CHAPTER I

INTRODUCTION TO THE ROLE OF P4-ATPASES AND OXYSTEROL BINDING PROTEIN HOMOLOGUES IN PROTEIN TRANSPORT

The plasma membrane and membranes of organelles compartmentalize eukaryotic cells to allow formation of specialized microenvironments in the cytosol and organelle lumens. Specific reactions including glycosylation, proteolytic cleavage and degradation take place in the organellar compartments. The functional identity of each organelle is conferred by their unique set of proteins and lipids which must be transported from common site of synthesis to multiple destinations (Owen *et al*., 2004). Proteins are transported between organelles of the secretory and endocytic pathways by transport vesicles. Vesicles are often identified by the cytosolic coat proteins help form them, with the best characterized examples being COPI, COPII and clathrin. Clathrin is required to bud vesicles from the *trans*-Golgi network (TGN), endosomes, and plasma membrane for receptor mediated endocytosis. Several years ago our lab disvoered that a budding yeast protein called Drs2 is required for budding of specific class of exocytic vesicles from the TGN (Chen *et al*., 1999) and clathrin coated vesicles mediating vesicle transport between the TGN and early endosomes (Liu *et al*., 2008b). Drs2 is a type IV P-type ATPase (P4-ATPase) and lipid flippase that helps generate membrane asymmetry in biological membranes by flipping specific lipids from one leaflet to the other. Moreover *DRS2* is an essential gene at low temperature (Ripmaster *et al*., 1993). Yeast strains harboring a distruption of DRS2 *(drs2Δ)* grow well from 23C to 37C, but cannot grow at temperatures below 23C. My research is focused on

understanding why *drs2Δ* cells exhibit this unusually strong cold-sensitive growth defect. I performed a *drs2Δ* bypass suppressor screen to select extragenic mutations that restored growth to *drs2Δ* cells at low temperature. We hypothesized that characterization of these suppressor mutants would illuminate the mechanism underpinning the cold-sensitive growth of *drs2Δ* cells. I discovered that mutations in *KES1*, an oxysterol binding protein homologue, suppress the cold-sensitive growth of *drs2Δ*. My research is primarily devoted to characterizing the relationship between Drs2 and Kes1.

P-type ATPases

P-type ATPases are a large group of membrane proteins that act as pumps to transport heavy metal ions, cations or phospholipids across biological membranes (Moller *et al.*, 1996; Kuhlbrandt, 2004). The P-type designation is given to these pumps because they form an aspartyl phosphate catalytic intermediate during ATP hydrolysis. The large family of P-type ATPases is divided into five major groups based on phylogenetic relationships and transport substrate specificity (Axelsen and Palmgren, 1998). Table 1 lists the major subdivisions of the P-type ATPase family with representative members.

Most P-type ATPases transport cations across membranes against the electrochemical gradient and have charged residues within transmembrane segments 4 and 6 that form a binding pocket for the cation. The mechanism of transport, called the Post-Albers cycle, describes the sequence of reaction steps

for the Na^+/K^+ -ATPase and has been used as model for other P-type ATPases. These pumps cycle through two main conformational states called E1 and E2 that are controlled by ligand and nucleotide binding, as well as phosphate transfer and release (Kuhlbrandt, 2004; Moller *et al.*, 2005).

Table 1-1. The P-Type ATPase superfamily

Subfamily	Subtype	Representative members
Type I	A	*E.coli* Kdp K^+ ATPase
	B	Heavy Metal pumps, Cu^+-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^+-ATPase
Type IV		Phospholipid translocases
Type V		Yeast Cod1/spf1, Ypk9/Park9

These conformational changes drive the active transport of cations. In the E1 state, these ATPases expose high affinity ion-1 binding sites to the cytosol. After the binding of ion-1, Mg^{2+} and ATP, the enzyme is phosphorylated at the

highly conserved aspartic acid residue, to form E1-P. Later in the E2-P state, the ATPase has reduced affinity to ion-1 and releases it to the extracellular or luminal side of the membrane. Now the ligand binding site has high affinity for ion-2, which binds from the luminal or the extracellular side and is subsequently relocated to the cytosol upon dephosphorylation of the aspartic acid (E2-P → E2 → E1). The ATPase is then ready to start another cycle. High-resolution X-ray crystal structures of sarcoplasmic reticulum Ca^{2+}-ATPase (Toyoshima and Inesi, 2004), Na^{+}/K^{+}-ATPase (Morth *et al.*, 2007) and H^{+}-ATPase (Pedersen *et al.*, 2007) suggest that they share a common Post-Albers catalytic cycle. However, only atomic resolution structures of the sarcoplasmic reticulum-Ca^{2+}-ATPase (SER-Ca^{2+}) in multiple conformational states (Fig 1) are available so far (Toyoshima *et al.*, 2000; Toyoshima and Nomura, 2002; Toyoshima and Inesi, 2004).

Four domains are identifiable in each crystal structure (Fig 1-1A). The actuator (A) domain is formed by the cytosolic N-terminal tail and the loop between transmembrane helices 2 and 3. The phosphorylation (P) and nucleotide-binding (N) domains are formed by the large cytosolic loop between membrane segments 4 and 5. The membrane domain (M) comprises 10 transmembrane segments and short connecting loops on the extracellular / lumenal or the cytosolic side of the membrane. Separation, rotation, and gathering of the three cytosolic domains (A, P and N), as well as displacement and bending of the

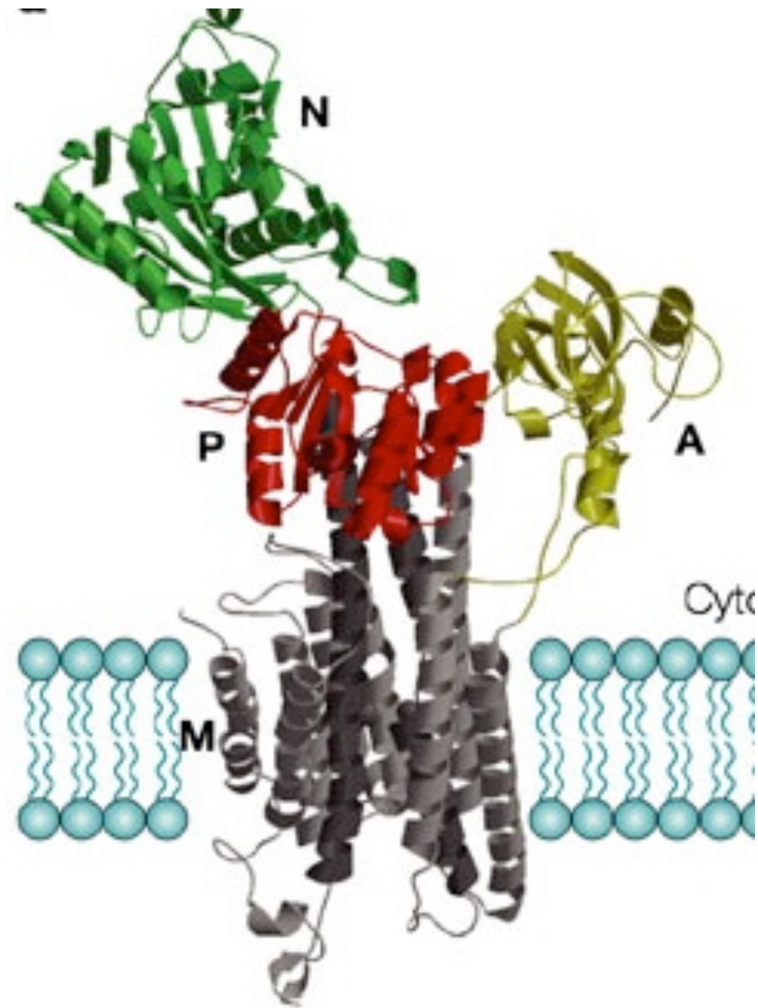
transmembrane segments, occur concomitantly with the sequential steps of the ATPase cycle (Fig 1-1B) (Toyoshima and Inesi, 2004).

