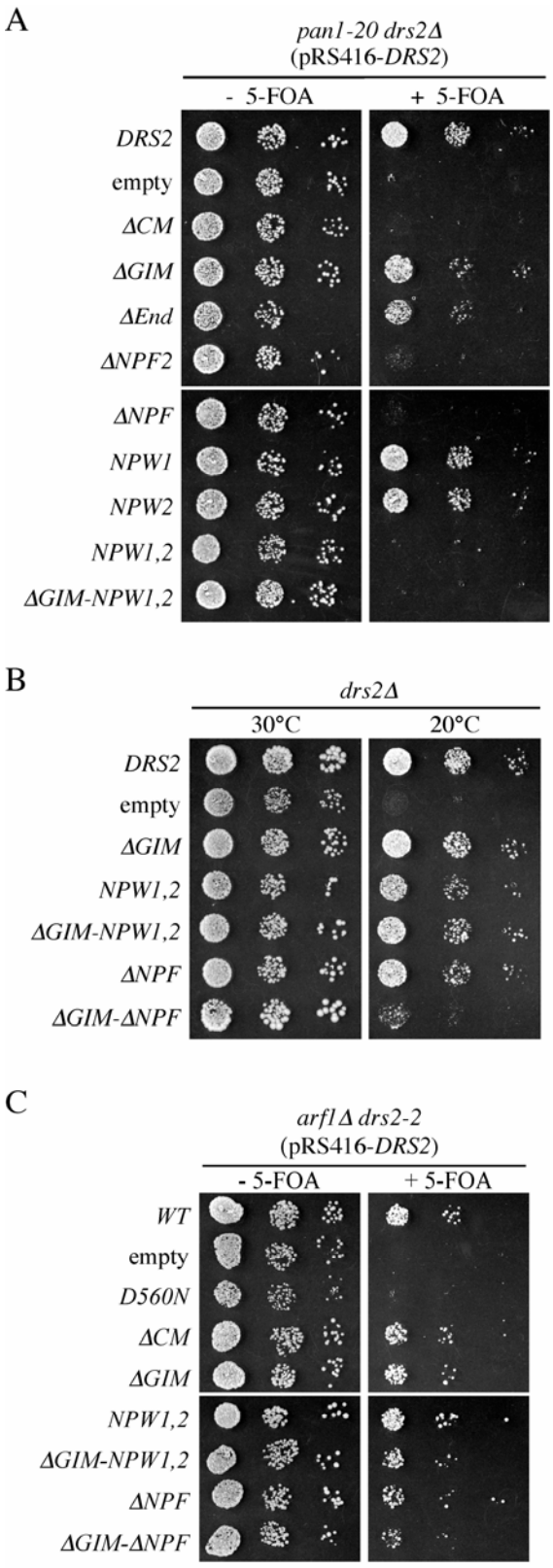

Figure 2-3: Mutation of Drs2p NPFXD motifs causes synthetic lethality with *pan1-20*.

Figure 2-3: Mutation of Drs2p NPFXD motifs cause synthetic lethality with *pan1-20*.
(A) Serial dilutions of ZHY823 (*drs2*Δ *pan1-20* pRS416-DRS2) expressing the indicated constructs from *LEU2*-based plasmids were tested for growth at 30°C on medium with or without 5-FOA. Failure to grow on the +5-FOA medium indicates synthetic lethality between *pan1-20* and the *drs2* allele expressed from the *LEU2* plasmid.
(B) The experiment shown in Figure 2-2C was repeated to include ΔGIM combined with the NPW point mutations.
(C) The *drs2-NPW1,2* allele is not synthetically lethal with *arf1Δ*. Serial dilutions of CCY2808 (*arf1Δ drs2-2* pRS416-DRS2) expressing the indicated constructs from *LEU2*-based plasmids were tested for growth at 30°C on medium with or without 5-FOA.

To determine how mutations of the C-tail affect expression of Drs2p, we performed a Western Blot with whole cell lysates from the strains indicated in Figure 2-2C. Most of the mutants were expressed at lower levels than wild-type Drs2p (*DRS2*), with Δ*CT* being most affected at significantly less than 10% expression (Figure 2-2D). However, co-expression of Δ*CT* and the ATPase dead *D560N* allele from two separate plasmids complements the cold-sensitive (cs) growth defect of *drs2*Δ, which indicates that this small amount of Δ*CT* provides sufficient ATPase activity to support Drs2p function reasonably well (Chantalat *et al.*, 2004). Importantly, all other C-tail mutant proteins were more stable than Δ*CT*. Therefore, while protein stability might be a factor that influences the ability of C-tail mutants to complement *drs2*Δ*,* each mutant should supply sufficient Drs2p ATPase activity for *in vivo* function, and loss of specific sequence within the Drs2p C-tail is primarily responsible for reduced Drs2p function. Particularly relevant is the observation that Δ*NPF* and Δ*GIM*-Δ*NPF* are expressed at a similar level, but the Δ*GIM*-Δ*NPF* double mutant is much more defective than either single mutant (Figures 2-2C and 2D).

Moreover, we were surprised to find that deletion of even one NPFXD motif was sufficient to cause synthetic lethality with *pan1-20* (Figure 2-3A). For this experiment, viability of a *drs2*Δ *pan1-20* strain was maintained by the presence of wild-type *DRS2* on a *URA3*-based plasmid (pRS416-*DRS2*). Various mutant *drs2* alleles were introduced into this strain on *LEU2* plasmids and cells capable of losing the wild-type *DRS2-URA3* plasmid were selected on medium containing 5 fluoro-orotic acid (5-FOA). Growth in the

absence of 5-FOA shows the phenotype of the *pan1-20* single mutant while the 5-FOA plate shows the *drs2 pan1-20* double mutant phenotype. The *drs2*Δ (empty) and Δ*CM* alleles are synthetically lethal with *pan1-20* although the Δ*GIM* allele is not. Deletion of the C-terminal 22 residues *(*Δ*End*) had little effect on Drs2p function by this assay, but constructs bearing additional deletions removing one (Δ*NPF2*) or both NPFXDs (Δ*NPF*) failed to support growth of a *pan1-20 drs2*Δ strain. To better define the role of the NPFXDs in this genetic interaction, we mutated them to NPWXD. In this case, each individual *drs2-NPW* allele supported growth of the *drs2*Δ *pan1-20* strain (*NPW1* and *NPW2*), but the *drs2-NPW1,2* double mutant (*NPW1,2*) failed to complement the *drs2*Δ *pan1-20* synthetic lethality (Figure 3A). This allele-specific genetic interaction indicates that a yeast strain compromised for Pan1p activity relies on an NPFXD-dependent function of Drs2p to sustain life.

Because the Δ*NPF2* C-terminal truncations showed a stronger phenotype in the *pan1-20* synthetic lethality test than the *NPW2* point mutation, we considered the possibility that other sequences in the C-terminal 44 residues contribute to Drs2p function independently of the NPFXDs. This possibility was tested by combining the Δ*GIM* and *NPW1,2* mutations *(*Δ*GIM-NPW1,2*) and comparing the ability of this new allele to complement *drs2*Δ relative to the alleles used in Figure 2-2C. As shown in Figure 2-3B, the Δ*GIM-NPW1,2* allele complemented the cs growth defect of *drs2*Δ while Δ*GIM*-Δ*NPF* again failed to complement. These data indicate that C-terminal 44 amino acids have an NPFXD-independent function that appears to act redundantly with the

Gea2p interaction motif (GIM). Alternatively, it is possible that the mutant NPW motif retains some function.

To further test if the genetic interaction is specific to *pan1-20*, we also examined the synthetic lethality between *drs2* mutants and *arf1*Δ (Figure 2-3C). As expected, the ATPase dead *drs2-D560N* allele failed to support growth of a *drs2-2 arf1*Δ strain. The *drs2-*Δ*CT* allele causes slow growth when combined with *arf1*Δ (data not shown), but *drs2* alleles carrying deletions of CM, GIM or NPFXD motifs do not substantially perturb growth of *arf1*Δ cells. Therefore, the synthetic lethal interaction between *drs2-npf* alleles and *pan1-20* is specific.

Functional Requirement for the Dnf1p NPFXD Motif

Interestingly, Dnf1p contains an NPFXD within its N-terminal cytosolic tail (N-tail) that is not present in the closely related Dnf2p. In addition, Dnf1p, but not Dnf2p, has redundant functions with Drs2p at the TGN, suggesting that the NPFXD-dependent endocytosis of Dnf1p may be required for its Golgi function. To test whether the NPFXD is important for Dnf1p function, we mutated NPF to NAI, the sequence found in Dnf2p (Figure 2-4A). Because there is no significant phenotype associated with deleting *DNF1* alone, we tested the *dnf1-NAI* allele for complementation of growth defects associated with the *drs2*Δ *dnf1*Δ double mutant. On minimal medium, *drs2*Δ *dnf1*Δ grows well at 30˚C but is strongly cs and ts for growth (Figure 2-4B, empty). Transformation with wild-type *DRS2* completely complements these growth defects (Figure 2-4B, *DRS2*),

showing the robust growth of a *dnf1* single mutant. Transformation with wild-type *DNF1* allowed growth at 24˚C and 37˚C, although these *drs2*Δ *DNF1* cells grew more slowly than the *DRS2 dnf1*Δ cells. In contrast, *dnf1-NAI* weakly complemented the cs growth defect and failed to complement the ts growth defect. Therefore, the NPFXD motif plays an important role in the ability of Dnf1p to compensate for the loss of Drs2p.

If the important role of the Dnf1p NPFXD is for Sla1p-dependent endocytosis, then a *drs2*Δ *sla1*Δ mutant should show a more severe growth defect than *drs2*Δ*,* similar to what was observed for *drs2*Δ *dnf1-NAI*. Deletion of *SLA1* alone causes a ts growth defect; however, we could detect a slightly more severe growth defect *of sla1*Δ *drs2*Δ at 37˚C (Figure 2-4C). However, the *sla1*Δ single mutant grows well at low temperatures. Therefore, we tested whether *sla1*Δ would exacerbate the cs growth of *drs2*Δ by using a slightly lower, more restrictive temperature than used in Figure 2-4B. Indeed, *drs2*Δ *sla1*Δ grew much more slowly at 23˚C than *drs2*Δ or *sla1*Δ. Even though *sla1*Δ may have pleiotropic effects on trafficking of several proteins, the uniquely strong cs growth defect of *drs2*Δ *dnf1*Δ suggests the effect of *sla1*Δ we are scoring in this assay is reduced Dnf1p function.

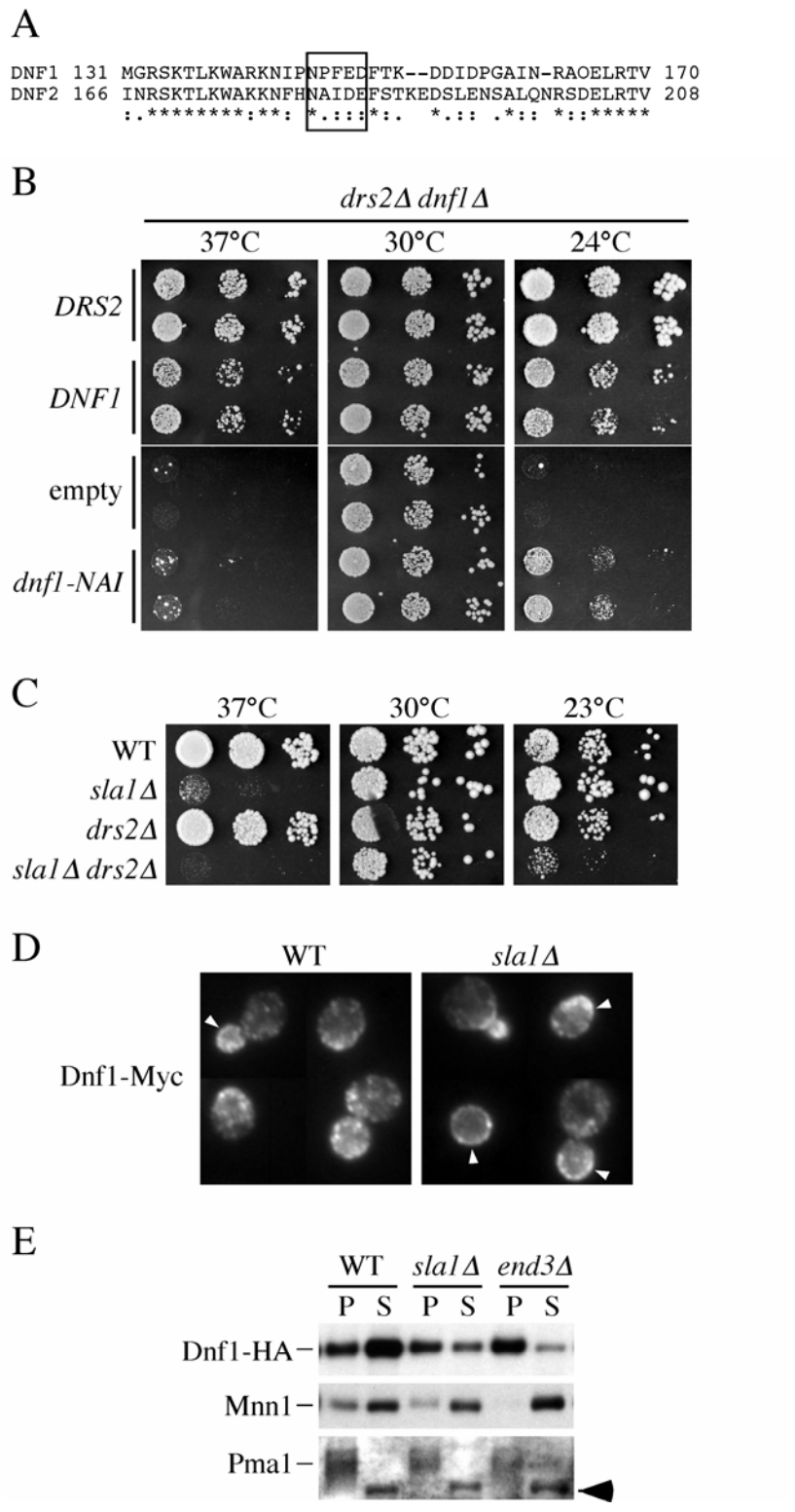

Figure 2-4: An NPFXD/Sla1p interaction contributes to Dnf1p function and endocytosis.

Figure 2-4: An NPFXD/Sla1p interaction contributes to Dnf1p function and endocytosis.
(A) Sequence alignment of Dnf1p and Dnf2p in the region surrounding the Dnf1p NPFXD motif.
(B) Plasmids bearing wild-type *DRS2* (pRS315-DRS2), *DNF1* (pRS313-DNF1), empty vector and NPF to NAI mutated *DNF1* (pRS313-Dnf1-NAI) were introduced into *drs2*Δ*dnf1*Δ (ZHY2149D). Cell growth on minimal medium at 37°C, 30°C and 24°C was examined.
(C) Growth of wild-type (BY4742), *sla1*Δ (KLY011), *drs2*Δ (ZHY615M2D) and *sla1*Δ *drs2*Δ (KLY035) was examined at 37°C, 30°C and 23°C.
(D) Fluorescence microscopy of wild-type and *sla1Δ* cells expressing Dnf1-Myc and stained with a mouse monoclonal anti-Myc antibody. Arrowheads indicate regions of labeled plasma membrane.
(E) Distribution of Dnf1p-HA between the plasma membrane and internal membranes. Wild-type, *sla1Δ* and *end3Δ*cells expressing HA tagged Dnf1p were osmotically lysed and centrifuged at 400 x g to clear the cell debris. The supernatants were subsequently centrifuged at 13,000 x g for 15min to generate pellet (P) and (S) fractions. Samples from each fraction were immunoblotted for Dnf1p-HA, the plasma membrane H^+-ATPase (Pma1p) and the Golgi protein Mnn1p. The arrow indicated a background protein that distributes in the S fraction.