Fig 1-1A. Crystal structure of P-type Ca^{2+}-ATPases of the sarcoplasmic reticulum (SERCAs) in the E1 state (Toyoshima *et al.*, 2000; Kuhlbrandt, 2004).

Fig 1-1B. Schematic representation of the catalytic cycle of P-type ATPases.

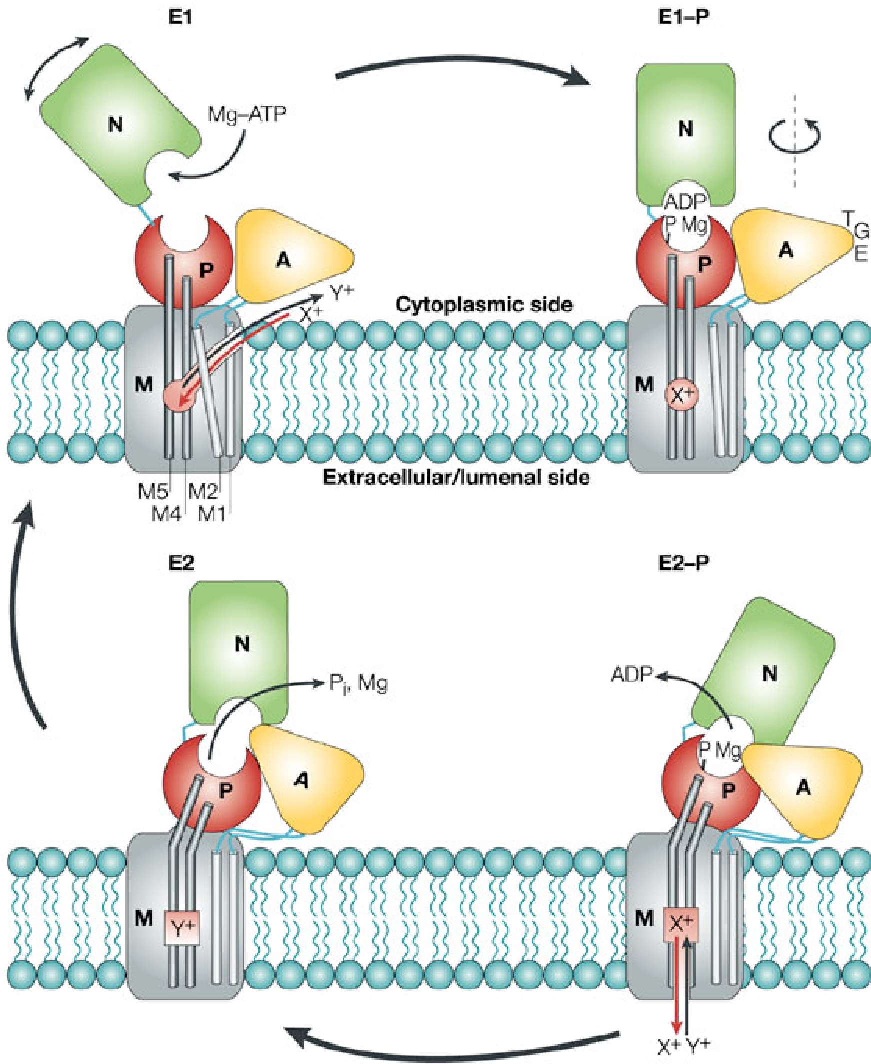
In the E1 state, ion 1 (X^+) from the cytoplasm binds to its high-affinity site in the membrane (M)-domain. Binding of X^+ causes phosphorylation of a crucial aspartic acid residue in the P domain by Mg^{2+} ATP, which is delivered to the phosphorylation site by the nucleotide-binding (N)-domain, resulting in the formation of E1–P state. In E1–P to E2–P transition, the P-domain reorientates and the actuator (A)-domain rotates to bring its TGE loop into close contact with the phosphorylation site. The A-domain rotation and P-domain reorientation cause the movement of M-domain which disrupts the high affinity X^+ binding site and closes the cytosolic ion-access channel. X^+ is released into the extracellular or luminal side. The ion-binding site now has a high affinity for ion-2 (Y^+), which binds from the outside or lumen. Dephosphorylation of Asp residue releases the ion 2 Y^+ into the cytosol, the enzyme returns to the E1 state to start another cycle (Kuhlbrandt, 2004).

1-1A



Kuhlbrandt W, 2004 Nat Rev Mol Cell Biol , 282-295.

1-1B



Kuhlbrandt W, 2004 Nat Rev Mol Cell Biol , 282-295.

The P domain has the signature sequence of P-type ATPases (DKTGT[L,I,V,M][T,L,S]) containing the phosphorylated aspartic acid residue (Andersen, 1995). Hence, it forms the catalytic core. ATP binds to the nucleotide-binding domain (N) inducing a 20° tilt of the P domain and a clamping down motion of the N domain to bring ATP closer to the phosphorylation site in the P domain. The A domain rotates 110° horizontally to make close contact with P domain during the catalytic cycle and causes great conformational changes in transmembrane segments during this movement. In the cases of Ca^{2+} ATPase and Na^{+} /K^{+} -ATPase, the ligand binding sites are buried inside the M domain. For SERCAI, in the E1 state, Ca^{2+} binding sites are accessible from the cytosol and this binding leads to phosphorylation of the aspartic residue in the P domain. The byproduct ADP stays bound for a short time and in this E1-P-Ca^{2+}ADP state, ligand is inaccessible from either side of the membrane. Then the pump releases ADP and moves to a low energy level E2-P-Ca^{2+} state that opens the ligand binding site to the lumenal side of the endoplasmic reticulum, against a concentration gradient. The conformational changes in the binding site allows the release of Ca^{2+} and binding of two counter transporting H^{+} ions. Hydrolysis of the aspartyl phosphate residue, catalyzed by the actuator (A) domain, causes movement of E2-P-H^{+} to the E2-H^{+} state. Mg^{2+} and inorganic phosphate (Pi) dissociate, and the enzyme reverts to the E1 state, in which the counter-transported H^{+} ligands are released into the cytosol (Fig 1-1B) (Toyoshima and Inesi, 2004).

Mutational (Clarke *et al.*, 1989) and structural studies (Toyoshima and Inesi, 2004; Sugita *et al.*, 2005) have shown that, within the membrane-bound region of the Ca^{2+}-ATPase, the Asn-768, Glu-771, Thr-799, Asp-800, and Glu-908 side chains, and two water molecules, contribute oxygen atoms for Ca^{2+} coordination on site I. Ca^{2+} binding on neighboring site II is stabilized by Glu-309 (M4), Asn-796, and Asp-800 (M6) side chain, as well as by Val-304, Ala-305, and Ile-307 (M4) main-chain carbonyl oxygen atoms. Cation interaction with negatively charged amino acids in the transmembrane domain appears to be required to stabilize the structure of the membrane domain (Obara *et al.*, 2005). These charged residues form the Ca^{2+}/H^{+} binding site (e.g. E309, E771, D800, E908) in the center of the Ca^{2+} ATPase transmembrane domain and are essential for Ca^{2+} transport. Charged residues in the membrane domain are conserved in all cation pumps, however, not all P-type ATPases have this characteristic.

Type IV P-type ATPases

Type IV P-type ATPases (P4-ATPases) are unusual among P-type ATPases in that they appear to pump phospholipid molecules across a membrane rather than cations (Moller *et al.*, 1996). The best characterized P4-ATPases, Drs2p from *Saccharomyces cerevisiae* and its close homologue Atp8a1 from mammals, appear to flip phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the luminal leaflet of the Golgi (Drs2) (Natarajan *et al.*, 2004; Alder-Baerens *et al.*, 2006) or chromaffin granules (Atp8a1) (Zachowski *et al.*, 1989) to

the cytosolic leaflet. This directional flipping to the cytosolic leaflet is consistent with an E2 → E1 transition in the Post-Albers cycle. Atp8b1 and Drs2p have nonpolar residues (I508, L1022, V1055, G1148) in the analogous positions where charged residues form cation binding sites in cation pumps (Muthusamy *et al.*, 2009a). Therefore, it is unlikely that P4-ATPases transport cations. This structure is more consistent with a hydrophobic transport substrate and supports the proposed activity of P4-ATPases in transporting phospholipids. If the structure of the TM domain is conserved, it is hard to imagine how the ligand-binding pocket of the transmembrane domain could accommodate a large phospholipid compared to a small cation. Flippases must provide a sizeable hydrophilic pathway for the polar headgroup to pass through the membrane as well as accommodate the hydrophobic nature of the lipid backbone. In addition, they must be able to discriminate phospholipids based on atoms in the lipid headgroup and glycerol backbone (Lenoir *et al.*, 2009). Whether the phospholipid molecule moves through the center of the membrane domain, similar to the path followed by cations, or moves at the protein/lipid interface is unknown.

It is also unknown if P4-ATPases have a counter-transport ion or molecule coupled to the E1 → E2 transition. If the phospholipid moves at the protein/lipid interface of the P4-ATPases, there may not be a structural requirement for countertransport of an ion. However, PS carries a net charge of −1 and so there may be a requirement for ion movement to balance the electrogenic charge

displacement (Muthusamy *et al.*, 2009a). It is also possible that the structural requirement for counter ion transport in P4-ATPases is removed by the presence of one or more residues in the ligand-binding pocket that function as a constitutive counter-ligand, comparable with those of proton pumps (AHA2) (Pedersen *et al.*, 2007). Aspartic acid 684 is the central proton donor/acceptor of proton pump AHA2 of *Arabidopsis thaliana*. Conformational movements in the membrane region after the E1-P transition, brings Arginine 655 closer to Asp 684. Thus, Arg655 may act as a built-in counter-ion during dephosphorylation and the E2 to E1 transition, neutralizing the deprotonated negatively charged Asp 684. The presence of Arg 655 as a constitutive counter-ion makes the transition from E2-P to E2 extremely favorable and minimizes exposure of Asp 684 to the extracellular side (Pedersen *et al.*, 2007).

A. P4-ATPases and membrane asymmetry

An asymmetric distribution of phospholipids between the two leaflets of the bilayer is a fundamental characteristic of the eukaryotic plasma membrane (Zachowski, 1993). Aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), are restricted to the cytosolic leaflet of the PM, and phosphatidylcholine and sphingolipids are restricted to the exoplasmic leaflet (Balasubramanian and Schroit, 2003). There are three classes of enzymes called flippases, floppases and scramblases that are involved in transport of membrane lipids. Scramblases allow energy independent, bidirectional movement of lipid

without specificity for the lipid species (Dekkers *et al.*, 2002). The protein responsible for scramblase activity is not yet identified. By contrast, flippases and floppases move lipids in energy dependent, directional and lipid specific manner (van Meer *et al.*, 2006). Flippases and floppases are thought to be P4-ATPases and ATP binding cassette proteins, respectively. Exposure of phosphatidylserine on the outer leaflet of the PM has been studied in physiological phenomena like blood clotting, acrosome activation in sperm, apoptosis, vesicle mediated transport, polarized growth of cells and pathological conditions like host viral interactions, diabetes and sickle cell anemia.

B. Functions of P4- ATPases in mammals

The fourteen members of the P4-ATPase family in humans are expressed in a tissue specific pattern (Table 2) and a substantial number of reports indicate that mutations in P4-ATPases like ATP8B1, ATP8B3, ATP10A and ATP10D, can result in human disease or mouse pathology (Folmer *et al.*, 2009).

Atp8b1 is the best characterized P4-ATPase linked to human disease. Mutations in ATP8B1 cause Progressive Familial Intrahepatic Cholestasis type 1 (PFIC1) and Benign Recurrent Intrahepatic Cholestasis type 1 (BRIC) (Bull *et al.*, 1998), which is a milder form of the disease. PFIC patients develop symptoms progressively to end-stage liver disease before adulthood. In contrast BRIC patients have episodic occurrence of disease with spontaneous clearance of

symptoms without any liver damage (van Mil *et al.*, 2001). Patients have clinical symptoms like itching, jaundice, pancreatitis, diarrhea and hearing loss and pathological symptoms like loss of microvilli, dilated canaliculi, proliferated pericanalicular microfilaments and coarse granular bile instead of amorphous bile structure. Impaired bile flow leads to cholestasis (accumulation of bile salts) that in turn affects fat digestion and absorption. Atp8b1 is localized in apical membrane of epithelial cells including the canalicular membranes of hepatocytes (Bull *et al.*, 1997; Knisely, 2000; Kurbegov *et al.*, 2003).

Table 2: The table content is adopted from Folmer et al, 2009, P4-ATPases, Lipid flippases and their role in disease

Mammalian P4-ATPases	Human disease or mouse pathology	Primary expression
ATP8A1		skeletal muscle and thyroid
ATP8A2	Tumorigeneis	Testis, low in heart and brain
ATP8B1	Familial intra hepatic cholestasis	Small intestine, pancreas, and liver,
ATP8B2		Brain, Bladder and Uterus
ATP8B3	Compromised sperm penetration	Testis
ATP8B4	Alzheimer disease	Ubiquitous, brain, liver, kidney and testis
ATP9A		Ubiquitous
ATP9B		Testis
ATP10A	Insulin resistance and Obesity	Brain, kidney, lung, pancreas
ATP10B		Brain and testis
ATP10D	Obesity	Moderate in liver, kidney, spleen and ovary
ATP11A		Liver, heart, kidney and muscle
ATP11B		Kidney
ATP11C		Liver, pancreas and kidney

To better understand how Atp8B1 deficiency causes cholestasis, a mouse model was generated (Pawlikowska *et al.*, 2004). In contrast to human patients, mice harboring a common disease causing allele of ATP8B1 (ATP8B1 G308V/G308V) exhibited mild symptoms. However, when these mice are fed with cholic acid supplemented diets, they displayed a four fold increased level of serum bile salts relative to wild-type controls (Groen *et al.*, 2007). Moreover, perfusion of taurodeoxycholate, a human bile salt, in ATP8B1 deficient mice resulted in reduced biliary output and mild cholestasis (Paulusma *et al.*, 2006). It appears that mutations in ATP8B1 cause loss of membrane asymmetry in bile canalicular membranes, and thus membrane organization is disturbed. This membrane structure is vulnerable to bile salt extraction of membrane contents including PS and cholesterol (Paulusma *et al.*, 2006). Hence Atp8b1 deficient mice had significant amount of PS in their bile whereas PS is essentially absent from wild-type bile. PS appearance in the bile suggests that there could be a significant amount of PS in the outer leaflet of the bile canalicular membrane.

Consistent with its potential PS flippase activity, expression of Atp8b1 in UPS-1 (Chinese hamster ovary mutant cell line) cells increases the internalization of NBD-PS (1-palmitoyl-2- [6-(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] hexanoyl] -*sn*-glycero phospho-l-serine) (Ujhazy *et al.*, 2001). The UPS-1 cells normally expose high levels of endogenous PS on the outer leaflet, but Atp8b1 expression reduced PS exposure by 17-25% based on annexin V binding,

suggesting that Atp8b1 pumped the endogenous PS to the inner leaflet (Paulusma *et al.*, 2008). These data indicate that Atp8b1 is a potential PS flippase in hepatocytes. However, it is also formally possible that Atp8b1 may interfere with the expression, localization or activity of other proteins which are directly responsible for PS flippase activity.