NPFXD-dependent Endocytosis of Dnf1p and Drs2p

To directly determine whether the endocytosis of Dnf1p was dependent on Sla1p, the localization of Dnf1p-Myc was examined in both wild-type and *sla1*Δ cells by indirect immunofluorescence (Figure 2-4D). In wild-type cells, Dnf1p-Myc is localized to both the plasma membrane and internal membranes with a polarized distribution, which could be transient endocytic and/or exocytic vesicles. Dnf1p is concentrated at the emerging bud site, small buds and the mother-daughter neck of dividing cells (Hua *et al.*, 2002; Pomorski *et al.*, 2003); Figure 2-4D). Relative to wild-type cells, *sla1*Δ cells showed an accumulation of Dnf1p-Myc on the plasma membrane, although the change in the localization pattern was subtle by this method. To more quantitatively address the distribution of Dnf1p, a fractionation approach was used (Figure 2-4E). Cells expressing HA tagged Dnf1p were converted to spheroplasts, osmotically lysed, centrifuged at 400 X g to pellet unlysed cells and large membranes, and then centrifuged at 13,000 X g to produce pellet (P) and supernatant (S) fractions. In wild-type cells, most Dnf1p-HA was found in the S fraction, which was relatively devoid of plasma membrane as judged by the distribution of the plasma membrane H^+-ATPase, Pma1p. However, with *sla1Δ* and *end3Δ*, the amount of Dnf1p-HA in the S fraction was diminished with a concomitant increase in the plasma membrane P fraction. As a control, the distribution of an integral membrane glycoprotein of Golgi complex, Mnn1p, was examined, and no difference was observed between wild-type and *sla1Δ* and *end3Δ* cells. Moreover, the HA-tagged Dnf1-NAI mutant showed a 1.4-fold increase in the pellet fraction relative to wild-type

HA tagged Dnf1p (our unpublished data). The *end3Δ* strain showed a more substantial redistribution of Dnf1o to the P fraction than *sla1Δ* or wild-type cells expressing Dnf1-NAI. In total, these experiments indicate that the NPFXD/Sla1p interaction significantly contributes to endocytosis of Dnf1p, but other endocytosis signals likely exist in this protein.

Even though Drs2p localizes to the TGN with Kex2p (Chen *et al.*, 1999), the fact that Drs2p contains two NPFXD motifs suggests that it might travel to the plasma membrane and get rapidly endocytosed by the NPFXD/Sla1p pathway to maintain a steady-state TGN localization. If this is the case, we would expect to see accumulation of Drs2p on the cell surface when either the NPFXD motifs were mutated or *SLA1* was deleted. To test this hypothesis, wild-type or NPFXD mutated GFP-Drs2 fusion proteins were expressed in either wild-type or *sla1*Δ cells (Figure 2-5). The *GFP-DRS2* construct used here fully complements the *drs2*Δ cs growth defect and localizes appropriately to the TGN based on its co-localization with Sec7-RFP (Chen *et al.*, 2006). We fused GFP to the N-terminus of Drs2p to avoid interference with the potential trafficking signals near the C-terminus. Surprisingly, neither truncation of the C-terminal region containing the two NPFXD motifs (GFP-ΔNPF) nor mutation of both NPFXD motifs to NPWXD (GFP-NPW1,2) caused accumulation of Drs2p on the plasma membrane of wild-type cells (Figure 2-5, WT). Similar results were obtained for *sla1*Δ cells, with neither the wild-type nor the ΔNPFXD GFP-Drs2p being mislocalized (Figure 2-5, *sla1*Δ).

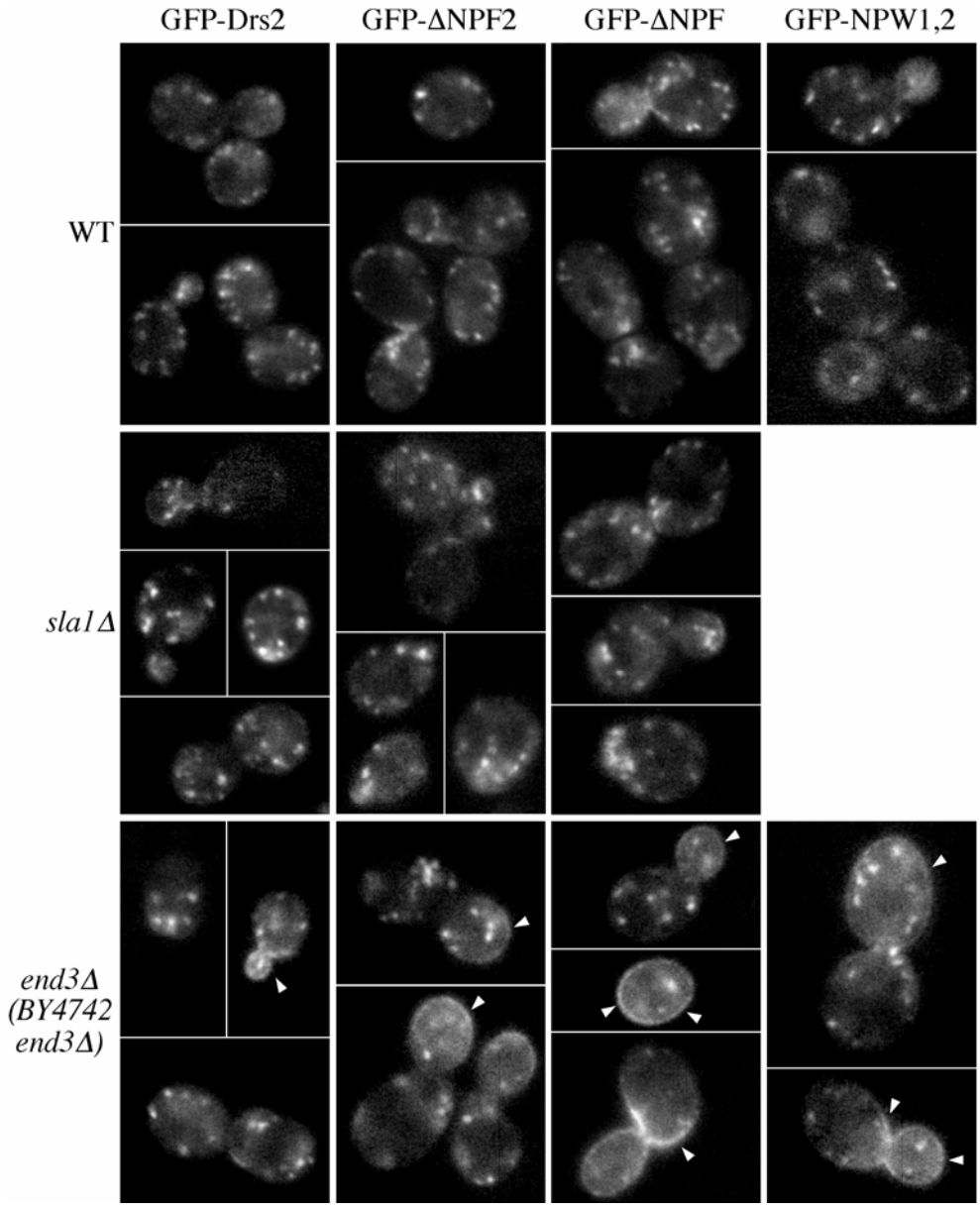

Figure 2-5: Drs2p does not rely on its NPFXDs and Sla1p for endocytosis unless the endocytic machinery is compromised.

A series of plasmids harboring GFP tagged wild-type (pGFP-*DRS2*) or NPFXD mutated *drs2* alleles (pGFP-ΔNPF2, pGFP-ΔNPF, pGFP-NPW1,2) were co-transformed with pRS425-*CDC50* into wild-type (BY4742), *sla1*Δ (KLY011) or *end3*Δ (BY4742 YNL084C) cells. Transformants were grown to early-log phase at 30 °C and imaged by fluorescence microscopy at room temperature. Arrowheads indicate plasma membrane fluorescence.

To test whether Drs2p trafficked to the plasma membrane and was retrieved by endocytosis signals other than NPFXD motifs, we expressed GFP-Drs2p in cells carrying a disruption of *END3* (*end3*Δ), which should elicit an efficient block in endocytosis of all proteins that transit the cell surface. Only a modest amount of wild-type GFP-Drs2p was trapped on the *end3*Δ plasma membrane, most noticeably in small buds of a small percentage of cells (arrowheads Figure 2-4, *end3*Δ, GFP-Drs2). Surprisingly, we found that deletion of one NPFXD motif (GFP-ΔNPF1) caused substantial accumulation of Drs2p on the *end3*Δ cell surface. Further truncation to remove the second NPFXD motif (GFP-ΔNPF) exacerbated this phenotype. Mutation of both NPFXDs to NPWXDs also resulted in accumulation of GFP-Drs2p on the *end3*Δ plasma membrane (Figure 2-5).

These results were unexpected and suggested that Drs2p did not normally travel to the plasma membrane, but deletion of the NPFXD motifs caused mislocalization of Drs2p to the plasma membrane where it could be trapped behind the *end3* block. This was, however, contradictory to a published report showing Drs2p-GFP accumulates on the plasma membrane upon deletion of verprolin (*vrp1*Δ), a protein required for proper organization of cortical actin patches and the internalization step of endocytosis (Saito *et al.*, 2004). In addition, NPFXD-mediated endocytosis reportedly requires End3p function (Tang *et al.*, 1996). To resolve these discrepancies, we examined localization of GFP-Drs2 in *vrp1*Δ as well as *end4-1* (also known as *sla2)*, another endocytosis mutant. GFP-Drs2 accumulated at the plasma membrane of both *vrp1*Δ and *end4-1* cells (Figure 2-6), in agreement with previously published *vrp1*Δ data (Saito *et al.*, 2004). This result

led us to suspect that the *end3*Δ strain from the yeast knockout collection contained an extragenic suppressor that specifically restored function of the NPFXD/Sla1p pathway. By backcrossing the original *end3*Δ strain to wild-type cells, new *end3*Δ strains were isolated that exhibited a tighter temperature-sensitive growth phenotype. Using a backcrossed *end3*Δ strain (KLY201), wild-type GFP-Drs2 was readily detected on the plasma membrane (Figure 2-6).

The observation that GFP-Drs2p localizes to the TGN in wild-type cells, but accumulates on the plasma membrane of *end3*, *vrp1* and *end4* suggests that Drs2p cycles between the exocytic and endocytic pathways. Neither deletion of *SLA1*, nor deletion of the Drs2-NPFXD motifs led to accumulation at the plasma membrane. Therefore, Drs2p must contain a second endocytosis signal that acts independently of Sla1p, but requires the actin-based endocytic machinery. Because GFP-Drs2p was only trapped on the plasma membrane of the original *end3*Δ strain when the NPFXD motifs were mutated, this strain must contain an extragenic mutation that suppresses Sla1p/NPFXD-mediated endocytosis but does not suppress *end3*Δ defects in endocytosis mediated by other signals. Thus, the original *end3*Δ strain (BY4742 *end3*Δ) was useful because it demonstrated an active role for the Drs2p NPFXD motifs in endocytosis.

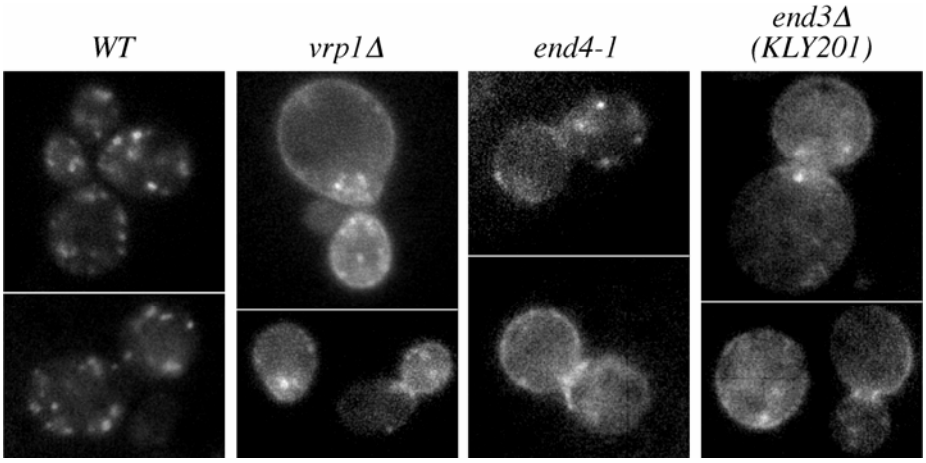

Figure 2-6: Localization of GFP-tagged Drs2p to the plasma membrane of endocytosis mutants.
pGFP-*DRS2* was co-transformed with pRS425-*CDC50* into wild-type (BY4742), *vrp1*Δ (BY4742 YLR337C), *end3*Δ (KLY201) and *end4-1* (TGY1912) cells. Transformants were grown to early-log phase at 27°C and examined by fluorescence microscopy.

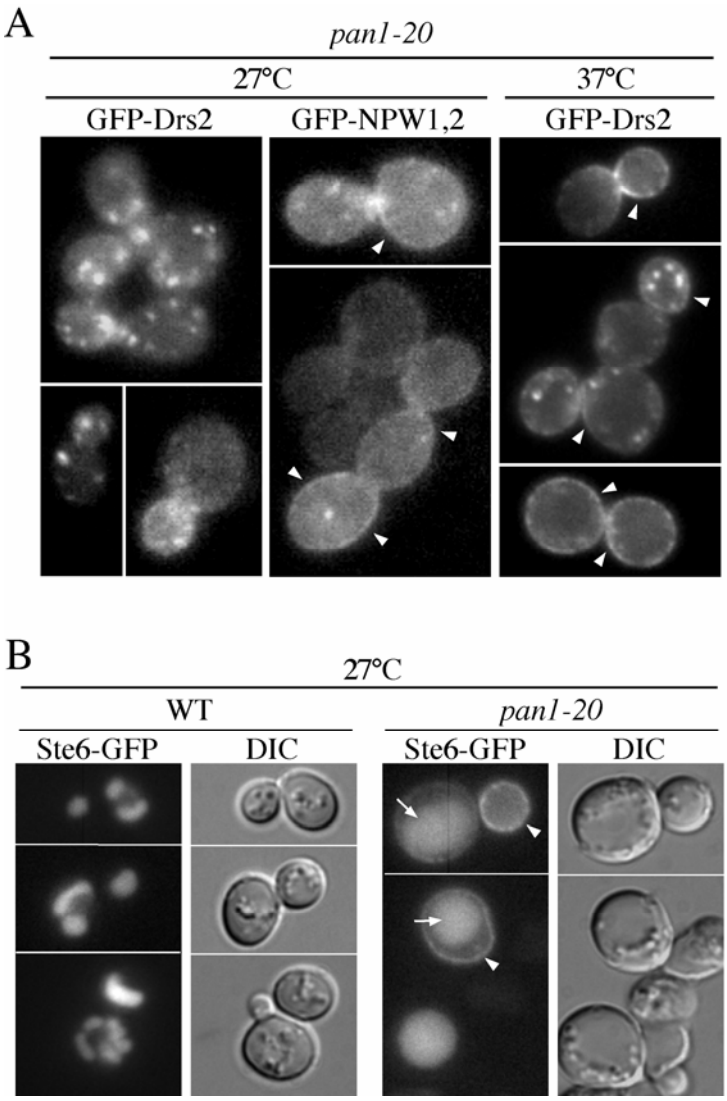

Figure 2-7: The *pan1-20* mutant exhibits a constitutive defect in Ub-dependent endocytosis and a temperature conditional defect in NPFXD-dependent endocytosis.
(A) Localization of GFP-Drs2p and GFP-Drs2-NPW1,2p in *pan1-20* (TGY1906) cells. This *pan1-20* strain expresses wild-type Drs2p from its endogenous locus to support viability. Cells were grown to mid-log phase at 27°C with or without shifting to 37°C for 1 h before imaging. Arrowheads indicate plasma membrane fluorescence.
(B) Ste6p-GFP localization in wild-type and *pan1-20* cells at 27°C. Wild-type (SEY6211) and *pan1-20* (TGY1906) cells transformed with a plasmid harboring Ste6p-GFP (pSM1493) were grown at 27°C to mid-log phase and examined by fluorescence microscopy. Arrows indicate vacuoles. Arrowheads indicate plasma membrane fluorescence.

Cargo-selective Endocytosis Defect of *pan1-20*

Mutation of both NPFXD motifs in Drs2p results in synthetic lethality with *pan1-20* (Figure 2-3), suggesting that *pan1-20* must maintain an active Sla1p/NPFXD-dependent endocytosis pathway at the permissive growth temperature. Otherwise, it is difficult to understand how mutation of an NPFXD endocytosis signal would further exacerbate growth of *pan1-20*. To test the Pan1p requirement for endocytosis of Drs2p, we examined the localization of GFP-Drs2p and GFP-Drs2-NPW1,2p in *pan1-20* at the permissive (27˚C) and nonpermissive (37˚C) growth temperatures. GFP-Drs2p localized appropriately to the TGN at 27˚C, but in stark contrast, GFP-Drs2-NPW1,2p was primarily localized to the plasma membrane. After a 1-h shift to 37˚C, both GFP-Drs2p and GFP-Drs2-NPW1,2p accumulated on the plasma membrane. Kex2-GFP, another TGN resident, did not accumulate on the plasma membrane of *pan1-20* at either temperature (unpublished observations). These results indicate that at the permissive temperature, *pan1-20* cells are defective in the Sla1p/NPFXD-independent endocytosis pathway but retain a functional Sla1p/NPFXD-dependent pathway. Both pathways are blocked at the nonpermissive temperature, causing accumulation of wild-type GFP-Drs2p at the plasma membrane.