Knocking down expression of ATP8A2 (ML-1) in nontumorigenic, anchorage-independent growth (AIG) cells induced a tumorigenic phenotype (Sun *et al.*, 1999). Atp8b3 mRNA is expressed in the testis in spermatocytes and spermatids (Wang *et al.*, 2004) and localizes to the acrosomal region of mouse sperm cells. Sperm capacitation was reduced in Atp8b3$^{-/-}$ mouse. Capacitation refers to the cascade of events that occurs in spermatozoa to prepare them for binding to, and penetration of, the zona pellucida of oocyte. In this sequence of events, membrane remodeling plays a crucial role (Gadella *et al.*, 2008). PS exposure was detected only after capacitation in control mice whereas PS exposure was already detectable before capacitation in Atp8b3$^{-/-}$ sperm cells. Although in vitro fertilization studies indicated that Atp8b3$^{-/-}$ sperm cells required more time and a higher concentration of spermatozoa for fertilization, these mice did not have severe fertility problems (Wang *et al.*, 2004). The role of ATP8B3 in human fertility is not known.

Heterozygous mice with maternal inheritance of ATP10A mutations (also called as ATP10C) exhibit high-fat diet induced obesity and insulin resistance. Atp10c deletion mice have an altered insulin-stimulated response in adipose tissue and the skeletal muscle and GLUT4-mediated glucose uptake is the specific metabolic pathway associated with it (Dhar *et al.*, 2006). A mutant allele of ATP10D is linked to metabolic disorder including obesity, hyperglycemia and hyperinsulinemia in a particular strain of mice (C57BL/6J) that are fed with a high fat diet. ATP11C has been mapped to a chromosomal region, Xq27, associated with X-linked inherited disorders, including hypoparathyroidism, albinism, deafness, and thoracoabdominal syndrome (Andrew Nesbit *et al.*, 2004). In addition ATP11a overexpression induces resistance to anticancer drugs and knockdown of endogenous ATP11a causes increased sensitivity to those anticancer drugs. ATP11a expression level in cancer patients could play a role in prognosis of the treatment using that particular drug (Zhang *et al.*, 2005). Thus although some P4-ATPases have been implicated in human disease and lipid metabolism, the molecular mechanisms are not well established. However, studies in yeast have helped to define the cellular function of these ATPases.

C. Role of P4-ATPase in Yeast

There are five P4-ATPases in *Saccharomyces cerevisiae*. Among them, *DRS2*, *DNF1*, *DNF2* and *DNF3* form an essential group of genes whereas *NEO1* alone is an essential gene (Hua *et al.*, 2002). Some degree of functional

redundancy between *DRS2* and the *DNF* genes was suggested by the observation that any one of these genes is sufficient to support viability. However, additive phenotypic defects are exhibited in strains carrying combined deletions of *DRS2* and *DNF* genes compared to single deletion mutants. *DNF1* and *DNF2* are the most closely related genes in this family, sharing 69% sequence identity and 83% similarity (Hua *et al.*, 2002). *DRS2* is an essential gene in yeast at low temperatures (Ripmaster *et al.*, 1993; Chen *et al.*, 1999), suggesting that the Dnf proteins cannot compensate for an essential function of Drs2 at or below 23C (Hua *et al.*, 2002). Drs2 and Dnf3 localize to *trans*-Golgi network (TGN) at steady state, whereas Dnf1 and Dnf2 reside in the plasma membrane and other intracellular organelles. Neo1 resides in Golgi compartments (Hua and Graham, 2003) and also accumulates in the late endosomes (Wicky *et al.*, 2004) of class E *vps* (vacuolar protein sorting) mutants, suggesting that Neo1p normally trafficks through the endosome.

Similar to the Na^+/K^+ ATPase, several yeast P4-ATPases have noncatalytic ß subunits, encoded by the *CDC50*, *LEM3* and *CRF1* genes, that are glycosylated proteins with two transmembrane domains. *CDC50* was identified in a genetic screen for cold sensitive cell-division cycle mutants (Moir *et al.*, 1982). *CDC50* deletion causes a cold-sensitive growth defect and phenocopies *drs2*Δ defects, but the mechanistic basis of the cold-sensitive defect is not known. Cdc50p forms a complex with Drs2p and this complex is necessary for these

proteins to exit from the ER. Drs2p and Cdc50p then colocalize in the TGN and early endosome (Saito *et al.*, 2004; Chen *et al.*, 2006).

Another ß subunit *LEM3* was first recovered in a yeast genetic screen to identify ligand-effect modulators (LEMs), proteins that modulate the cellular response to hormone in the glucocorticoid signal transduction pathway (Sitcheran *et al.*, 2000). Later, *LEM3* was identified in a screen to select for mutants that were resistant to edelfosine, an alkylphosphocholine anticancer and antimalarial drug. The *lem3*Δ mutants showed defects in internalization of a short chain, fluorescent NBD-labeled phosphatidylcholine and phosphatidylethanolamine reporters (Hanson *et al.*, 2003). *lem3/ros3* (Ro-sensitive 3) was also recovered in a screen for Ro09-0198 (Ro) sensitive strains. Ro specifically binds phosphatidylethanolamine (PE) that is exposed to the outer leaflet of the plasma membrane and cause cytolysis. Decreased internalization of fluorescence-labeled analogs of PE and PC was found in strains carrying a disruption of the *ROS3* gene (Kato *et al.*, 2002). *lem3*Δ phenocopies *dnf1*Δ*dnf2*Δ, and Lem3 forms complexes with Dnf1 and Dnf2 that are required for ER export. Crf1 chaperones Dnf3 and a *cdc50*Δ*lem3*Δ*crf1*Δ strain is inviable, a phenocopy of *drs2dnf1,2,3*Δ (Saito *et al.*, 2004; Furuta *et al.*, 2007).

The Tanaka group has identified *lem3* point mutations that exhibit normal association with Dnf1 and Dnf2, an apparent normal localization of Dnf1p and nearly normal uptake of NBD-PE and NBD-PC, but display a synthetic growth defect with *cdc50* (Noji *et al.*, 2006). This result suggested that Lem3p may regulate Dnf1 or Dnf2 function in more ways than just chaperoning their ER exit, or that Lem3 has other functions independent of Dnf1 and Dnf2. The Lem3p-Dnf1p complex may play a role distinct from its plasma membrane phospholipid translocase activity when it substitutes for the Cdc50p-Drs2p complex, its redundant partner in the endosomal/trans-Golgi network compartments (Noji *et al.*, 2006).

The precise role of the ß-subunits in the flippase function of Drs2 family members is not yet resolved. However, a recent study suggests that Cdc50p interacts specifically with the phosphorylated form of Drs2 (E1-P) and facilitates its catalytic cycle in a mutually dependent manner (Lenoir *et al.*, 2009). In humans, three proteins CDC50A, CDC50B, and CDC50C have been identified that are homologous to the yeast Cdc50p/Lem3p family. In UPS-1 cells, the Chinese hamster ovary (CHO) mutant cell line defective in the nonendocytic uptake of NBD-PS, ATP8B1 was retained in the ER when expressed alone, whereas coexpression with CDC50A or CDC50B resulted in rerouting of Atp8b1 to the plasma membrane and lysosome. In UPS-1 cells, both CDC50A and CDC50B stimulated ATP8B1-mediated internalization of NBD-PS and natural PS

by annexin V binding (Paulusma *et al.*, 2008). More studies are needed to better characterize the relationship between P4-ATPases and their ß subunits.

D. Role of P4-ATPase in other organisms

The cold-sensitivity of *drs2*Δ in yeast led to a study of P4-ATPase influence on chill tolerance in plants. Among 12 plant P4-ATPases (Ala1-Ala12), *ALA1* is most closely related to *DRS2* and complements the cold-sensitive defect of *drs2*Δ mutants at 15C. Down regulation of *ALA1* in *A. thaliana* reduces cold tolerance and plants are much smaller than the wild type when grown at low temperature (Gomes *et al.*, 2000). A family of *CDC50* homologs named *ALIS1* to *ALIS5* (for ALA-Interacting Subunit) is found in *A. thaliana* (Poulsen *et al.*, 2008). *ALISI* interacts with *ALA3*, which may represent the plant equivalent of Drs2p (Poulsen *et al.*, 2008). Moreover Ala3 complements the cold-sensitive defect of *drs2*Δ in yeast when co-expressed with Alis3. In plants, ALIS1, like ALA3, localizes to Golgi-like structures and is expressed in root peripheral columella cells (Poulsen *et al.*, 2008).

Two P4-ATPases from the rice blast fungus *Magnaporthe grisea, MgAPT2* and *MgPDE1* are involved in fungal pathogenicity. *MgPDE1* is required to form a plasma membrane outgrowth called appressoria (Balhadere and Talbot, 2001). MgApt2 is required for both foliar and root infection by the fungus, and for the rapid induction of host defense responses in an incompatible reaction.

Moreover, it helps to establish rice blast disease by the use of MgApt2-dependent exocytic processes that operates during plant infection (Gilbert *et al.*, 2006).

Six transbilayer amphipath transporters (Tat1-Tat6) are members of the P4-ATPase family in *C.elegans*. *tat-5* is the only ubiquitously expressed essential gene in the group and *tat-6* is a poorly transcribed recent duplicate of *tat-5*. *tat-2* through *4* exhibit tissue-specific, developmentally-regulated expression patterns and these genes become essential for reproductive growth during sterol starvation (Lyssenko *et al.*, 2008). Worms deficient in *tat-1* expose PS abnormally on their cell surfaces, and cells that normally survive in the adult worm are randomly lost through a mechanism dependent on PSR-1, a PS-recognizing phagocyte receptor, and *CED-1*. *CED1* contributes to recognition and engulfment of apoptotic cells (Darland-Ransom *et al.*, 2008). There are six P4-ATPases in *D. melanogaster* (Okamura *et al.*, 2003), although they are not yet characterized individually.

E. Evidence for the lipid flippase function of P4-ATPases

1. Lipid Flippase Function for Mammalian P4-ATPases

In 1984, Seigneuret and Devaux were the first to describe the ATP-dependent, inward movement of spin-labeled PE and PS across the erythrocyte membrane, which they called an aminophospholipid translocase (APLT) activity. The addition of different exogenous phospholipids has unique impacts on the

shape of erythrocytes that depends on whether they are a substrate of the APLT (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985, 1989). Erythrocytes with a normal discoid shape become crenated immediately on addition of spin-labeled PC, PS or PE as the lipids incorporate into the outer leaflet. With further incubation, erythrocytes with PS or PE analogues convert back to the discoid shape and eventually end up with a uniconcave shape. These shape changes correlate with translocation of the lipid to the inner leaflet. However erythrocytes with the PC analogue, which is not an APLT substrate, remain crenated during further incubation (Seigneuret *et al.*, 1984). These results strengthened the view that an APLT specifically translocates aminophospholipid to the inner leaflet of plasma membrane to establish the observed membrane asymmetry. However the identity of the erythrocyte membrane APLT is still uncertain. A partially purified vanadate-sensitive, PS-stimulated ATPase from erythrocytes (presumably Atp8a1) has been reconstituted in artificial liposomes. An ATP-dependent translocation of PE and PS, but not PC, from the inner to outer leaflet of the liposomes was observed. ATP and magnesium-dependent translocation of spin labeled PS was also observed in bovine chromaffin granules (Zachowski *et al.*, 1989). A vanadate sensitive, PS-stimulated ATPase was purified from the chromaffin granules (ATPase II/Atp8a1) and a cDNA clone was isolated. However, Atp8a1 was not reconstituted into liposomes to demonstrate its ability to translocate PS.

2. Lipid Flippase function in Yeast

Genetic and biochemical studies in *Saccharomyces cerevisiae* also support the flippase function for P4-ATPases. NBD-PC and NBD-PE are efficiently translocated across the yeast plasma membrane. Removal of the plasma membrane resident P4-ATPases Dnf1p and Dnf2p or its chaperone Lem3 abolishes inward translocation of fluorescent NBD-labelled PE and PC, and causes aberrant exposure of endogenous PE at the cell surface (Kato *et al.*, 2002; Pomorski *et al.*, 2003; Alder-Baerens *et al.*, 2006; Riekhof *et al.*, 2007; Stevens *et al.*, 2008). NBD-PS is more weakly flipped across the plasma membrane and most available data indicates that *dnf1,2*Δ and *lem3*Δ strains translocate NBD-PS normally. The same P4-ATPases have been implicated in the transport of lyso-PE and lyso-PC as bona fide native phospholipid substrates across the plasma membrane (Riekhof *et al.*, 2007). The distribution of endogenous PC across the plasma membrane of yeast has not been measured. However, the ability of wild-type yeast to take up NBD-PC may suggest that PC is restricted to the inner leaflet of the plasma membrane and that the outer leaflet is primarily composed of sphingolipids, as NBD-sphingomyelin is not taken up (Pomorski *et al.*, 2003). While these data are consistent with the proposed PC/PE flippase activity of Dnf1 and Dnf2, it is possible that disruption of the *DNF* and *LEM3* genes indirectly influences the expression, localization or activity of an undefined lipid transporter.

To reduce the pleiotropic effect that arises from disrupting *DRS2* and test its potential flippase function, our lab constructed a temperature-sensitive *drs2* allele. TGN membranes were purified from a strain carrying the temperature-sensitive *drs2* allele grown at permissive temperature to minimize pleiotropic defects by maintaining the normal structure of TGN. These membranes exhibited significant reduction in NBD-PS translocation activity when shifted to the nonpermissive temperature (Natarajan *et al.*, 2004), whereas TGN membranes from wild-type cells retain the NBD-PS flippase activity at both temperatures. Moreover, a weaker activity was detected for NBD-PE, but not for NBD-PC from TGN membranes of WT cells. Subsequent studies showed that Drs2p, together with Dnf3p, is required to maintain APLT activity in post-*Golgi* secretory vesicles (Alder-Baerens *et al.*, 2006). These findings strongly support the proposed lipid flippase activity of P4-ATPase. Thus far, no specific ion requirement has been detected for the plasma membrane or TGN flippase activities, arguing against an ion requirement to drive lipid translocation by a secondary mechanism (Natarajan *et al.*, 2004). However, ultimate proof that P4-ATPases translocate phospholipid across the bilayer directly requires reconstitution of this activity with a pure enzyme in a liposome. Recently, Drs2p was successfully purified and reconstituted into liposomes, and was found to be sufficient to catalyze phospholipid translocation (Zhou and Graham, 2009)