The Sla1p/NPFXD-independent endocytosis pathway blocked in *pan1-20* at all temperatures is mostly likely dependent on Ub-dependent endocytosis signals. To test this possibility, we examined the localization of Ste6p-GFP in *pan1-20* cells. Ste6p is a ABC transporter that uses a Ub-dependent signal for endocytosis (Kolling and Hollenberg,

1994; Kelm *et al.*, 2004; Krsmanovic *et al.*, 2005). In wild-type cells, Ste6-GFP accumulates in the vacuole over time and so the cells show primarily vacuolar patterns of fluorescence. However, Ste6-GFP accumulated at the plasma membrane of *pan1-20* cells at 27˚C (Figure 2-7B). Thus, Ub-dependent endocytosis is abrogated in *pan1-20* at permissive growth temperatures.

Additional Endocytosis Signals in the Drs2p N-tail

The dependence of Drs2p on its NPFXD signals for TGN localization in *pan1-20* and *end3*Δ (suppressor) cells, but not wild-type cells strongly suggested that Drs2p must contain an additional endocytosis signal that is Ub dependent. A search for this signal using the PESTfind algorithm identified two "potential" and one "poor" PEST sequence along with 11 lysines in the N-tail of Drs2p (Figure 2-8A). Several other "poor" PEST sequences were found throughout Drs2p although none were in the C-tail. Deletions removing two (ΔN2, amino acids 1-72) or all three possible PEST sequences (ΔN3, amino acids 1-103) in the N-tail were constructed and the localization of GFP-Drs2-ΔN proteins was examined in wild-type, *sla1*Δ and *pan1-20* cells. As predicted, GFP-Drs2-ΔN2 and GFP-Drs2-ΔN3 were localized to the TGN in wild-type and *pan1-20* cells at 30 ˚C and mislocalized to the plasma membrane in *sla1*Δ cells (Figure 2-8B). Both GFP-Drs2-ΔN2 and GFP-Drs2-ΔN3 complemented the cs growth defect of *drs2*Δ, indicating that this deletion did not perturb Drs2p ATPase activity or function in protein transport. These data indicate that Drs2p has an endocytosis signal(s) in the N-tail that is

recognized by components of the endocytic apparatus specifically disrupted by the *pan1-20* mutation. Combining the ΔN3 and NPW mutations caused accumulation of a small amount of GFP-Drs2p on the plasma membrane of wild-type cells (Figure 2-8C). The localization of most Drs2-ΔN3-NPW1,2 to intracellular compartments suggests that these mutations have not eliminated all of the endocytosis signals in Drs2p. In addition, the observation that Drs2-ΔN3-NPW1,2 does not accumulate on the plasma membrane as much as Drs2-ΔN3 expressed in *sla1Δ* cells suggests that Sla1p contributes more to endocytosis than just the recruitment of NPFXD cargo.

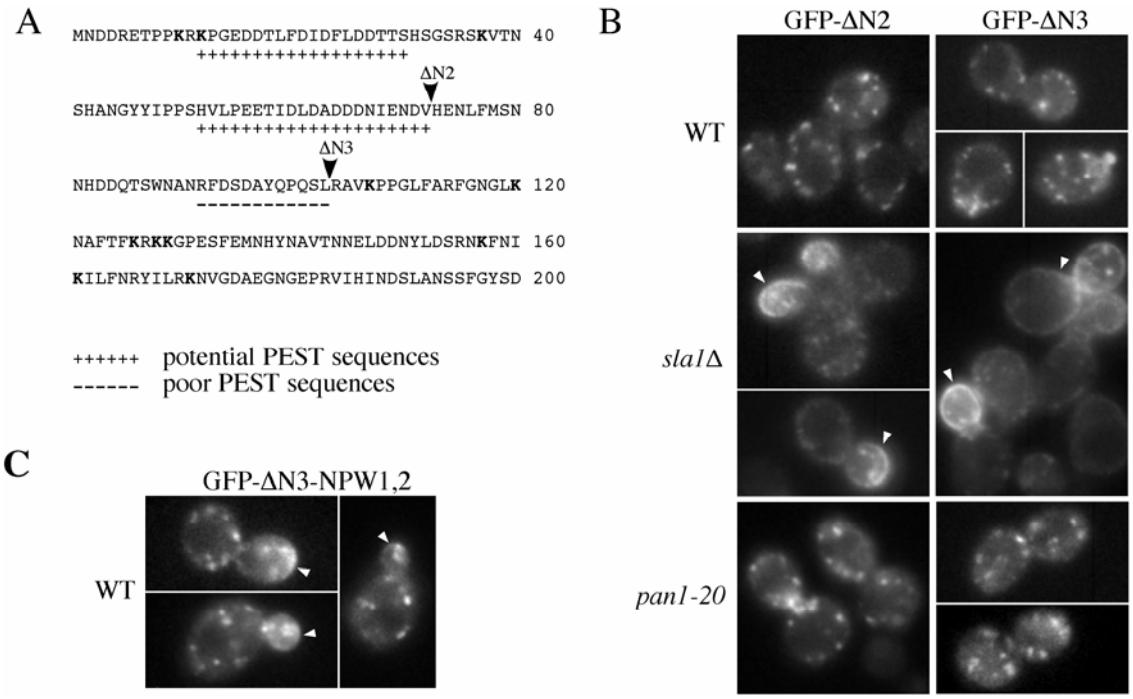

Figure 2-8: Sequences in the Drs2p N-tail bearing PEST-like motifs mediate endocytosis redundantly with the NPFXD motifs.

(A) The N-tail of Drs2p contains two potential PEST sequences and one poor PEST sequence. Arrowheads indicate the N-terminal truncation boundaries for the GFP-DRS2-ΔN2 and -ΔN3 alleles.

(B) Localization of the GFP-Drs2p with N-terminal truncations in wild-type, *sla1*Δ and *pan1-20* cells. pGFP-*DRS2,* pGFP-Drs2-ΔN2 and pGFP-Drs2-ΔN3 were co-transformed with pRS425-*CDC50* into wild-type (BY4742), *sla1*Δ (KLY011) or *pan1-20* (TGY1907) cells. Transformants were grown to early-log phase at 30 °C and imaged by fluorescence microscopy at room temperature. Arrowheads indicate plasma membrane fluorescence.

(C) Localization of the GFP-Drs2p with both the N-terminal truncation and the NPW1,2 mutations in wild-type cells. pGFP-Drs2-ΔN3-NPW1,2 was co-transformed with pRS425-*CDC50* into wild-type cells. Transformants were grown to early-log phase at 30 °C and imaged by fluorescence microscopy at room temperature. Arrowheads indicate plasma membrane fluorescence.

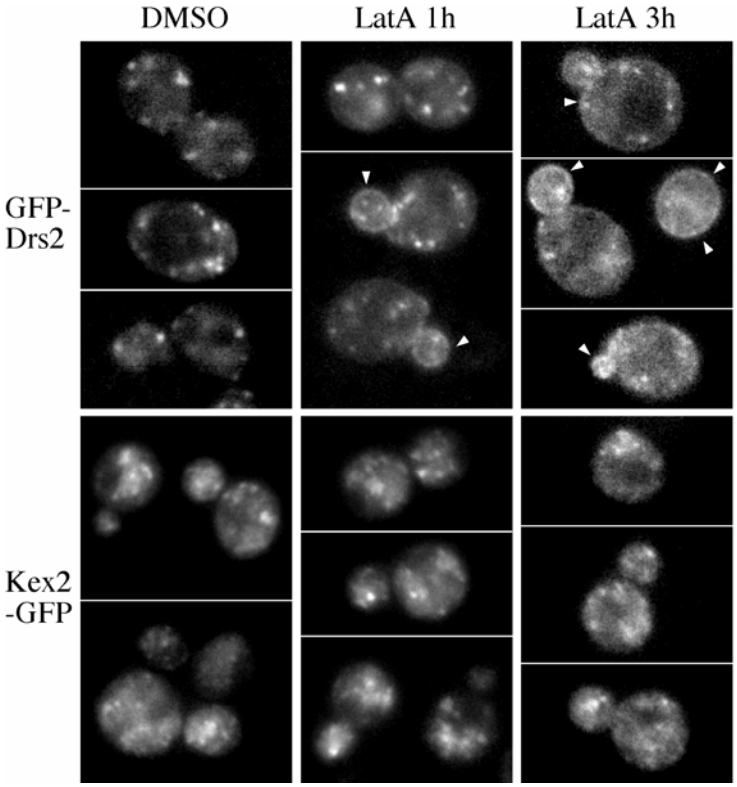

Figure 2-9: Drs2p accumulates slowly on the plasma membrane after disrupting endocytosis.

Wild-type cells expressing either GFP-Drs2p or Kex2p-GFP were grown to early-log phase at 30˚C and then incubated in SD medium containing 200 μM latrunculin A (Lat-A) or dimethylsulfoxide at 30°C for the times shown. Arrowheads indicate plasma membrane fluorescence.

Slow Exit of Drs2p from the TGN

The *vrp1* and *end* mutants used above are constitutively defective for endocytosis and so these studies do not indicate how frequently GFP-Drs2p travels to the plasma membrane. To determine the kinetics of GFP-Drs2p transport to the plasma membrane, wild-type cells expressing GFP-Drs2 were treated with latrunculin A (lat-A), an inhibitor of actin assembly and endocytosis, and imaged over time (Figure 2-9). After 1 h of treatment, GFP-Drs2p was still primarily retained intracellularly in small puncta, although it could be detected on the plasma membrane (Figure 2-9, Lat-A 1 h). After 3 h of treatment, the cell surface GFP-Drs2p further increased, concomitant with a reduction in the intensity of GFP-Drs2p intracellular fluorescence, and approached the distribution observed in the *end* mutants (Figure 2-9, Lat-A 3 h). Actin assembles on Golgi membranes and so we considered the possibility that perturbation of actin caused mislocalization of all late Golgi proteins, comparable with clathrin mutants. Therefore, Kex2p-GFP was also examined in this experiment. Even when overexpressed, Kex2p-GFP did not show any plasma membrane staining after 3 h of lat-A treatment (Figure 2-9, Kex2-GFP). These data demonstrate that unlike Kex2p, another TGN-localizing, NPFXD-containing protein, Drs2p slowly cycles between the TGN and the plasma membrane.

Drs2p and Cdc50p Do Not Significantly Transit the Late Endosome

Several different TGN proteins cycle through the late endosome as part of their normal trafficking itinerary (Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993; Cooper and Stevens, 1996; Brickner and Fuller, 1997; Foote and Nothwehr, 2006). We tested if Drs2p transited through the late endosome/prevacuolar compartment (PVC) by examining its localization in class E *vps* mutants, which block protein and lipid transport out of the PVC and result in the formation of an enlarged endosomal compartment adjacent to the vacuole called the class E compartment (Conibear and Stevens, 1998). Proteins that traffic through the PVC accumulate in the class E compartment of *vps4* or *vps27* cells. We expressed GFP-Drs2p in *vps4*Δ or *vps27*Δ and labeled the class E compartments with the endocytic tracer FM4-64 (Vida and Emr, 1995) before analysis by fluorescence microscopy. Although a small amount of GFP-Drs2 was detected in class E compartments at steady-state, the large majority of this protein did not colocalize with the FM4-64 and remained in small puncta (Figure 2-10A). In addition, we could not detect Drs2-Myc (C-terminal tag) in the class E compartment of *vps27*Δ by immunofluorescence (data not shown).

The Drs2p chaperone Cdc50p has been described as a late endosomal protein because Cdc50-GFP (GFP fused to C-terminus of Cdc50p) accumulated in the class E compartment (Misu *et al.*, 2003). However, this phenotype does not discriminate between a TGN or endosomal protein, and is inconsistent with our observations with

GFP-Drs2p. Therefore, we examined the localization of GFP-Cdc50p (GFP fused to the N-terminus of Cdc50p) in the *vps* mutants. This fusion protein is functional based on complementation of the cs growth defect of *cdc50*Δ. However, we were unable to detect a significant amount of colocalization between FM4-64 and GFP-Cdc50 in the class E compartment of *vps27*Δ or *vps4*Δ cells (Figure 2-10B). Because neither GFP-Drs2p nor GFP-Cdc50p fluorescence collapsed into the class E compartments of either mutant, the PVC does not appear to be a significant destination in the trafficking itinerary of these proteins. We also considered the possibility that NPFXD motifs play a role in retrieval of Drs2p from early endosomes back to the TGN. If so, deletion of the NPFXD motifs may force Drs2p to transit through the PVC more frequently. To test this possibility, we examined localization of GFP-Drs2p-ΔNPF and GFP-Drs2p-NPW in the *vps* cells. Neither GFP-Drs2p-ΔNPF nor GFP-Drs2p-NPW accumulated in the class E compartments (our unpublished data), indicating that potential sorting signals that prevent Drs2p trafficking to the PVC do not lie in the last 44 amino acids.

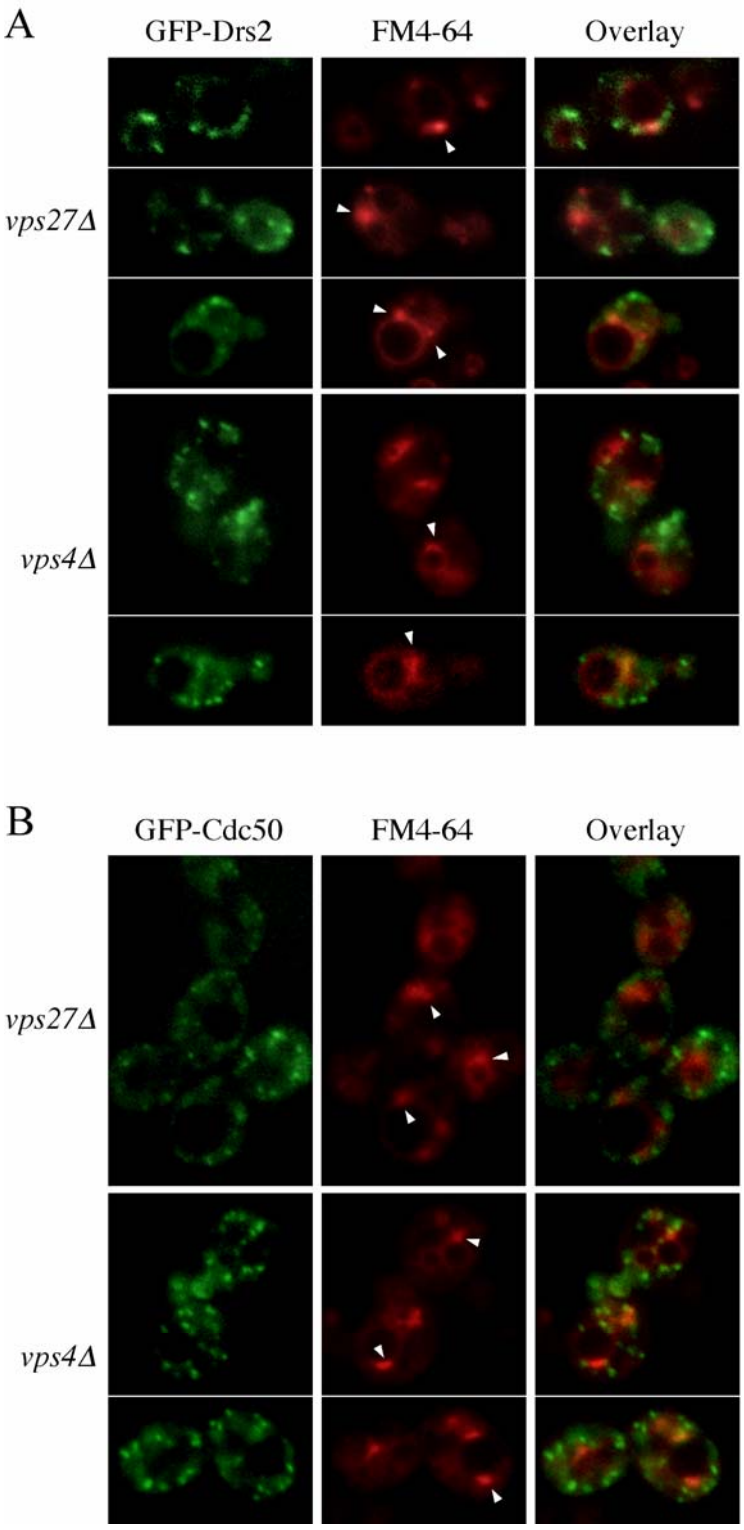

Figure 2-10: GFP-Drs2p and GFP-Cdc50p do not accumulate in the prevacuolar compartment of class E *vps* mutants.