Role of Drs2-Cdc50 in Protein Transport and Vesicle Biogenesis

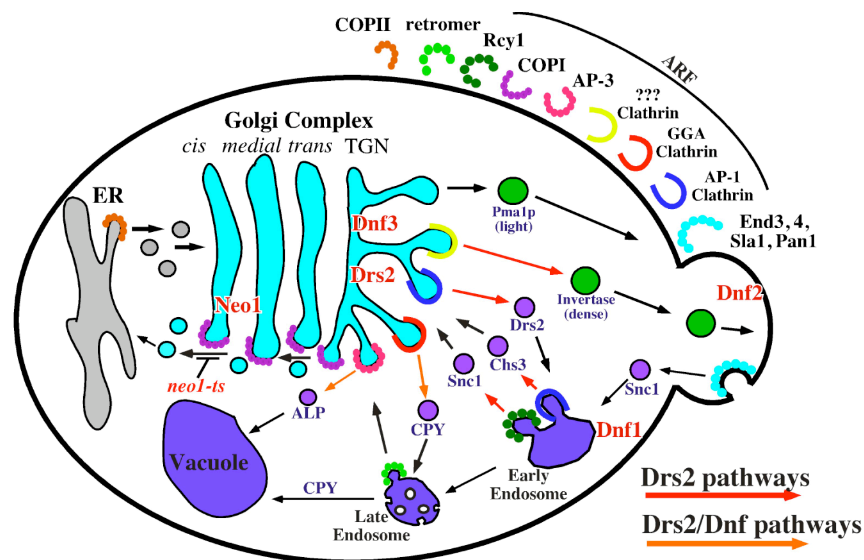
Drs2p was first characterized in vesicle-mediated protein transport by a genetic screen for mutants defective in *ARF*-dependent vesicle biogenesis (Chen *et al.*, 1999). Other participants of clathrin-coated vesicle budding were also identified in this screen, including clathrin (Chen and Graham, 1998), Swa2p (yeast auxilin) (Gall *et al.*, 2000), as well as Cdc50p. Arf-GTP recruits coat proteins, such as clathrin and its adaptors, to Golgi membranes (Kawasaki *et al.*, 2005). Genetic interactions were found between clathrin, Arf and Drs2 mutations, but not between Drs2 and COPI or COPII subunits (Chen and Graham, 1998). In addition, *drs2*Δ cells exhibits TGN defects comparable with those exhibited by clathrin mutants.

Clathrin-coated vesicles bud from the TGN, early endosomes and plasma membrane (Fig 1-2) (Schmid, 1997). Clathrin uses adaptor proteins including AP-1, AP-2 and GGAs (Golgi associated, Gamma-ear containing, Arf-binding) to sort cargo into different pathways. The AP-1 complex (β1-, γ-, μ1-, σ1-adaptin) is recruited by Arf to the TGN and early endosome membranes, and mediates bidirectional traffic between these organelles (Liu *et al.*, 2008b). Another tetrameric adaptor AP-3 (β3-, δ-, μ3-, σ3-adaptin) is required for alkaline phosphatase transport from TGN to vacuole, but functions independently of clathrin (Odorizzi *et al.*, 1998). The AP-2 clathrin adaptor acts in receptor-mediated endocytosis at the plasma membrane of most cells (Schmid, 1997). However, there is no function identified for AP-2 in yeast (Yeung *et al.*, 1999).

Arf1 recruits AP-4 to the TGN and sorts cargos to lysosomes independently of clathrin in mammalian cells (Robinson, 2004), but AP-4 is absent in yeast. A set of functionally overlapping, monomeric clathrin adaptor proteins, Gga1p and Gga2p, localize to the TGN and sort cargos into a late endosome pathway (Black and Pelham, 2000; Costaguta *et al.*, 2001). In addition, a class of clathrin co-adaptors called epsin-related proteins, Ent3p and Ent5p, bind to the common gamma ear motif in AP-1 and GGA, and appear to facilitate the function of these clathrin adaptors (Fig 1-2) (Copic *et al.*, 2007).

Drs2 also acts in a bidirectional protein transport pathway between the TGN and early endosome in pathways mediated by AP-1/clathrin and Rcy1. Rcy1 is an F-box protein that plays a poorly defined role in early endosome to TGN transport (Chen *et al.*, 2005; Furuta *et al.*, 2007). Synthetic lethality or defective growth was noticed in strains with combinations of AP-1 and GGA mutations suggesting that they are acting in parallel pathways to endosomes. The function of at least one of these pathways is essential to sustain wild-type growth rates (Costaguta *et al.*, 2001). *drs2*Δ *gga1,2*Δ showed a severe growth defect similar to *apl4*Δ *gga1,2*Δ (AP-1 Gga double mutants). However, no additional growth defect was noticed when an AP-1 mutation was combined with *drs2*Δ suggesting Drs2 and AP-1 are in same pathway (Liu *et al.*, 2008b). Consistent with this prediction, disruption of AP-1 or deletion of *DRS2* perturbs the Chs3p trafficking pathway leading to enhanced and unregulated cell surface expression and

increased trafficking into the late endosomes (Liu *et al.*, 2008b). Normally chitin synthase III (Chs3p) is retained intracellularly by recycling between the TGN and early endosomes in wild-type cells. When cells enter the G1 phase of the cell cycle, a novel coat complex called exomer trafficks the Chs3p to incipient bud sites at the plasma membrane (Wang *et al.*, 2006).



Muthusamy et al 2009, BBA

Fig 1-2. P4-ATPase requirements for vesicle-mediated protein transport in the secretory and endocytic pathways. Transport pathways are defined by cargo protein (purple) traveling the route and the vesicle coat protein (labeled in the upper right quadrant) required for sorting and transport of the cargo. The coat requirement for early endosome to TGN recycling pathway traveled by Snc1 is indicated by Rcy1, although it is not known if Rcy1 is a vesicle coat constituent. Pathways with known P4-ATPase requirements are indicated with colored arrows.

Drs2 itself appears to be a cargo of AP-1/clathrin-coated vesicles that bud from the TGN (Liu *et al.*, 2008b). Drs2p is rerouted to the plasma membrane of AP-1 mutants where it can be trapped by an endocytosis block. Drs2 has several endocytosis signals, like NPFXD motifs and PEST-like sequences, that mediate its endocytosis (Liu *et al.*, 2007). Removal of the endocytosis block in the AP-1 mutants allows rapid retrieval of Drs2p to the TGN via early endosomes without trafficking through late endosomes. These data suggest that Drs2 is a cargo of AP-1/clathrin coated vesicles budding from TGN that are targeted to the early endosomes Fig 1-2, and that AP-I is not required for transport of Drs2 from early endosomes to the TGN (Liu *et al.*, 2008b). The latter pathway appears to be mediated by Rcy1p. Inactivating Drs2p activity also causes its rerouting to the plasma membrane, comparably to inactivating AP-1. Interestingly, Gea2p (Arf GEF), AP-1 and clathrin are efficiently recruited to the TGN in *drs2Δ* cells, indicating that Drs2p is not required for recruitment of coat, adaptor and accessory proteins (Liu *et al.*, 2008b). In spite of the normal coat recruitment, it appears that AP-1/clathrin coated vesicles are not generated in *drs2*Δ cells. Moreover, Drs2p is required for budding of one specific class of exocytic clathrin-coated vesicle from the TGN as inactivation of *drs2-ts* drastically reduced this vesicle population (Gall *et al.*, 2002).

The proposed mechanism of Drs2p contribution to vesicle formation is based on the bilayer-couple hypothesis (Sheetz *et al.*, 1976). Flippases can induce

positive curvature in membranes by translocating phospholipid molecules from the exofacial leaflet to the cytosolic leaflet. Contraction of the exofacial leaflet combined with expansion of the cytosolic leaflet will induce bending towards the cytosol. The positive membrane curvature would then be captured by coat, accessory and adaptor proteins to bud the vesicles. Vesicle budding defects of *drs2*Δ cells provide evidence for a bilayer-couple mechanism for vesicle biogenesis at the TGN. Early evidence in support of this concept was the dramatic changes in erythrocyte membrane shape induced by the APLT (Daleke and Huestis, 1989). In addition, APLT-dependent translocation of phospholipid across the plasma membrane has been shown to stimulate endocytosis (Pomorski *et al.*, 2003).

However, early studies suggested that coat proteins were sufficient to generate membrane curvature. Clathrin can self assemble and form vesicles from liposomes in the presence of Arf, GTP and adaptor proteins in the absence of transmembrane proteins (Zhu *et al.*, 1999). However, to drive membrane curvature sufficiently to stabilize it, the rigidity of the coat proteins should supercede the resistance given by mechanically bending the membrane. Theoretical calculations indicate that the rigidity of clathrin triskelia is similar to the resistance of lipid membranes to bending (Nossal, 2001). If true, clathrin alone could stabilize an already curved membrane, and thus prevent it from collapsing back to a planar form, but clathrin assembly alone would not provide

sufficient energy to bend membranes. Other endocytic accessory proteins that contain domains with intrinsic curvature, such as BAR (Bin, amphiphysin, Rvs) and ENTH domains, are also implicated in membrane deformation (Itoh and De Camilli, 2006). The degree to which coat, accessory proteins and flippases contribute to membrane deformation *in vivo* is not clear. However, the observation that AP-1/clathrin is recruited to TGN membranes in *drs2*Δ cells, but appears to be nonfunctional, suggests that the flippase makes a critical contribution.

Drs2 and Cdc50 are also required for recycling proteins from the early endosome to the TGN. The SNARE protein Snc1p continuously cycles between the TGN, plasma membrane, early endosome and back to the TGN (Lewis *et al.*, 2000). In wild-type cells, the kinetically slow step in this path is endocytosis and so most of GFP-Snc1 is observed primarily at the plasma membrane of the bud. Both *drs2Δ* and *cdc50*Δ mutants accumulate GFP-Snc1p in cytoplasmic punctate organelles. For *cdc50*Δ mutants, GFP-Snc1 was shown to accumulate in early endosomes (Furuta *et al.*, 2007) and this is likely to be the case for *drs2*Δ as well. The F-box protein Rcy1p, the sorting nexin Snx4p, Ypt31/32p (rab11 homologues), Arf and the Arf GTPase activating protein (ArfGAP) Gcs1p are all required at this same early endosome to TGN transport step traveled by Snc1p (Muthusamy *et al.*, 2009a). Moreover, Cdc50p uses this route as well since *rcy1Δ* causes accumulation of Cdc50p in the early endosomes (Furuta *et al.*, 2007).

There is suggestive evidence that this Rcy1 pathway may be mediated by COPI (Robinson *et al.*, 2006). Although more studies are needed to determine if these vesicles are COPI-coated, clathrin-coated, or use a novel coat structure. Rcy1 co-immunoprecipitates with Drs2p and Cdc50p, and the *rcy1*Δ mutant has a cold-sensitive growth defect, a phenocopy of *drs2*Δ or *cdc50*Δ. *rcy1*Δ and *rcy1*Δ *cdc50*Δ exhibits a similar growth phenotype, suggesting they are acting in same pathway from endosome to TGN (Furuta *et al.*, 2007). Drs2p/Cdc50 along with Rcy1p may help to generate and bud vesicles from the early endosomes that travel to the TGN.

Although *DRS2* and the *DNF* genes show some functional redundancy, Dnf ATPases cannot compensate for loss of Drs2 in the exocytic, AP1 and Rcy1 pathways. However protein trafficking pathways from TGN to the late endosome and TGN to the vacuole exhibited functional redundancy between Drs2 and Dnf1 (Hua *et al.*, 2002). The AP-3 dependent transport of ALP from TGN to vacuole is strongly perturbed in *drs2*Δ*dnf1*Δ mutants while the *dnf1*Δ or *drs2*Δ single mutants have little to no effect on this pathway (Hua *et al.*, 2002). Deletion of the GGAs causes partial secretion of the immature form (p2 CPY) of the vacuolar protein carboxypeptidase Y (CPY) and kinetically delays the maturation of p2 CPY. Deletion of Drs2 causes a threefold kinetic delay in CPY maturation, but only when cells are shifted to 17C. However, *drs2*Δ*dnf1*Δ cells exhibit a comparable defect at permissive temperature, 30C. These findings suggest that the

other P4-ATPases cannot support this pathway at low temperature in the absence of Drs2. My research is focused on determining why *DRS2* is essential at low temperatures.

Relationship of the P4-ATPases to Sterols

Cholesterol may influence the activity of membrane proteins, and is known to play a crucial role in the kinetics of ion pumping by the Na^+,K^+-ATPase (Cornelius, 1995). It is also possible that membrane proteins would affect the lipid content of the membrane, and therefore the physical properties of the membrane. For example the membrane of bile canaliculi must resist the detergent action of excreted bile. Tight packing of lipids that comprise the membrane and other unknown factors of the canalicular membrane could be responsible for the resistance to detergent action of bile. Enrichment of canalicular membrane with cholesterol and sphingomyelin was noticed in rats fed diosgenin- (a steroid sapogenin which is the product of hydrolysis by acids, strong bases, or enzymes of saponins) and these rats exhibited resistance to bile salt-induced hepatic damage (Amigo *et al.*, 1999). The canalicular membranes are more sensitive to the detergent action of hydrophobic bile salts in ATP8b1 deficient mice (Paulusma *et al.*, 2006). Significantly increased activity of ectoenzymes like alkaline phosphatase and aminopeptidase were found in the canalicular membrane of Atp8b1 deficient mice and liver samples from PFIC patients, which indicates membrane damage. Moreover, enhanced biliary recovery of cholesterol and

sphingomyelin was noticed in Atp8b1 deficient mice compared to wild type without increased expression or activity of the canalicular sterol (cholesterol) transporting Abcg5/Abcg8 heterodimer (Paulusma *et al.*, 2006). Surprisingly, mice deficient for both Atp8b1 and Abcg8 excrete wild-type levels of cholesterol into bile (Groen *et al.*, 2007; Groen *et al.*, 2008). This observation suggests that the disorganized structure of the Atp8b1$^{(-/-)}$ bile canalicular membrane fails to retain cholesterol leading to its nonspecific extraction into the bile. There is a possibility that increased concentration of PS in the outer leaflet leads to loose packing of the membrane and this reduced lipid packing caused the membrane to be more sensitive to bile salt extraction of cholesterol and other components. Increased extraction of cholesterol appears to also affect the activity of bile salt export pumps, ABCB11/ABCC2 (Abcb11 exports bile salt and Abcc2 exports organic anion in to the bile). This process causes cholestasis as Abcb11/Abcc2 activities are strongly dependent on membrane cholesterol concentrations (Paulusma *et al.*, 2009).

A. Relationship between sterols and P4-ATPases in yeast and *C.elegans*

Ergosterol is important for bulk membrane function, membrane rigidity, fluidity, and permeability (Parks and Casey, 1995). Ergosterol is synthesized endogenously and differs from cholesterol by containing two more double bonds at C5 and C7 positions, and a methyl group at position C24. The structural requirements of ergosterol for endocytosis require several late steps in ergosterol

synthesis and endocytosis is perturbed in erg mutants (*erg2Δ*, *erg6Δ*, and *erg2Δerg6Δ*) affecting these steps (Munn *et al.*, 1999). The tryptophan permease Tat2p is trafficked from the Golgi apparatus to the vacuole at high tryptophan, and to the plasma membrane at low tryptophan in the cell. However, in the *erg6* mutant, Tat2p is missorted to the vacuole at low tryptophan concentration. Thus, sterol composition is crucial for protein sorting late in the secretory pathway (Umebayashi and Nakano, 2003). There is a synthetic lethal relationship between mutants distrupting latter steps of ergosterol synthesis and *cdc50*Δ (Kishimoto *et al.*, 2005) or *drs2*Δ in *S. cerevisiae,* suggesting that a strong relationship between P4-ATPase and sterol content of the membranes exists. Accumulation of lipid particles in cells which are deficient for Cdc50, the noncatalytic subunit for Drs2p was reported (Fei *et al.*, 2008). Moreover, *tat-2* through *tat-4* becomes essential for reproductive growth during sterol starvation in *C.elegans* (Lyssenko *et al.*, 2008). The Influence on protein trafficking by sterol and P4-ATPAse and sterol related phenotypes in P4-ATPAse suggest a direct relationship between sterol and the P4-ATPases.