Figure 2-10: GFP-Drs2p and GFP-Cdc50p do not accumulate in the prevacuolar compartment of class E *vps* mutants.
*vps27*Δ and *vps4*Δ cells carrying pGFP-*DRS2* and pRS425-*CDC50* (A) or pGFP-*CDC50* and pRS425-*DRS2* (B) were grown to early-middle log phase at 30˚C, labeled with10 μg/ml FM4-64 on ice for 20 min, washed with fresh medium, and then chased at 30°C for 30 min before analysis. GFP and FM4-64 images were acquired separately and merged to show the coincidence of the two patterns. Arrowheads indicate the prevacuolar compartment.

Discussion

Trafficking of Drs2p and Dnf1p

TGN-dwelling proteins have evolved different mechanisms to maintain their steady-state localization in this organelle, although transport to the endosomal system and retrieval back to the TGN appears to be a common theme. Recycling mechanisms are critical for localization of Golgi proteins because a growing body of evidence indicates that Golgi cisternae are not stable structures but instead mature from *cis* to *trans* by changing the content of resident enzymes (Losev *et al.*, 2006; Matsuura-Tokita *et al.*, 2006). Cisternae mature with a half-time of 1 to 2 min in the yeast system and the TGN is apparently consumed into transport carriers with multiple destinations. Therefore, TGN resident proteins must recycle back to younger cisternae every few minutes to maintain Golgi residence. Some mammalian TGN proteins, such as the mannose-6-phosphate receptor, TGN38 and furin, cycle to the plasma membrane and endosomes before returning to the TGN. These proteins require endocytic signals for removal from the plasma membrane and retrograde sorting signals to mediate endosome to TGN transport (Thomas, 2002; Ghosh *et al.*, 2003; Traub, 2005). In yeast, the vacuolar hydrolase sorting receptors Vps10p and Mrl1p rapidly cycle between the TGN and the late endosome / PVC (Cereghino *et al.*, 1995; Cooper and Stevens, 1996; Andrew Nesbit *et al.*, 2004). The TGN markers Kex2p and Ste13p are thought to primarily cycle between the TGN and early endosomes, occasionally transiting the PVC but not the plasma membrane

(Abazeed *et al.*, 2005; Foote and Nothwehr, 2006).

None of the yeast TGN proteins mentioned above accumulate on the plasma membrane when endocytosis is blocked (Cooper and Bussey, 1992; Roberts *et al.*, 1992; Wilcox *et al.*, 1992; Bryant and Stevens, 1997). In contrast, most (but not all) Drs2p accumulates on the plasma membrane of mutants constitutively defective for endocytosis. However, acute inactivation of endocytosis by latrunculin A treatment of cells causes a rather slow accumulation of Drs2p on the plasma membrane over the course of ~3 h. These findings suggest that Drs2p is inefficiently incorporated into exocytic vesicles, and is rapidly endocytosed upon arrival at the plasma membrane. The slow delivery to the plasma membrane and rapid endocytosis leads to an undetectable amount of Drs2p on the plasma membrane of wild-type cells unless endocytosis is inhibited. It is formally possible that disruption of endocytosis causes an aberrant incorporation of Drs2p into exocytic vesicles leading to plasma membrane accumulation. However, other TGN proteins do not share this fate in endocytosis mutants arguing against a reduced fidelity of sorting TGN residents from exocytic cargo. Moreover, Drs2p can be found in exocytic vesicles that accumulate in the *sec6* mutant, providing a method independent of disrupting actin or blocking endocytosis to show targeting of some Drs2p to the plasma membrane (Alder-Baerens *et al.*, 2006). Thus, it is much more likely that disrupting endocytosis simply traps Drs2p on the plasma membrane as it undergoes its normal trafficking itinerary.

After endocytosis, Drs2p must also be efficiently removed from the endocytic

pathway and transported from the early endosome to the TGN, because we fail to see significant accumulation of Drs2p or its Cdc50p subunit in the late endosome of class E *vps* mutants. This is similar to the recycling pathway described for the SNARE protein Snc1p and chitin synthase (Chs3p). In contrast, class E vps mutants accumulate most of Vps10p, Mrl1p, Kex2p and Ste13p in the PVC (Cereghino *et al.*, 1995; Brickner and Fuller, 1997; Bryant and Stevens, 1997; Andrew Nesbit *et al.*, 2004). Moreover, we never detect GFP-Drs2p in the vacuole, which is a common destination for many proteins that use Ub for an endocytosis signal because this modification also directs proteins into the multivesicular body pathway at the late endosome for delivery to the vacuole lumen (Katzmann *et al.*, 2002; Hicke and Dunn, 2003). If Drs2p is ubiquitinated, it must either avoid the late endosome to escape vacuolar delivery, or the Ub must be removed to allow retrieval of Drs2p to the TGN/early endosomal system. A previous report suggested that Drs2p is a late endosomal protein based on a relatively minor localization of Drs2p-GFP to the prevacuolar compartment of a class E *vps* mutant (Saito *et al.*, 2004). We disagree with this interpretation because at the rate of recycling suggested by the cisternal maturation model, the majority of Drs2p would relocate to the PVC in class E vps mutants if even a small percentage of molecules transited the PVC in each round of recycling (as observed for Ste13p and Kex2p). In contrast, a majority of Cdc50-GFP (C-terminally tagged) was reported to localize to the PVC (Misu *et al.*, 2003) while we found that GFP-Cdc50p (N-terminally tagged) was primarily excluded from the PVC. We suggest that the GFP-Cdc50p fusion protein more faithfully represents the localization of

the endogenous Drs2p-Cdc50p complex as these data are more consistent with the localization data for Drs2p. In summary, it is likely that Drs2p rapidly cycles between the TGN and early endosome as suggested for several other TGN proteins, and slowly cycles in the TGN → plasma membrane → early endosome → TGN loop.

Dnf1p is localized to both the plasma membrane and cytoplasmic punctate structures typically found near the plasma membrane. The steady-state localization pattern of Dnf1p and functional studies suggest that this protein cycles in a plasma membrane → endosome → TGN → plasma membrane pathway (Hua *et al.*, 2002). This hypothesis was confirmed by studies of the Tanaka group (Saito *et al.*, 2004) and those reported here, which extend these studies to demonstrate that endocytosis of Dnf1p is dependent on the NPFXD motif and Sla1p. Interestingly, the majority of Dnf1p was reported to fractionate in membranes with the same density as the plasma membrane (Pomorski *et al.*, 2003; Alder-Baerens *et al.*, 2006), which we have confirmed (our unpublished data), but most Dnf1p appears to be inside the cell by immunofluorescence localization and is separable from the plasma membrane by differential centrifugation techniques. We suggest that these data could be explained by efficient incorporation of Dnf1p into exocytic vesicles at the TGN (and/or endosome) and efficient endocytosis at the plasma membrane, thus giving a primary steady-state localization to vesicles trafficking to and from the plasma membrane.

Synthetic Lethality between *drs2* and *pan1*

Endocytosis plays an important role in remodeling the plasma membrane, internalizing extracellular nutrients, down-regulating signal transduction pathways by internalizing receptors, and retrieving proteins required for Golgi/endosome function that have escaped to the plasma membrane. The synthetic lethality between *pan1-20* and *drs2-npw1,2* provides the best example we are aware of for the essential role of endocytosis in retrieving Golgi proteins. The synthetic lethality between *drs2*Δ and *pan1-20* was initially surprising because Drs2p is required for Golgi/early endosome function while Pan1p acts at the internalization step of endocytosis. However, the relationship between Drs2p and Pan1p is now clearer. The Ub-dependent endocytosis defect of *pan1-20* likely depletes the TGN/endosomal system of several proteins required for the essential function of these organelles in protein transport. In spite of this deficit, Golgi/early endosome function can be maintained as long as Drs2p is present in these organelles. In *pan1-20*, Drs2p localization to the TGN/early endosome requires its endocytosis by the NPFXD/Sla1p pathway, thus explaining the inviability of *pan1-20 drs2-npw1,2* (shown here) and *pan1 sla1*Δ (Tang *et al.*, 1996).

We have also considered the possibility that the *pan1-20 drs2-npw1,2* synthetic lethality could be explained by a requirement for Drs2p in the internalization step of endocytosis. However, Dnf1p and Dnf2p appear to provide a much more important contribution to endocytosis than Drs2p (Hua *et al.*, 2002; Pomorski *et al.*, 2003). In addition, Dnf1p should be present in the plasma membrane of *pan1-20* cells and capable

of engaging Sla1p through its NPFXD motif, thus satisfying a potential requirement to couple a phospholipid translocase to the endocytic machinery. Surprisingly, Drs2p does not appear to be active on the plasma membrane because *end4* cells accumulate Drs2p on the plasma membrane, but there does not appear to be a loss of phosphatidylserine translocation across the plasma membrane when *DRS2* is deleted in *end4* cells (Marx *et al.*, 1999). Therefore, it is much more likely that TGN/early endosome residence of Drs2p is required to sustain viability of *pan1-20* cells. Moreover, endocytosis of Dnf1p is required in order for this protein to compensate for loss of Drs2p, further arguing for critical Golgi functions for these proteins. Class I myosins (Myo3p and Myo5p) are required for endocytosis in yeast and *CDC50* was recovered in a screen for multicopy suppressors of a *myo3*Δ *myo5-360* double mutant growth defect (Misu *et al.*, 2003). This suppression could also be explained by an increased Drs2p/Cdc50p concentration in the Golgi/early endosome that overcomes the loss caused by the endocytosis defect.

It is also possible that Pan1p has some unrecognized function in the TGN/early endosome system because *end3*Δ*, end4*Δ and *vrp1*Δ mutants have a defect in both the Ub- and NPFXD-dependent endocytic pathways, accumulate Drs2p on the plasma membrane, and yet these mutants are viable while *pan1*Δ is not. In addition, *drs2*Δ *end4-1* and *drs2*Δ *sla1*Δ double mutants are viable but the *drs2*Δ *pan1-20* double mutant is dead (Chen *et al.*, 1999); this study). One potential explanation for this discrepancy is that Pan1p contributes to the clathrin-dependent localization of TGN proteins independently of End4p, End3p and Sla1p, perhaps though its interaction with the

clathrin assembly protein AP180. In this case, loss of Pan1p function would accelerate transport of TGN proteins to the plasma membrane while trapping them there behind the endocytosis block. Moreover, it seems that the redistribution of GFP-Drs2p from the TGN to the plasma membrane occurs faster and more completely in *pan1-20* after shift to the nonpermissive temperature relative to latrunculin A-treated cells. This potential influence of Pan1p on Golgi localization seems to be specific to Drs2p because Kex2p is not mislocalized to the plasma membrane of *pan1-ts* cells. However, further work is required to determine whether or not these differences are significant and whether Pan1p has any functions beyond the internalization step of endocytosis.

Interactions between Cargo and the Endocytic Machinery

The nature of the *pan1-20* mutation is also of interest because it causes cargo-selective defects in endocytosis at the permissive growth temperature, and the mechanism for recognition of Ub-dependent endocytic cargo is incompletely understood. Pan1p, End3p, End4p and Sla1p are all recruited to the same endocytic sites and are considered a coat complex for clathrin/actin-based endocytosis. The Ub-dependent signal typically requires phosphorylation prior to ubiquitination by the Rsp5p ubiquitin ligase (Rotin *et al.*, 2000; Hicke and Dunn, 2003), then recognition of the ubiquitinated cargo by epsins (Ent1p, Ent2p) and Ede1p, another Eps15-related protein, for inclusion into forming vesicles (Shih *et al.*, 2002). Any of these steps could be defective in the *pan1-20* strain at the permissive temperature and so it will be informative to test whether

Drs2-npw1,2p accumulates on the *pan1-20* plasma membrane in a phosphorylated and/or ubiquitinated form. Interestingly, the *pan1-20* mutation is a frameshift very near the carboxyl-terminus within the proline-rich domain of Pan1p (Beverly Wendland, personal communication), suggesting this mutation abrogates interaction with a Src homology (SH)3-containing protein. One possibility is that this region could potentially interact with the SH3 domain of Rvs167p (amphiphysin/endophilin). A point mutation in the SH3 domain of Rvs167p causes a synthetic lethal interaction with *sla1*Δ (Friesen *et al.*, 2006), as does the *pan1-20* allele. Moreover, Sla1p forms a complex with Rvs167 that interacts with Rsp5p, comparable to the Cin85-Endophilin interaction with the Cbl ubiquitin ligase in mammalian cells (Stamenova *et al.*, 2004). This network of interactions may play a critical role in coupling cargo recognition (by Sla1p, Rsp5p and epsins) to packaging of cargo into forming vesicles.

In summary, the observations reported here are novel in several respects. To our knowledge, the inviability of *pan1-20 drs2-npw2* represents the first example where mutation of an endocytosis signal in a single protein is lethal to a cell. This result highlights the importance of endocytosis in retrieving proteins whose primary function is in the Golgi/endosomal system. In addition, the selective defect of *pan1-20* in Ub-dependent endocytosis should facilitate a better understanding of how cargo is recognized and packaged into endocytic vesicles through characterization of effectors of the Pan1p praline-rich domain.

CHAPTER III

DRS2p IS A TGN TO EARLY ENDOSOME ANTEROGRADE CARGO REQUIRED FOR AP-1/CLATHRIN-COATED VESICLE FORMATION

Introduction

Clathrin was the first vesicle coat protein identified and is now known to mediate protein traffic from the plasma membrane to endosomes and between the *trans*-Golgi network (TGN) and endosomes. The spherical, cage-like clathrin coats are assembled from triskelions, which are three-legged structures containing three clathrin heavy chains and three light chains (Brodsky *et al.*, 2001). Clathrin does not bind directly to cargo proteins, but instead interacts with pathway-specific adaptors (AP-1, AP-2 etc) that associate with the cytosolic sorting signals of cargo proteins (Owen *et al.*, 2004). In addition to adaptors, a large variety of accessory factors, such as phosphoinositides, Arf, epsins, amphiphysins, AP180 and dynamins, have been identified that also contribute to clathrin coated vesicle (CCV) formation (Traub, 2005). These accessory factors facilitate a set of sequential steps in vesicle formation: recruitment of adaptors to the appropriate membrane, assembly of coat proteins to promote membrane invagination as well as selective incorporation of cargo into the forming vesicles, and scission of the membrane to release the vesicle.

The AP-1 and GGA proteins are two major classes of adaptors that function in clathrin-mediated transport between the TGN and endosomes (Hinners and Tooze, 2003).

AP-1 is composed of two large subunits (β1 and γ), one medium subunit (μ1), and a small subunit (σ1). Three other heterotetrameric complexes with homology to AP-1 function as clathrin adaptors in other protein trafficking pathways (Robinson, 2004). AP-2 acts in clathrin-mediated endocytosis and AP-3 is implicated in protein transport from endosomes and the TGN to lysosomes. AP-4, absent in yeast, has been involved in sorting of cargos destined for lysosomes and the basolateral membrane in different cell types. GGA proteins (Golgi-localized, γ-ear-containing, ARF-binding proteins) are monomeric adaptors identified by searching sequence databases for homologs of the AP-1 γ subunit (Bonifacino, 2004). Mammalian cells express three GGA proteins and yeast express two. Like AP-1, GGAs localize to the TGN and endosomal membranes and depend on a small GTP binding protein called ADP-ribosylation factor (ARF) for membrane association. However, the precise pathways and protein sorting steps mediated by AP-1 and GGA are still poorly understood (Hinners and Tooze, 2003). In yeast, simultaneous inactivation of both AP-1 and GGA function causes a severe synthetic growth defect, suggesting that these adaptors mediate parallel transport pathways (Costaguta *et al.*, 2001; Hirst *et al.*, 2001). Current evidence favors a primary role for GGAs in the direct TGN to late endosome / prevacuole compartment (PVC) pathway (Black and Pelham, 2000; Costaguta *et al.*, 2001; Hirst *et al.*, 2001; Hinners and Tooze, 2003), while the primary role for AP-1 is proposed to be in the early endosome to TGN retrograde pathway (Valdivia *et al.*, 2002; Foote and Nothwehr, 2006). AP-1 is required for proper trafficking of several proteins within the TGN/endosome system, including

Kex2p, Ste13p, Chs3p, Tgl1p and Cps1p (Valdivia *et al.*, 2002; Ghosh *et al.*, 2003; Foote and Nothwehr, 2006; Phelan *et al.*, 2006; Copic *et al.*, 2007). Even though AP-1 is recruited to the TGN in yeast and mammals, and is present in clathrin coated buds forming on the TGN, no specific anterograde cargo requiring AP-1 has been defined in either system.

A genetic screen to identify accessory factors that function with *ARF* in vesicle biogenesis uncovered a novel requirement for a phospholipid translocase in clathrin function at the TGN. This *arf1* synthetic lethal screen recovered mutant alleles of *DRS2*, encoding the catalytic α subunit of this P-type ATPase, and *CDC50*, the noncatalytic β-subunit required for chaperoning the αβ complex from the ER to the Golgi (Chen *et al.*, 1999; Saito *et al.*, 2004; Chen *et al.*, 2006). Drs2p is directly responsible for an aminophospholipid translocase activity in the yeast TGN that flips phosphatidylserine (PS) and to a lesser extent phosphatidylethanolamine (PE) from the lumenal leaflet to the cytosolic leaflet (Natarajan *et al.*, 2004; Alder-Baerens *et al.*, 2006). Mutations in Drs2p that disrupt its ATPase or translocase activity phenocopy the effect of clathrin mutations on protein trafficking in the TGN and endosomal system. Drs2 and clathrin mutants cause mislocalization of TGN proteins, the accumulation of swollen Golgi cisternae, and loss of specific class of post-Golgi vesicles carrying exocytic cargo (Chen *et al.*, 1999; Gall *et al.*, 2002). In addition, *drs2Δ* cells exhibit a deficiency in CCVs that can be recovered in subcellular fractions containing assembled clathrin (Chen *et al.*, 1999). Moreover, Drs2p interacts directly with an ARF guanine nucleotide exchange factor Gea2p and Drs2

mutations that disrupt this interaction abrogate Drs2p function *in vivo* (Chantalat *et al.*, 2004; Liu *et al.*, 2007).

Drs2p has a steady-state localization to the TGN, although this protein slowly traffics to the plasma membrane where it is rapidly endocytosed and efficiently retrieved to the TGN from early endosomes (Chen *et al.*, 1999; Saito *et al.*, 2004; Liu *et al.*, 2007). As with other TGN proteins, Drs2 likely cycles rapidly to the early endosome and back to maintain TGN residence, a pathway linked to AP-1/clathrin function. Here we show that Drs2p interacts with AP-1 and loss of AP-1 causes rapid transport to the plasma membrane. However, retrieval of Drs2p back to the TGN is unaffected in AP-1 mutants and so steady-state localization is not perturbed. These data indicate that Drs2p requires AP-1 for anterograde transport to the endosome but not for endosome to TGN retrograde transport. We also show that Drs2p is required for AP-1/clathrin function, but does not appear to contribute significantly to the GGA/clathrin pathway. AP-1 and clathrin are recruited to the Golgi and endosomal membrane in the absence of Drs2p but appear to be incapable of budding CCVs. We propose that phospholipid translocation by Drs2p plays an essential role in generating the membrane curvature required for budding AP-1/clathrin-coated vesicles.

Materials and Methods

Media and Strains

Yeast were grown in standard rich medium (YPD) or synthetic defined (SD) minimal media containing the required nutritional supplements (Sherman, 1991). The calcoflour white (CW) sensitivity test was done on YPD 2% agar plates containing 100 μg/ml calcoflour white (F6259; Sigma-Aldrich, St. Louis, MO). Yeast transformations were performed using the lithium acetate method. Escherichia coli strains DH5α and XL1-Blue were used for plasmid construction and amplification.

Yeast strains used in this study are summarized in Table 3-1. The yeast knockout strain collection was purchased from Resgen, Invitrogen Corporation (Carlsbad, CA). Strains carrying multiple disruptions were generated by standard genetic crosses and tetrad dissection. The genotype of each clone was determined by PCR as described by the Saccharomyces genome deletion project (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html). The *DRS2* disruption plasmid pZH523 (Hua *et al.*, 2002) was linearized with *Sac*I and *Hpa*I and transformed into different strains to delete *DRS2*. Strains expressing Myc or HA tagged proteins were generated by PCR-based targeting into BY4741 or BY4742 strains using pPF6a-13Myc-HisMX6 or pPF6a-3HA-HisMX6 as the PCR template (Longtine *et al.*, 1998). Transformants were selected on SD plates without histidine and the integrated tags were confirmed by PCR. To avoid artifactual accumulation of GFP-Drs2p in the ER (Saito *et al.*, 2004),

pGFP-*DRS2* was co-transformed into yeast strains with a multicopy vector carrying *CDC50* (pRS425-*CDC50*) for experiments shown in Figure 3-4 and 3-5.

Microscopy

To visualize GFP or DsRed-tagged proteins, cells were grown to mid-log 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 using an Axioplan microscope (Carl Zeiss, Thornwood, NY). Fluorescent images were captured with a charge-coupled device camera and processed with Metamorph 4.5 software (Universal Imaging, Downingtown, PA). Chitin staining was performed with cells incubated in 1 mg/ml calcofluor white in water for 5 min, and washed 3 times prior to imaging. To study the kinetics of GFP-Drs2p transport to the plasma membrane, mid-log phase cells were collected and resuspended in SD medium containing 200 μM latrunculin A. Samples of cells were imaged at different time points. Brefeldin A (BFA, Sigma, B-7651) was dissolved in ethanol and used treat cells at 100 μg/ml in imaging buffer.

Immunological Methods

For chemical crosslinking experiments, approximately 50 OD_{600} units of each strain were harvested and converted to spheroplasts. After washing the spheroplasts three times with crosslinking buffer (1 M Sorbitol, 0.15 M NaCl, 0.1 M KH_2PO_4 pH 7.2), the cells were resuspended in crosslinking buffer supplemented with either 1 or 2 μm

dithiobis(succinimidyl)propionate (DSP) (Pierce, Rockford, IL) that was freshly prepared as a 20 mg/ml stock in dimethylsulphoxide. Crosslinking reactions were incubated at room temperature for 30 minutes. The 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, 1x complete protease inhibitor cocktail lacking EDTA (Roche Diagnostics, Basel, Switzerland), 1 mg/ml BSA) and incubated on ice for 15 minutes. The cell lysate was cleared by incubation with 30 µl protein G Sepharose (Amersham Biosciences, Sweden) at 4°C for 30 minutes and centrifugation at 20,000 x g for 20 minutes. The cleared lysate was incubated with 9 µg monoclonal anti-HA (HA-7, Sigma-Aldrich, St. Louis, MO) or anti-Myc (9E10, Sigma-Aldrich, St. Louis, MO) 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, 1x 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, 1x complete protease inhibitor cocktail lacking EDTA). Bound material was eluted with SDS/urea sample buffer (40 mM Tris-HCl pH 6.8, 8 M urea, 0.1 mM EDTA, 1% 2-mercaptoethanol, 5% SDS, 0.25% bromophenol blue) for 20 minutes at 50°C. The eluted material was separated by SDS-PAGE and immunoblotting was performed as previously described (Chen *et al.*, 1999) using anti-Drs2 (1:2000, (Chen *et al.*, 1999)), anti-Arf (1:2000), anti-Apl2 (1:1000, (Yeung *et al.*, 1999)), anti-HA (Sigma-Aldrich, St. Louis, MO) and anti-Myc (Sigma-Aldrich, St. Louis, MO) antibodies.

Table 3-1： Yeast Strains Used in Chapter III.

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Invitrogen
BY4742	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	Invitrogen
BY4741 YDR358W	BY4741 *gga1Δ::KanMX6*	Invitrogen
BY4742 YHR108W	BY4742 *gga2Δ::KanMX6*	Invitrogen
BY4741 YKL135C	BY4741 *apl2Δ::KanMX6*	Invitrogen
BY4741 YPR029C	BY4741 *apl4Δ::KanMX6*	Invitrogen
BY4741 YLR170C	BY4741 *aps1Δ::KanMX6*	Invitrogen
BY4741 YPL259C	BY4741 *apm1Δ::KanMX6*	Invitrogen
BY4741 YHL019C	BY4741 *apm2Δ::KanMX6*	Invitrogen
BY4741 YJL204C	BY4741 *rcy1Δ::KanMX6*	Invitrogen
BY4741 YJL036W	BY4741 *snx4Δ::KanMX6*	Invitrogen
ZHY615D1C	*MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 drs2Δ::KanMX6*	Hua *et al*., 2002
ZHY615M2D	BY4742 *drs2Δ::KanMX6* BY4742 *drs2Δ::LEU2*	Hua *et al*., 2002
KLY691	*MATa his3Δ1 leu2Δ0 ura3Δ0 gga1Δ::KanMX6 gga2Δ::KanMX6*	This Study
KLY741	*gga1Δ::KanMX6 gga2Δ::KanMX6 drs2Δ::LEU2*	This Study
KLY751	*MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 apl4Δ::KanMX6 drs2Δ::LEU2*	This Study
KLY371	*MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 apm1Δ::KanMX6 apm2Δ::KanMX6*	This Study
BY4742 YJL099W	BY4742 *chs6Δ::KanMX6*	Invitrogen
KLY022	*MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 drs2Δ::KanMX6 chs6Δ::KanMX6*	This Study
KLY492	*MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 apl4Δ::KanMX6 chs6Δ::KanMX6*	This Study
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al*., 1988
KLY281	BY4741 *APL4-3XHA::HIS3*	This Study
ZHYDRS2-MYC	BY4742 *DRS2-13MYC::KanMX6*	Hua *et al*., 2002
KLY1101	SEY6210 *SEC7-DsRed.T4::TRP*	Chen *et al*., 2006
KLY261	KLY1101 *drs2Δ::LEU2*	This Study
GGA2-GFP	BY4741 *GGA2-GFP::HIS3*	Invitrogen
APL1-GFP	BY4741 *APL1-GFP::HIS3*	Invitrogen
APL2-GFP	BY4741 *APL2-GFP::HIS3*	Invitrogen
APL6-GFP	BY4741 *APL6-GFP::HIS3*	Invitrogen
CLC-GFP	BY4741 *CLC-GFP::HIS3*	Invitrogen
KLY421	BY4741 *APL2-GFP::HIS3 drs2Δ::LEU2*	This Study
KLY451	BY4741 *GGA2-GFP::HIS3 drs2Δ::LEU2*	This Study

KLY391	BY4741 *CLC-GFP::HIS3 drs2Δ::LEU2*	This Study
KLY791	APL2-GFP X KLY1101	This Study
KLY795	GGA2-GFP X KLY1101	This Study
KLY792	CLC-GFP X KLY1101	This Study
KLY362	KLY451 X KLY261	This Study
KLY363	KLY421 X KLY261	This Study
KLY361	KLY391 X KLY261	This Study
KLY551	*MATa his3Δ1 leu2Δ0 ura3Δ0 APL2-GFP::HIS3 erg6Δ::KanMX6*	This Study
KLY561	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 APL6-GFP::HIS3 erg6Δ::KanMX6*	This Study
KLY351	KLY1101 *apl2Δ::KanMX6*	This Study
KLY701	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 apl4Δ::KanMX6 vps4Δ::KanMX6*	This Study
KLY711	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 apl4Δ::KanMX6 vps27Δ::KanMX6*	This Study
KLY761	BY4741 *rcy1Δ::KanMX6 vps27Δ::KanMX6*	This Study
KLY771	BY4742 *snx4Δ::KanMX6 vps27Δ::KanMX6*	This Study

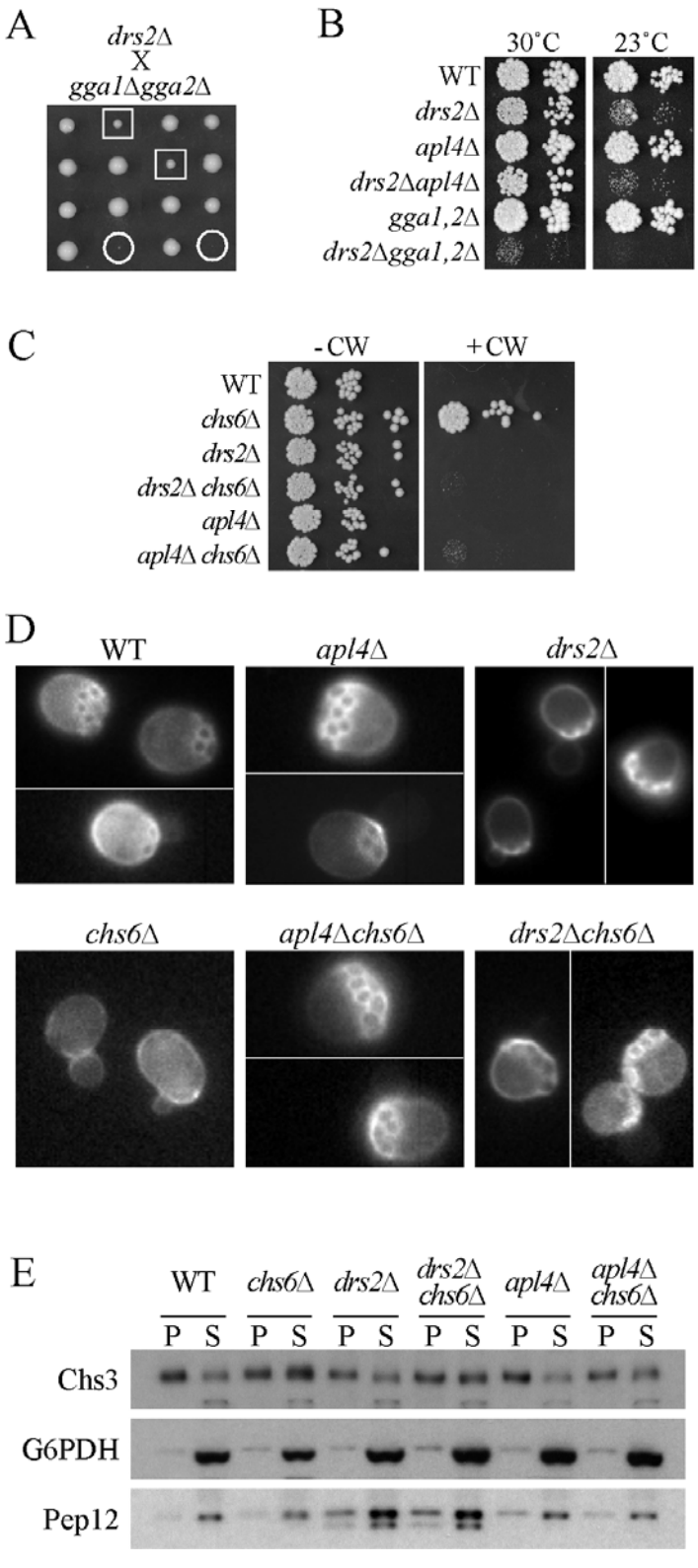

Figure 3-1: Drs2p functions in an AP-1 pathway.

Figure 3-1: Drs2p functions in an AP-1 pathway.
(A) Tetrad analysis of progeny derived from crossing *drs2Δ* with *gga1Δgga2Δ*. Circled progeny are *drs2Δ gga1Δgga2Δ* triple mutants. Boxed progeny are *drs2Δ gga2Δ* double mutants.
(B) Synthetic genetic interaction between *drs2* and *gga* alleles. Growth of the indicated strains (From Top to bottom: BY4742, ZHY615M2D, BY4742 YPR029C, KLY691, KLY751, KLY741) was assessed by spotting tenfold serial dilutions onto YPD plates and growing at 30°C and 23°C
(C) Cells deficient in Drs2p or AP-1 restore calcofluor white sensitivity to *chs6Δ* cells. Calcofluor white sensitivity was assayed on rich medium supplemented with 100 μg/ml calcofluor white.
(D) Chitin-ring staining is restored to *chs6Δ* cells by deletion of either AP-1 subunits or *DRS2*. Cell wall chitin was visualized by staining cells with 1 mg/ml calcofluor white.
(E) Localization of Chs3p to the plasma membrane of *chs6Δ* is restored by disruption of Drs2p or Apl4p. The distribution of proteins between the plasma membrane and the internal membranes was determined by centrifugation of cell lysates at 13,000 x g to pellet the plasma membrane. Golgi and endosomal membranes remain in the supernatant.

Results

Drs2p Is Required for AP-1 Function in the TGN-Early Endosome Pathway

Previously, we had implicated Drs2p in the formation of clathrin-coated vesicles from the TGN-endosomal system (Chen *et al.*, 1999; Gall *et al.*, 2002; Chantalat *et al.*, 2004). To address whether Drs2p preferentially acts in conjunction with AP-1 or Gga clathrin adaptors, we performed a synthetic genetic analysis between mutant alleles encoding these proteins. Cell growth is robust when either AP-1 or Gga-mediated transport is abolished, but disruption of both pathways causes a dramatic growth defect (Costaguta *et al.*, 2001; Hirst *et al.*, 2001). Thus, cells deficient for either adaptor are sensitized to perturbations in the function of the other. Therefore, if Drs2p is required for AP-1 function, *drs2Δ* should exhibit a strong synthetic growth defect with *gga1Δ gga2Δ* mutants. Conversely, if AP-1 function is lost in *drs2Δ* cells, then combining AP-1 null alleles with *drs2Δ* should have no additional consequence. To test this possibility, we crossed *drs2Δ* with *gga1Δ gga2Δ* and performed a tetrad analysis (Figure 3-1A). The *drs2Δ gga1Δ gga2Δ* triple mutant progeny were either inviable or extremely slow-growing (Figure 3-1A, circled and 3-1B), and the *drs2Δ gga2Δ* double mutants (Figure 3-1A, boxed) grew slower than *drs2Δ* or *gga2Δ* single mutant spores. In contrast, when we combined *drs2Δ* with *apl4Δ*, a deletion in the γ1 subunit of AP-1, the *drs2Δ apl4Δ* mutant showed a similar growth rate to the *drs2Δ* single mutant at all temperatures tested (Figure 3-1B). For example, *drs2Δ* grew poorly at 23˚C, but even at this

semi-permissive temperature, loss of AP-1 has no additional consequence. These results suggest that Drs2p has selective function in AP-1/clathrin mediated traffic but does not appear to act in the Gga pathway.

The best defined role for AP-1 in yeast is in the trafficking of chitin synthase III (Chs3p), 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 cycling between the TGN and early endosome, but a portion is transported to the plasma membrane in a cell-cycle regulated process that requires the Chs5p and Chs6p (ChAPs/exomer) coat complex (Santos *et al.*, 1997; Ziman *et al.*, 1998; Trautwein *et al.*, 2006; Wang *et al.*, 2006). Thus, *chs6*Δ cells retain nearly all Chs3p intracellularly and have reduced chitin incorporation at the bud site, which makes this mutant resistant to toxic effects of calcofluor white (CW), a chitin-binding fluorescent compound (Figure 3-1C and (Valdivia *et al.*, 2002; Phelan *et al.*, 2006)). Deletion of any AP-1 subunit bypasses the Chs6p requirement, restoring cell surface transport of Chs3p, the appearance of chitin rings and CW sensitivity to an AP-1 *chs6Δ* double mutant (Valdivia *et al.*, 2002). Similar to the *chs6Δ apl4Δ* strain, the *chs6Δ drs2Δ* double mutant failed to grow on the medium containing CW (Figure 3-1C), and formed chitin rings morphologically indistinguishable from those in the wild-type and *drs2Δ* cells (Figure 3-1D). Subcellular fractionation of membranes by differential centrifugation (9,000g) was also used to distinguish Chs3p in the PM and intracellular membrane pools (Figure 3-1E). This analysis again indicated that disruption of *DRS2* in the *chs6Δ* background, like

disruption of *APL4*, caused redistribution of Chs3p from internal organelles to the plasma membrane fractions, but did not alter the steady-state subcellular distribution of other cytosolic (G6PDH) and endosomal (Pep12p) markers. Together, these data indicate that deletion of *drs2*Δ bypasses *chs6*Δ as effectively as deletion of an AP-1 subunit, consistent with a requirement for Drs2p in forming AP-1/clathrin coated vesicles.

Drs2p Associates with AP-1, but Is Not Required to Recruit AP-1 to Membranes

AP-1 recruitment to Golgi or endosomal membranes of mammalian cells requires the small GTP-binding protein ARF. The observation that Drs2p interacts directly with an ARF-GEF by its C-terminal tail prompted us to further test if ARF or its effector AP-1 can associate with Drs2p. To do so, cells expressing a functional Myc tagged version of Drs2p were spheroplasted and treated with dithiobis(succinimidyl) -prionate (DSP) before lysis. DSP is a bifunctional, cleavable chemical crosslinking reagent that can permeate the cell. Drs2p was immunoprecipitated from the cell lysate, the crosslinks were disrupted by reduction, and the immunoprecipitate was probed by immunoblotting with affinity-purified polyclonal antibodies to Arf1p and Apl2p, the AP-1β subunit. Apl2p, but not Arf1p, co-immunoprecipitated with Drs2p-Myc (Figure 3-2A). However, Apl2p could not be detected in control immunoprecipitates from cells lacking *DRS2-Myc*. In addition, chemical crosslinking followed by immunoprecipitation of HA tagged Apl4p, the AP-1 γ subunit, specifically isolated endogenous Drs2p (Figure 3-2B). These results indicate that AP-1 can form a complex with Drs2p in yeast cells;

however, only a small percentage (0.5%) of Drs2p co-immunoprecipated with AP-1 and so this complex is likely to be transient *in vivo*.

The requirement for Drs2p in the AP-1/clathrin-dependent Chs3p pathway and the association of Drs2p with AP-1 suggested that Drs2p might be required to recruit AP-1 and clathrin onto TGN/endosomal membranes. To test this possibility, we compared the localization of AP-1β-GFP (Apl2p-GFP) in wild-type and *drs2Δ* mutant cells. Both strains showed a pattern of spots typical of the yeast Golgi and endosomal compartments with no indication that membrane association of AP-1 was perturbed in *drs2*Δ (Figure 3-2C). We also analyzed the distribution of Apl2p relative to the TGN marker Sec7p. The overlap between Apl2p and Sec7p was extensive in both wild-type and *drs2Δ* cells: in wild-type cells, 51% of Apl2-GFP puncta were coincident with Sec7-DsRed puncta and in *drs2Δ* cells, 73% of Apl2-GFP puncta colocalized with Sec7-DsRed puncta. The increased association of Apl2-GFP with Sec7-positive membrane is likely caused by normal recruitment of AP-1 to the TGN combined with a defect in the release of AP-1/clathrin-coated vesicles (Chen *et al.*, 1999; Gall *et al.*, 2000). Localization of GFP tagged clathrin light chain (Clc1-GFP) and GGA2 (GGA2-GFP) were also examined in these strains and their membrane association appeared to be unaffected (Figure 3-2C). Thus, Drs2p is not required for recruitment of AP-1 or clathrin to the TGN membrane, even though it appears to be essential for AP-1/clathrin function.

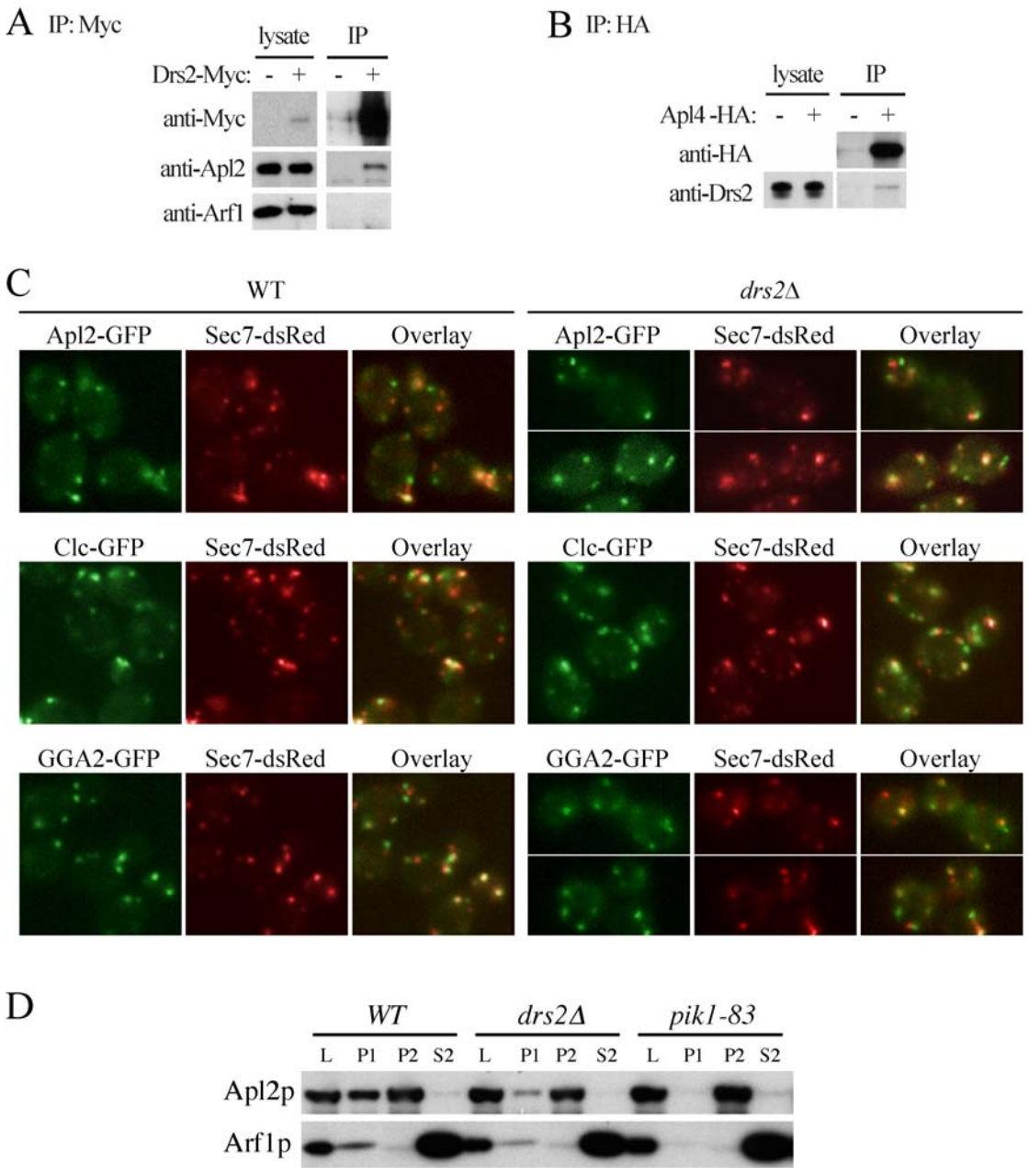

Figure 3-2: Drs2p interacts with AP-1 but is not required for AP-1 or clathrin recruitment to Golgi membranes.

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Chemical crosslinking identifies a complex containing Drs2p and AP-1.

(A) Cells expressing Myc-tagged Drs2p or control cells without the tag were treated with crosslinker DSP, lysed, and anti-Myc monoclonal antibodies were used to purify the Drs2p-Myc. The crosslinker was cleaved by reduction and bound material was visualized by immunoblotting. For the "lysate" lanes, approximately 0.5% of the amount of material used for the crosslinking reaction was loaded.

(B) Cells expressing HA-tagged Apl4p and control cells without the tag were treated as described in (A). Anti-HA monoclonal antibodies were used to immunoprecipitate the Apl4p-HA.

(C) Drs2 is not required for Golgi recruitment of AP-1 or clathrin.

Wild-type and *drs2Δ* strains expressing the indicated fusion proteins are imaged by fluorescence microscopy.

(D) Membrane association of AP-1 is not affected by deletion of Drs2.

Wild-type, *drs2Δ* and *pik1-83* cells grown at 30°C were lysed (Lysate, L) and subjected to centrifugation at 13,000 x g for 20 min to generate pellet (P1) and supernatant fractions. The supernatant fraction was further centrifuged at 100, 000 x g for 1 hour to generate pellet (P2) and supernatant (S2) fractions. Different fractions were then immunoblotted for Apl2p and Arf1p.

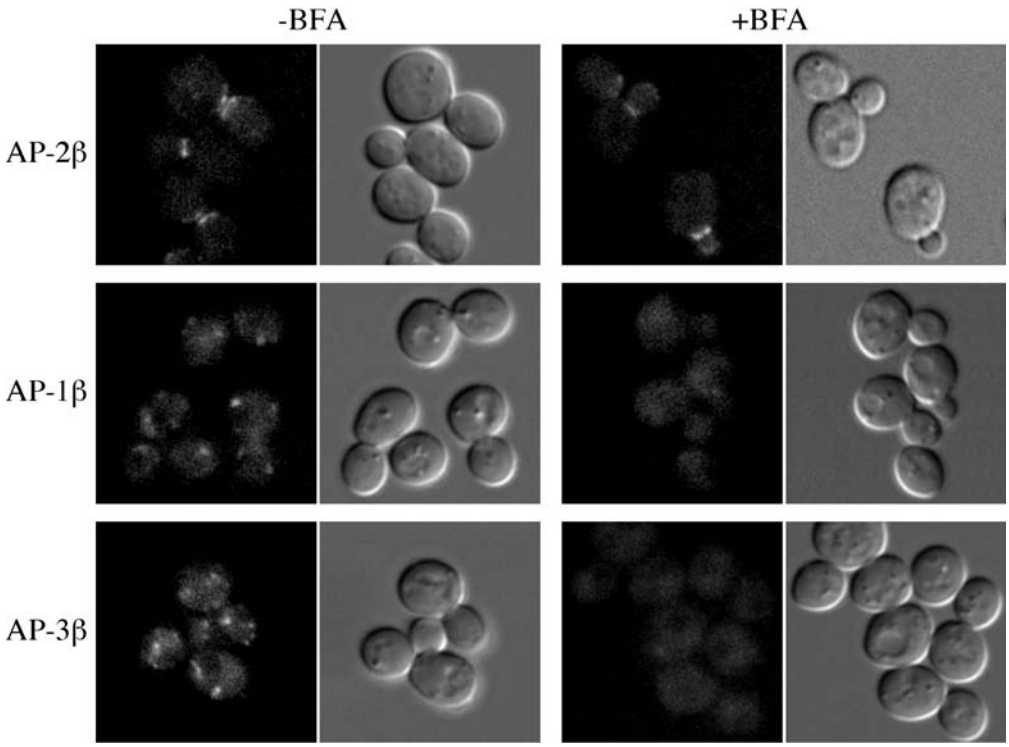

Figure 3-3: The punctate localization pattern of AP-1 and AP-3, but not AP-2, is dependent on Arf. Cells expressing GFP tagged Apl1p (AP-2 β), Apl2p (AP-1 β) or Apl6p (AP-3 β) were grown to log phase, treated with 100 μg/ml BFA and the visualized by fluorescence microscopy.

Membrane association of AP-1 was also examined in wild-type and *drs2Δ* mutant cells by differential centrifugation. Because it is reported that phosphatidylinositol-4-phosphate is required for AP-1 recruitment to the Golgi in mammalian cells (Wang *et al.*, 2003), a *pik1* mutant, the yeast Golgi resident phosphatidylinositol 4-kinase, was also tested. Cell lysates (L) were sequentially centrifuged at 10,000 x g and 130,000 x g to collect P1 and P2 pellets, respectively, and 130,000 x g supernatants (S2), which were probed for AP-1 (Apl2p) and ARF. Apl2p was found primarily in the membrane fractions (P1 and P2) while ARF was found primarily in the cytosol (S2) fractions (Figure 3-2D). No significant difference in the fractionation profile for AP-1 or ARF was detected between wild-type and *drs2Δ* strains, and we also failed to detect any release of Apl2p from the *pik1-83* membranes.

In mammalian cells, AP-1 can be released from membranes by brefeldin A (BFA), a drug that binds to an Arf GEF-Arf1-GDP complex and prevents Arf1 activation (Peyroche *et al*., 1999). Wild-type yeast are resistant to BFA, but an *erg6Δ* mutant, defective in one of the final steps of ergosterol biosynthesis, is sensitive (Graham et al., 1993; Peyroche and Jackson, 2001). To be certain that the punctate localization pattern of Apl2-GFP required ARF-dependent membrane localization, we introduced the *erg6Δ* mutation into cells expressing Apl2p-GFP and treated the cells with BFA. In these cells, Apl2p-GFP was released from membranes within 1min of treatment with BFA and was dispersed throughout the cytosol (Figure 3-3). We also introduced *erg6Δ* into strains expressing GFP tagged Apl1p, the AP-2 α subunit, and GFP tagged Apl6p, the AP-3 β

subunit, and treated these cells with BFA. BFA also caused release of GFP-Apl6p (AP-3) but not GFP-Apl1p (AP-2) (Figure 3-3). Hence, as with mammalian cells, the dynamic association of AP-1 and AP-3 with membranes requires a BFA-sensitive ARF-GEF (i.e. Gea1p, Gea2p or Sec7p) while AP-2 does not. These data also indicate that the punctate pattern of Apl2p-GFP represents an ARF-dependent membrane-associated pool and Drs2p is not required for recruiting AP-1 to the membrane.

Disruption of AP-1 Alters Drs2p Trafficking but not Its Steady-state Localization to the TGN

Our previous work suggested that Drs2 primarily traffics between the TGN and early endosome, a pathway associated with AP-1 function (Liu *et al.*, 2007). To test if Drs2p localization requires AP-1, we first examined the steady-state localization of GFP-Drs2p in wild-type and AP-1-deficient cells. GFP-Drs2p localizes to several discrete puncta in wild-type cells, representing the TGN and possibly early endosomes. This pattern was not altered in cells lacking AP-1 function (Figure 3-4A, *apl2Δ*). Furthermore, similar levels of overlap between GFP-Drs2p and Sec7p-DsRed were observed for wild-type and *apl2Δ* cells: in wild-type cells 81% of Drs2p-GFP puncta were coincident with Sec7-DsRed puncta, while in *apl2Δ* cells 82% of Drs2p-GFP puncta colocalized with Sec7-DsRed puncta. Thus, the steady-state localization of GFP-Drs2p is not affected by AP-1 disruption.

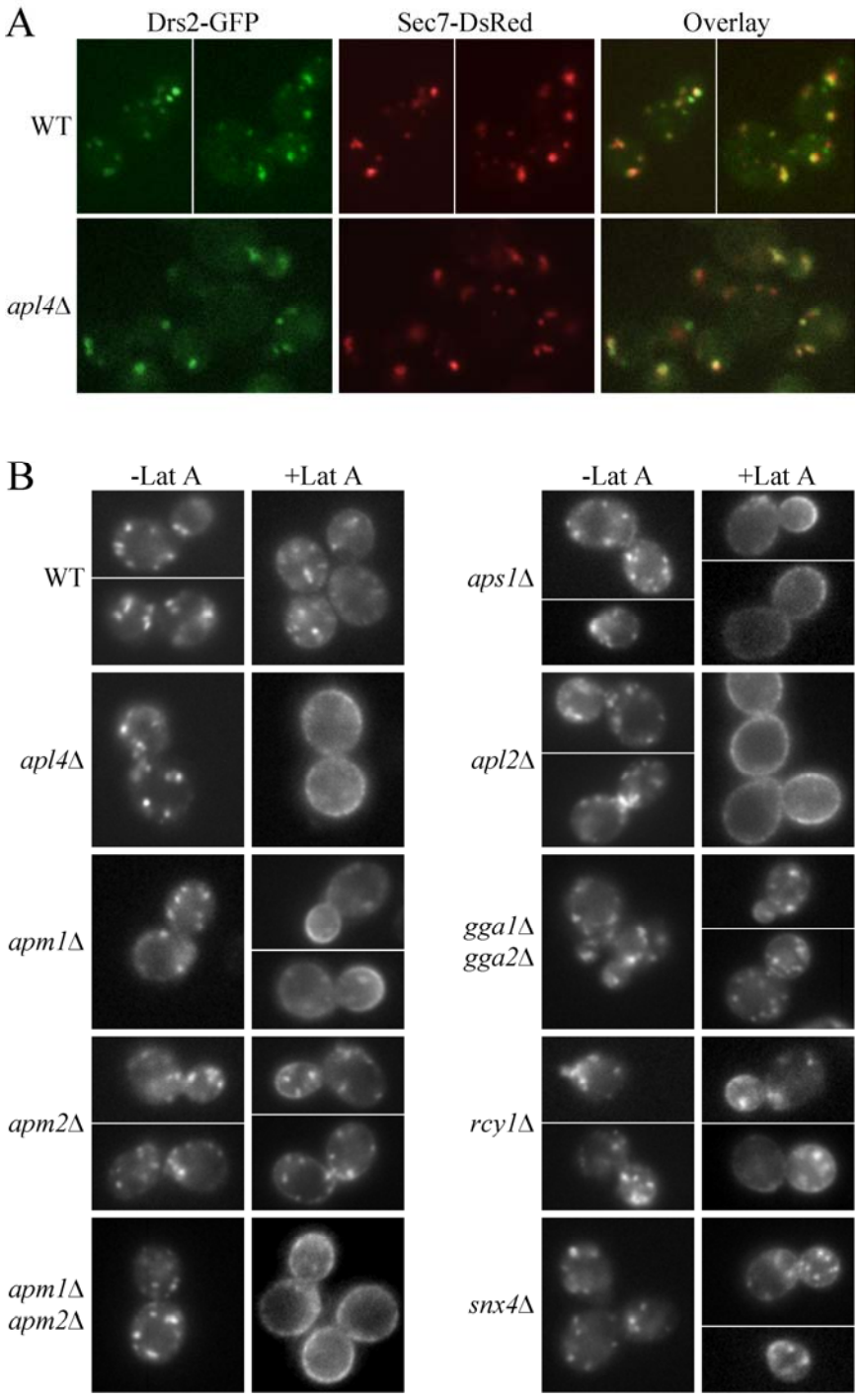

Figure 3-4: Loss of AP-1 perturbs Drs2p trafficking but not its steady-state localization.

Figure 3-4: Loss of AP-1 perturbs Drs2p trafficking but not its steady-state localization.
(A) Colocalization of Drs2p-GFP and Sec7-DsRed in wild-type and AP-1 deficient (*apl4Δ*) cells.
(B) Disruption of AP-1 causes rapid accumulation of Drs2p on the plasma membrane when endocytosis is blocked. The indicated strains (BY4741, BY4741 YLR170C, BY4741 YPR029C, BY4741 YKL135C, BY4741 YPL259C, KLY691, BY4741 YHL019C, BY4741 YJL204C, KLY371, BY4741 YJL036W) expressing GFP-Drs2p were incubated with or without 200 μM latrunculin A (Lat-A) at room temperature for 30 minutes and then examined by fluorescence microscopy.

We then addressed whether deletion of AP-1 has an effect on trafficking of GFP-Drs2p. Drs2p slowly cycles in the TGN → plasma membrane → early endosome → TGN loop. Acute inactivation of endocytosis by treating cells with latrunculin A (Lat A), an inhibitor of actin assembly and endocytosis, causes a rather slow accumulation of Drs2p on the plasma membrane over the course of about three hours. If GFP-Drs2p rapidly cycles between the TGN and early endosome in an AP-1 pathway, we would expect an increased rate of Drs2p transport to either the plasma membrane or late endosome in an AP-1 mutant. To test this possibility, we treated wild-type and AP-1 mutant cells expressing GFP-Drs2p with Lat A and imaged over time. In wild-type cells GFP-Drs2p was primarily retained intracellularly in small puncta after 30 minutes of Lat A treatment (Figure 3-4B, WT). In stark contrast, deletion of the AP-1 β subunit (*APL2*), γ subunit (*APL4*), or σ subunit (*APS1*) caused accumulation of nearly all GFP-Drs2p on the plasma membrane after 30 minutes of treatment. This cell surface accumulation of GFP-Drs2p was much less pronounced in *apm1Δ* or *apm2Δ* cells, which harbor deletions of the two alternative AP-1 μ subunits. However, deletion of both *APM1* and *APM2* leads to plasma membrane accumulation comparable to deletion of other AP-1 subunits (Figure 3-4B), suggesting that the two μ subunits play redundant roles in Drs2p trafficking. To determine whether trafficking of Drs2p also requires Gga functions, we treated the *gga1Δ gga2Δ* strain with Lat A and found that these cells slowly accumulated GFP-Drs2p on the plasma membrane at a rate similar to wild-type cells. Thus, the fast cell surface accumulation of Drs2p after Lat A treatment is specific to AP-1 mutants.

AP-1 has been proposed to play a role in retrograde transport from early endosome back to the TGN. To determine if the Lat A-induced fast accumulation of Drs2p on the plasma membrane in AP-1-deficienct cells was an indirect consequence of perturbing early endosome to TGN recycling, we examined the trafficking of GFP-Drs2p in two other mutants implicated in early endosome to TGN retrieval (Figure 3-3B, *rcy1Δ* and *snx4Δ*). However, inactivation of Rcy1p, an F-box protein, only slightly increased the rate of GFP-Drs2p accumulation on the cell surface after Lat A treatment, while deletion of Snx4p, a sorting nexin had no effect on the rate of GFP-Drs2p plasma membrane accumulation. These data suggest a direct requirement for AP-1 in removing Drs2p from the exocytic pathway for delivery to the early endosome.

As a cargo of the AP-1 mediated early endosome to TGN retrograde pathway, Chs3p normally avoids transport to the late endosome / prevacuole compartment (PVC) but transits this compartment frequently in AP-1 mutants. This perturbation is revealed in the class E *vps* AP-1 double mutants (*apl4Δvps4Δ* and *apl4Δvps27Δ*) which trap Chs3p in enlarged endosomal compartments adjacent to the vacuole. To test if AP-1 disruption perturbs retrograde transport of Drs2p in a manner similar to Chs3p, localization of GFP-Drs2p and Chs3p-GFP were monitored in *apl4Δ vps4Δ* and *apl4Δ vps27Δ* cells. As previously observed, Chs3-GFP collapses into a few aberrant structures next to the vacuole in both *apl4Δ vps4Δ* (Figure 3-5) and *apl4Δ vps27Δ* (unpublished results). In contrast, GFP-Drs2p does not accumulate in the PVC of these double mutants and shows a localization pattern indistinguishable from that in the wild-type cells. Moreover,

GFP-Drs2p also appears to localize normally in *rcy1Δ vps27Δ* and *snx4Δ vps27Δ* strains (Figure 3-5). Thus, GFP-Drs2p does not appear to require AP-1, Rcy1 or Snx4 for its exclusion from the late endosome. In summary, these data indicate that AP-1 is required for anterograde transport of Drs2p from the TGN back to the early endosome, does not require AP-1 for the retrograde trip back to the TGN.

Snc1p is a v-SNARE that actively cycles along the TGN→ plasma membrane → early endosome → TGN loop. Drs2p, Rcy1p, and Snx4p are all required for efficient Snc1p recycling. In wild-type cells, GFP-Snc1p primarily localizes to the plasma membrane concentrating in the regions of polarized growth, with a few intracellular punta corresponding to endosomal/TGN compartments. Deletion of *DRS2* or *RCY1* causes accumulation of GFP-Snc1p on internal structures, whereas GFP-Snc1p is missorted to the vacuole in *snx4Δ* cells. To test if AP-1 has any function in the Snc1p recycling pathway, we expressed GFP-Snc1p in *apl4Δ* and *apl4Δvps27Δ* cells (Figure 3-6 A). GFP-Snc1p localized normally in both mutants. We next tested whether loss of AP-1 function would affect the kinetics of GFP-Snc1p trafficking to the plasma membrane. Since most GFP-Snc1p is already present on the cell surface in wild-type and *apl4Δ* cells, we instead treated *drs2Δ* and *drs2Δ apl4Δ* strains with Lat A (Figure 3-6 B). Both strains have little GFP-Snc1p present on the PM before Lat A treatment and they exhibit similar kinetics of Snc1p-GFP accumulation on the PM after treatment. Collectively, these results demonstrate that Snc1p trafficking does not seem to require AP-1 function.

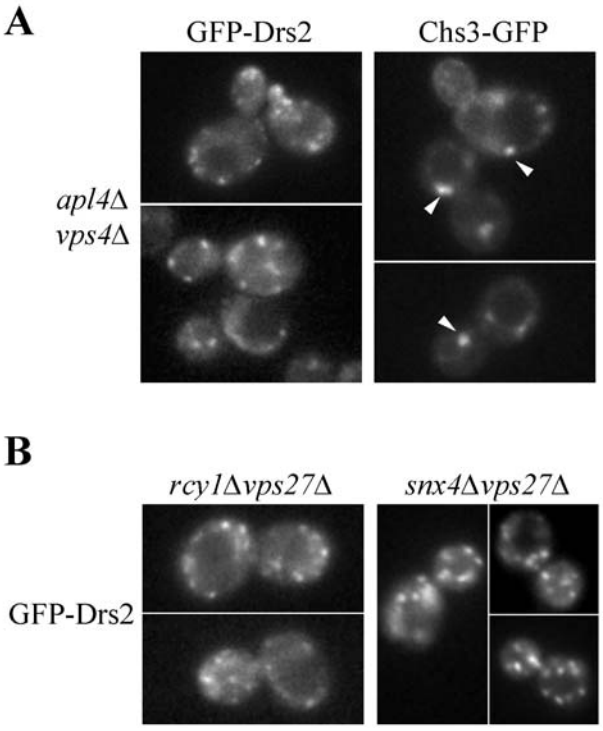

Figure 3-5: GFP-Drs2p is not trapped in the late endosome/PVC in AP-1, *rcy1* or *snx4* mutants.

(A) In *apl4Δ vps27Δ* cells, Chs3p-GFP accumulates in the class E compartments, while GFP-Drs2p appears to localize normally to the TGN.

apl4 Δvps27Δ strains expressing GFP-Drs2p or Chs3p-GFP are imaged by fluorescence microscopy. Arrowheads indicate the class E compartments.

(B) Localization of GFP-Drs2p in *rcy1Δ vps27Δ* and *snx4Δ vps27Δ* cells.

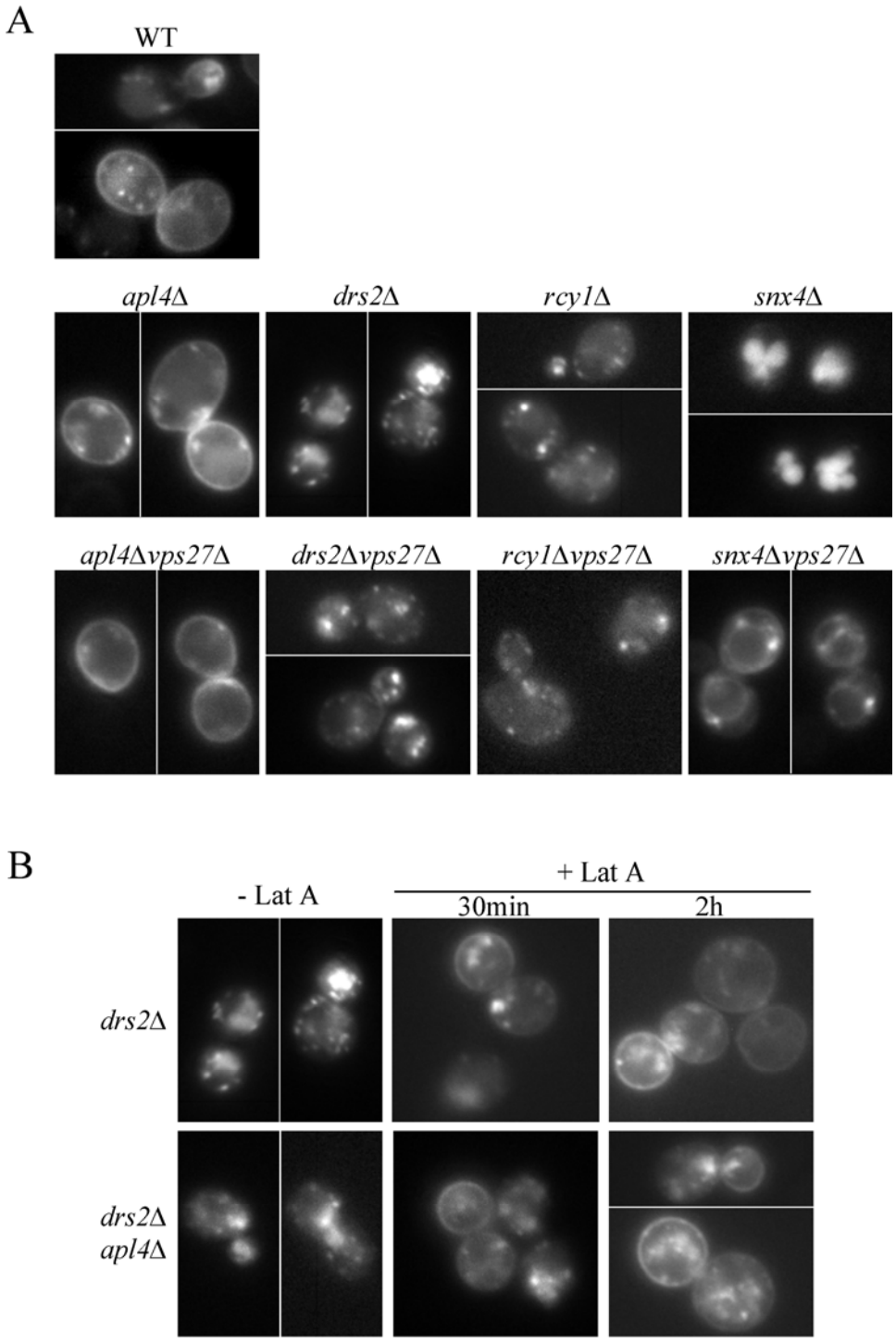

Figure 3-6: Loss of AP-1 does not perturb Snc1p trafficking and its steady-state localization.

Figure 3-6: Loss of AP-1 does not perturb Snc1p trafficking and its steady-state localization.
(A) Localization of Snc1p-GFP in wild-type cells and various mutants that disrupt protein trafficking in the TGN-endosomal system.
(B) Deletion of AP-1 from *drs2Δ* cells does not alter the trafficking kinetics of Snc1p-GFP. *drs2Δ* and *drs2Δ apl4Δ* cells expressing GFP-Drs2p were incubated with or without 200 μM latrunculin A (Lat-A) at room temperature for 30 min or 2 h and then examined by fluorescence microscopy.

Discussion

A major goal of this study is to address the relationship between the clathrin adaptor AP-1 and Drs2p, a potential phospholipid translocase, in clathrin mediated protein trafficking. Our results demonstrate that Drs2p and AP-1 function together to mediate protein transport between the TGN and early endosome, and that these proteins interact directly or indirectly. Deletion of *DRS2* does not seem to affect recruitment of AP-1 to the TGN membrane, suggesting that Drs2p is required at late stages in AP/clathrin-coated vesicle formation. In addition, disruption of AP-1 causes missorting of Drs2p to the plasma membrane as judged by fast plasma membrane accumulation of Drs2p after acute inactivation of endocytosis. These results define Drs2p as an anterograde cargo for AP-1.

Potential Roles Drs2p Might Play in Budding Clathrin-Coated Vesicles

Previous studies have demonstrated that Drs2p, a phospholipid translocase, plays a role in the CCV formation from the TGN and endosomal membranes (See Introduction). Several lines of evidence indicated that the translocase activity of Drs2p is required for its function in vesicle biogenesis. Acute inactivation of *drs2-ts* by temperature shift abrogates the translocation of NBD-PS, a fluorescent phosphatidylserine derivative, across the purified TGN membrane as well as the formation of a clathrin-dependent class of exocytic vesicles (Gall *et al.*, 2002; Natarajan *et al.*, 2004). Moreover, an ATPase-dead allele of Drs2 (*drs2-D560N*) fails to complement *drs2Δ* defects in dense exocytic vesicle

formation and the recycling of the exocytic v-SNARE Snc1p (Chen *et al.*, 1999; Chantalat *et al.*, 2004; Liu *et al.*, 2007).

Two models have been proposed to explain how the phospholipid translocase activity of Drs2p contributes to vesicle biogenesis (Chen *et al.*, 1999). One possibility is that Drs2p may affect the recruitment of peripherally associated proteins by increasing the concentration of some specific phospholipid on the cytosolic leaflet. However, our results rule out this possibility by showing that membrane association of coat components required for vesicle formation, such as AP-1, GGA2, AP-3 and clathrin, is independent of Drs2p. In addition, we had previously shown that recruitment of the Arf-GEF Gea2p to the TGN does not require Drs2p (Chantalat *et al.*, 2004). In fact, we observed a modest clustering of Clc-GFP in *drs2Δ* haploid cells. Therefore, in contrast to current models for CCV budding, our data argue that membrane recruitment of Arf-GEF, Arf, AP-1 and clathrin is insufficient to bud AP-1/clathrin-coated vesicles in the absence of Drs2p.

An alternative possibility is that the transbilayer movement of lipids mediated by Drs2p helps to bend the membrane to facilitate vesicle budding. According to the bilayer-couple hypothesis of Sheetz and Singer, the two leaflets of membrane are physically coupled together and a relative increase in surface area of one leaflet would spontaneously induce membrane curvature (Sheetz and Singer, 1974; Sheetz *et al.*, 1976). Both in erythrocytes and pure phospholipid model systems, bilayer asymmetry induced by insertion of additional lipids in one of the membrane leaflets leads to dramatic shape changes (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985; Daleke and Huestis,

1989; Farge and Devaux, 1992). When the surface area of outer leaflet is increased relative to the inner leaflet, the closed lipid bilayer system (e.g. liposomes, erythrocytes) will become crenated, tubulated or vesiculated. When the surface area of inner leaflet is increased relative to the outer leaflet, the membrane becomes concave. A large group of proteins, which have the potential to induce the transbilayer area asymmetry, have been implicated in protein trafficking, including proteins with amphipathic helixes that could physically penetrate into one face of the bilayer (e.g. endophilin, amphiphysin, epsin (Itoh and De Camilli, 2006; Ren *et al.*, 2006)), lipid transfer proteins, lipid metabolic enzymes (e.g. sphingomyelinase (Zha *et al.*, 1998), lysophosphatidic acid transferases (Schmidt *et al.*, 1999; Weigert *et al.*, 1999)), as well as lipid translocases (Graham, 2004). By translocating phospholipids from the lumenal leaflet to the cytosolic leaflet, phospholipid translocases could increase the surface area of the cytosolic leaflet and bend the membrane towards the cytosol. Indeed, ATP-dependent inward translocation of exogenous PS and PE by phospholipid translocase on the plasma membrane of human erythroleukemia cells enhances bulk endocytosis (Farge *et al.*, 1999). Consistent with a putative role of Drs2p in regulating membrane curvature, Golgi membranes in the *drs2Δ* mutants are notable for their lack of fenestration or tubular regions (Chen *et al.*, 1999), whereas the Golgi in *arf1Δ* mutants is highly fenestrated or tubular in appearance (Gaynor *et al.*, 1998).

Drs2p Is an Anterograde Cargo of AP-1

In wild-type cells, Drs2p primarily cycles between the TGN and early endosomes, and only visits the plasma membrane occasionally (Liu *et al.*, 2007). Strikingly, in the AP-1 mutants, Drs2p rapidly traffics to and from the plasma membrane, but still maintian its steady-state TGN residence. How AP-1 affects the trafficking itinerary of Drs2p could be explained by two possibilities. First, AP-1 may mediate the retrieval of Drs2p from early endosome back to the TGN. In this case, loss of AP-1 would cause accumulation of Drs2p at the early endosome, from which Drs2p could potentially recycle to the plasma membrane. The second possibility is that AP-1 is required for the anterograde transport of Drs2p from the TGN to early endosomes. When AP-1 is disrupted, Drs2p is incorporated into exocytic vesicles targeted to the plasma membrane more frequently than in a wild-type cell. The second possibility is favored by several experimental results. The extensive colocalization between Drs2p and the TGN marker Sec7p remains in the AP-1 mutant, indicating that endocytosis and early endosome to TGN transport of Drs2p is unaffected by loss of AP-1. If Drs2p accumulated in the early endosome in AP-1 deficient cells, we would expect it to traffic more frequently to the late endosome/PVC, like Chs3p. However, Drs2p is not trapped in the class E compartments of *apl2Δvps27Δ* or *apl4Δvps4Δ* cells, suggesting that Drs2p does not transit through the late endosome/PVC, even in the absence of AP-1. Thus, inactivation of AP-1 does not seem to cause accumulation of Drs2p in the early endosome. The Snx4-Snx41-Snx42 complex and the F-box protein Rcy1p have both been implicated in the transport pathway from early

endosome back to the TGN (Wiederkehr *et al.*, 2000; Hettema *et al.*, 2003). In *rcy1Δ* cells the Drs2p/Cdc50p complex colocalizes extensively with Snc1p-positive structures, which are probably derived from early endosomes (Furuta *et al.*, 2007). These results, in combination with the physical interaction between Drs2p and Rcy1p, indicate that Rcy1p is involved in early endosome to TGN retrieval of Drs2p. However, disruption of Rcy1 only slightly increases the rate of Drs2p transport to the plasma membrane, while disruption of Snx4-Snx41-Snx42 has no apparent impact on Drs2p trafficking. Thus, the substantially enhanced plasma membrane trafficking of Drs2p in AP-1 deficient cells is more likely due to missorting of Drs2p at the TGN into secretory vesicles than a secondary effect of Drs2p accumulation at the early endosome.

A(F->A)ALP is a Ste13p based reporter protein which is thought to primarily traffic between the TGN and early endosome, but accumulates relatively slowly in the late endosome of class E *vps* mutants. AP-1 directly binds to the cytosolic tail of A(F->A)ALP and reduces its rate of trafficking to the late endosome (Foote and Nothwehr, 2006). Chs3p also accumulates rapidly in the late endosome of AP-1 mutants. These results suggest that AP-1 functions to retrieve proteins from the early endosome back to the TGN. However in this study, we demonstrate that AP-1 primarily mediates the anterograde transport of Drs2p from the TGN to the early endosome. Moreover, loss of AP-1 function restores the transport of Chs3p to the cell surface under conditions in which the normal export route of Chs3p is blocked (Valdivia *et al.*, 2002). Thus, AP-1 seems to function in both anterograde and retrograde pathways between the TGN and

early endosome. Remarkably, Drs2p appears to only use AP-1 for anterograde transport, while Chs3p may use AP-1 for both pathways. In fact, similar phenomena could also be observed for other clathrin adaptors such as GGA proteins. The GGA proteins are implicated in the direct TGN to late endosome transport of Pep12p (Black and Pelham, 2000) and Vps10p (Costaguta *et al.*, 2001), and a recent study demonstrated that GGA mutations have an impact on trafficking of Chs3p and A(F->A)ALP similar to AP-1 mutations. These results imply that GGA proteins may, like AP-1, play a role in TGN/early endosome pathways (Copic *et al.*, 2007). How the adaptors achieve this ability to perform a cargo-specific function is still not clear, but likely to involve the recognition of pathway-specific sorting signals.

Transport Pathways that Involve Drs2p

As we have discussed above, AP-1 and GGA proteins have redundant and non-redundant functions in clathrin-mediated transport between the TGN and endosomes. Simultaneous inactivation of AP-1 and GGA proteins causes a severe synthetic growth defect, while perturbation on either AP-1 or GGA mediated transport has little impact on cell growth (Costaguta *et al.*, 2001; Hirst *et al.*, 2001). Similar genetic interactions are found between Drs2 and the GGA proteins, but not between Drs2 and AP-1, suggesting that Drs2p is required for essential function of AP-1 but not that of the GGA proteins. However, Drs2p has a more substantial impact on protein trafficking than AP-1, since *drs2* mutants display more severe defects than AP-1 in both protein transport and cell

growth. Drs2p also play roles in AP-1 independent transport pathways, such as the AP-3 mediated alkaline phosphatase pathway to the vacuole and the Snc1p recycling pathway (Hua *et al.*, 2002; Liu *et al.*, 2007).

Interestingly, unlike other proteins that frequently transit the TGN (e.g. Kex2p, Chs3p, Vps10p, Snc1p), Drs2p is never trapped in the late endosome derived class E compartment in various mutants we tested (*vps4Δ, vps27Δ, apl2Δvps27Δ, rcy1Δvps27Δ, snx4Δvps27Δ*). It seems that the cells go to great lengths to prevent Drs2p from getting into the late endosome. The normal function of the late endosome/multivesicular body requires production of negative membrane curvature on its surface to facilitate the inward budding of vesicles. Considering that the proposed role for Drs2p is to induce positive membrane curvature, it is tempting to speculate that the presence of Drs2p on the late endosome/multivesicular body would be detrimental to late endosome function, and thus Drs2p is excluded from this compartment.

CHAPTER IV

SUMMARY

Drs2p, a P-type ATPase, is a phospholipid translocase at the yeast TGN that flips phosphatidylserine (PS) and to a lesser extent phosphatidylethanolamine (PE) from the luminal leaflet to the cytosolic leaflet (Natarajan *et al.*, 2004; Alder-Baerens *et al.*, 2006). Interestingly, mutations in Drs2p that disrupt its ATPase or translocase activity phenocopy the effect of clathrin mutations on protein trafficking in the TGN and early endosomal system (Chen *et al.*, 1999; Gall *et al.*, 2002). In addition, *drs2Δ* cells exhibit a deficiency in clathrin-coated vesicles that can be recovered in subcellular fractions containing assembled clathrin (Chen *et al.*, 1999). Moreover, Drs2p is engaged in extensive genetic and physical interactions with vesicle budding machinery acting in the TGN-early endosomal system (Chen *et al.*, 1999; Chantalat *et al.*, 2004; Saito *et al.*, 2004; Sakane *et al.*, 2006; Furuta *et al.*, 2007). Although all these evidences suggest that Drs2p is implicated in clathrin-mediated protein trafficking, two fundamental questions remain: 1) What mechanism does Drs2p use to facilitate clathrin coated vesicle biogenesis? 2) How is Drs2p function coupled to specific transport pathways? In this study, we attempted to answer these two questions by examining the trafficking itinerary, protein interactions and biological function of Drs2p.

Previously, two models have been proposed to describe the role Drs2p might play

in vesicle formation. One model proposes that Drs2p may help to recruit peripherally associated coat proteins by protein interactions or by concentrating specific phospholipid molecules on the cytosolic leaflet of the membrane. To test this model, we studied the membrane association of the coat componenets required for vesicle formation such as AP-1, AP-3 and clathrin and found that they are recruited to the Golgi and endosomal membrane in absence of Drs2p. In addition, we had previously shown that recruitment of the Arf-GEF Gea2p to the TGN does not require Drs2p (Chantalat *et al.*, 2004). The GGA/clathrin pathway appears functional in the absence of Drs2p, but AP-1/clathrin pathways require Drs2p activity. Therefore, in contrast to current models for CCV budding, our data argue that membrane recruitment of Arf-GEF, Arf, AP-1 and clathrin is insufficient to bud AP-1/clathrin-coated vesicles in the absence of Drs2p.

In an alternative model, phospholipid translocation by Drs2p may help to bend the membrane to facilitate vesicle budding. According to the bilayer-couple hypothesis of Sheetz and Singer, the two leaflets of membrane are physically coupled together and a relative increase in surface area of one leaflet spontaneously induces membrane curvature (Sheetz and Singer, 1974; Sheetz *et al.*, 1976). By translocating phospholipid from the lumenal leaflet to the cytosolic leaflet of the TGN or early endosomes, Drs2 would increase the surface area of the cytosolic leaflet relatively to the lumenal leaflet, and bend the membrane towards the cytosol. We propose that Drs2p imparts curvature to the membrane through a bilayer-couple mechanism that is captured by coat proteins to produce vesicles. To test this hypothesis, futher studies need to be done with purified and

reconstituted Drs2p in liposomes.

Although bending membrane is a common requirement for formation of all kinds of vesicles, Drs2p seems to only function in specific post-Golgi pathways. How Drs2p activity is coupled to these pathways remains unknown, but it is essential that Drs2p maintains its appropriate localization to the TGN/early endosome system. To do this, Drs2p primarily cycles between the TGN and the early endosome, and it occasionally traffics to the plasma membrane where it is rapidly endocytosed and efficiently retrieved to the TGN from early endosomes. Endocytosis of Drs2p is mediated by multiple signals, including two NPFXD motifs near the C terminus and PEST-like sequences near the N terminus for ubiquitin-dependent endocytosis. The NPFXD motifs can be specifically recognized by Sla1p, part of an endocytic coat/adaptor complex with clathrin, Pan1p, End3p, and Sla2p. When both ubiquitin- and NPFXD- dependent endocytic mechanisms are abrogated, Drs2p accumulates on the plasma membrane. In *pan1-20* temperature-sensitive mutants, mislocalization of Drs2p to the plasma membrane is lethal to the cell, demonstrating a requirement for Drs2p at the TGN and endosomes to sustain viability of *pan1-20*.

AP-1 and GGA proteins are two classes of clathrin adaptors that play both redundant and nonredundant roles in transport between the TGN and endosomes. AP-1 is required for the anterograde transport of Drs2p to the early endosome, while GGA proteins have little impact on Drs2p trafficking. Interestingly, Drs2p is implicated in AP-1/clathrin function but does not appear to contribute significantly to the GGA/clathrin

pathway. These observations indicate that Drs2p activity is tightly coupled to AP-1/clathrin coated vesicle budding, and that Drs2p becomes a cargo of these vesicles during their formation. Drs2p and AP-1 act in close proximity as they can be crosslinked in a complex. It remains to be determined whether Drs2p and AP-1 directly interact and if Drs2p has a sorting signal recognized by AP-1. Interestingly, Drs2p and clathrin are required for biogenesis of a class of dense exocytic vesicles, but Drs2p does not appear to be efficiently incorporated into these vesicles.

Despite these findings, many mysteries still remain. Although Drs2p is directly responsible for the translocation of NBD-PS across the TGN membrane, PS deficient mutants transport and process proteins normally in the secretory pathway, and still require Drs2p for vesicle formation. These results strongly suggest that Drs2p must pump some other substrates that may play a more critical role in transport vesicle formation. Drs2p appears to weakly translocate PE, and it is possible that the precise lipid translocated is less important than the physical displacement of lipid to distort the membrane. Purification and reconstitution of Drs2p in liposomes with defined lipid composition will help us to better understand the biochemical function of this protein. Moreover, the C-terminal tail of Drs2p has been found to be essential for its function and mediates a direct interaction with Gea2p, an ARF-GEF, by the Gea2p interacting motif (GIM). Adjacent to the GIM, there is another motif conserved among all Drs2p homologues that is homologous to a lipid-binding motif in Vps36p. Whether these interactions with proteins or lipids have a regulatory role for

Drs2p activity is not clear. More investigation will be needed to address these fundamentally important questions.

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