Proteins that bind Oxysterol

Oxygenated forms of cholesterol (oxysterols) are potent suppressors of cholesterol biosynthesis (Schroepfer, 2000). This observation prompted a search for proteins that bind oxysterol and mediate the oxysterol effect on cellular lipid metabolism. Cytosolic oxysterol binding proteins were identified (Taylor and

Kandutsch, 1985), but these proteins induce a large number of their effects by acting as ligands for nuclear receptors of the liver X receptor (LXR) family (Cummins and Mangelsdorf, 2006). However, not all effects of oxysterols on cell biology can be attributed to gene regulation. The search for additional oxysterol responsive proteins led to the discovery of the founding member of the oxysterol-binding protein family, OSBP (Levanon *et al.*, 1990). OSBP did not appear to directly affect transcription, as treatment of cells with 25 hydroxycholesterol did not result in OSBP translocation to the nucleus, but instead resulted in its association with Golgi membranes (Ridgway *et al.*, 1992). The OSBP homologues in yeast, *OSH4* and *OSH5* have been implicated as direct transporters of sterols (Raychaudhuri *et al.*, 2006). However, in other studies OSBP/ ORPs (OSBP related proteins) are suggested to be sterol sensors that in turn modulate cellular functions that include signal transduction, vesicular transport and lipid metabolism.

A. OSBP /ORP and their homologues

The signature motif in OSBP and ORPs is EQVSHHPP which is part of the osbp-related protein domain (Olkkonen and Lehto, 2004). The ORD (OSBP-related protein domain) is found in OSBP proteins from yeast to humans providing evidence for a fundamental function that originated very early in eukaryotic evolution. There are 12 family members in humans (Lehto *et al.*, 2001) and mice (Anniss *et al.*, 2002), 7 members in *S. cerevisiae* (Beh *et al.*, 2001), 5

members in *C. elegans (Sugawara et al., 2001)*, 4 members in *D. melanogaster* (Alphey *et al.*, 1998) and 12 members in *A. thaliana* (Skirpan *et al.*, 2006). In addition, extensive splice variants increase the number of encoded proteins in mice and humans. While some of the family members have ORD alone, designated as short OSBP related proteins (ORP), others have amino terminal extensions containing pleckstrin homology (PH) domain, FFAT motif or ankyrin repeats and are called long ORPs (Beh *et al.*, 2001; Loewen and Levine, 2005). These proteins are extensively studied in both yeast and mammalian cells.

OSBPs, ORPs and OSHs (OSBP binding protein homologue) are cytosolic proteins, but most of them associate with specific organelle membrane compartments like the Golgi complex, late endosomes, plasma membrane and ER. The PH domain of OSBP and *OSH1* interacts with phosphatidylinositol-4-phosphate (Levine and Munro, 1998) and facilitates Golgi targeting and function. It has been suggested that 25-hydroxycholesterol binding to the C-terminal domain of OSBP induces a conformational change that unmasks the PH domain, thus inducing a shift of the protein to Golgi membranes (Olkkonen *et al.*, 2006). When expressed as fragments detached from their protein context, the PH domains of several ORPs, unlike that of OSBP, can be targeted to a location different from that of the full-length protein (Lehto *et al.*, 2005) Thus, additional targeting information in determinants flanking the PH domain seems to play a role in the specific membrane association of several ‘‘long’’ ORP proteins. Ankyrin

repeats mediate protein- protein interaction and localization. The FFAT motif binds to VAMP-associated proteins (VAP), transmembrane proteins of the ER. Since the short mammalian ORP variants have a more cytosolic distribution than their long counterparts, they can be expected to be more mobile and to diffuse more freely through the cytosolic compartment than the long variants. Therefore, if ORPs serve as inter-compartmental lipid carriers, the short ORPs are the best candidates for this function (Schulz and Prinz, 2007).

B. Osh proteins in Yeast

S. cerevisiae contains seven oxysterol binding protein homologues called *OSH1* to *OSH7*. Each individual *OSH* gene is nonessential for growth, but deletion of all seven *OSH* genes is lethal suggesting that these genes perform at least one overlapping essential function. Expression of any single OSH gene, in the absence of all others, is sufficient for cell growth (Beh *et al.*, 2001). Thus, every OSH gene has the capability of providing the essential function of the entire OSH gene family, but what these essential overlapping functions are remains unidentified. This group contains three sets of paralogs, Osh1p (Swh1p) and Osh2p; Osh4p (Kes1p) and Osh5p (Hes1p); and Osh6p and Osh7p. Each pair shares a higher degree of similarity than with the other Osh proteins. Cells deficient in all seven osh proteins showed 80% reduction in sterol transfer from PM to ER using exogenously supplied radiolabelled sterols to cells and measuring their conversion to sterol esters in the ER (Li and Prinz, 2004; Raychaudhuri *et*

al., 2006). As yeast cannot utilize exogenous radiolabelled sterol during aerobic growth, strains that have an altered allele of a transcription factor (*upc2-1*) are typically used to allows aerobic sterol uptake in the previous experiment (Wilcox *et al.*, 2002).

Transport of newly synthesized sterol from the ER to the PM is ATP dependent, brefeldin A (BFA) insensitive and Sec18 independent suggesting a nonvesicular route or a BFA-insenitive, noncanonical vesicular route through the Golgi is involved in sterol transport (Baumann *et al.*, 2005). From these findings, only non-raft associated, free sterol is available for nonvesicular transfer between PM and ER. Moreover, sequestering free sterol during raft formation in the PM could be responsible for the concentration of sterol in PM. However, certain other temperature-sensitive sec mutants are capable of partially blocking ergosterol delivery to the PM, concomitant with a partial block in protein secretion (Schnabl *et al.*, 2005). In cells where all OSH function has been inactivated, the rate of transfer of newly synthesized ergosterol to the PM is reduced by ~20 fold. In mammalian systems, NPC2, sterol carrier protein-2/nonspecific lipid-transfer protein (SCP-2/nsLTP), and certain members of the steroidogenic acute regulatory protein related lipid transfer (START) domain family have been proposed as potential sterol carrier proteins[i] (Strauss *et al.*, 2002; Mukherjee and Maxfield, 2004). While yeast lack homologues of some mammalian lipid carriers, such as the SCP-2/nsLTP (Edqvist and Blomqvist, 2006), there is a similarity

between the crystal structures of the START domain and Kes1/Osh4 (Im *et al.*, 2005). This suggests that in yeast the Osh proteins could be sterol transporters.

There is also evidence implicating ORPs in cell signaling. Striking results showed that OSBP forms a complex with cholesterol, a serine/ threonine protein phosphatase and tyrosine phosphatase. This complex dephosphorylates pERK extracellular signal-regulated kinase (ERK), which is involved in the mitogen-activated protein kinase (MAPK) pathway regulation of cell proliferation, survival and differentiation. Cholesterol depletion or exposure of oxysterol induces disassembly of this complex and then OSBP moves to the Golgi (Wang *et al.*, 2005). In addition, the OSBP homologue in *C. elegans* may act in TGF-ß pathways to regulate body size of the worm (Sugawara *et al.*, 2001). ORP9L and ORP9S are substrates of PKCß (PDK-2) in mast cells and negatively regulate Akt phosphorylation in HEK293 cells (Lessmann *et al.*, 2007).

C. Osh4/Kes1

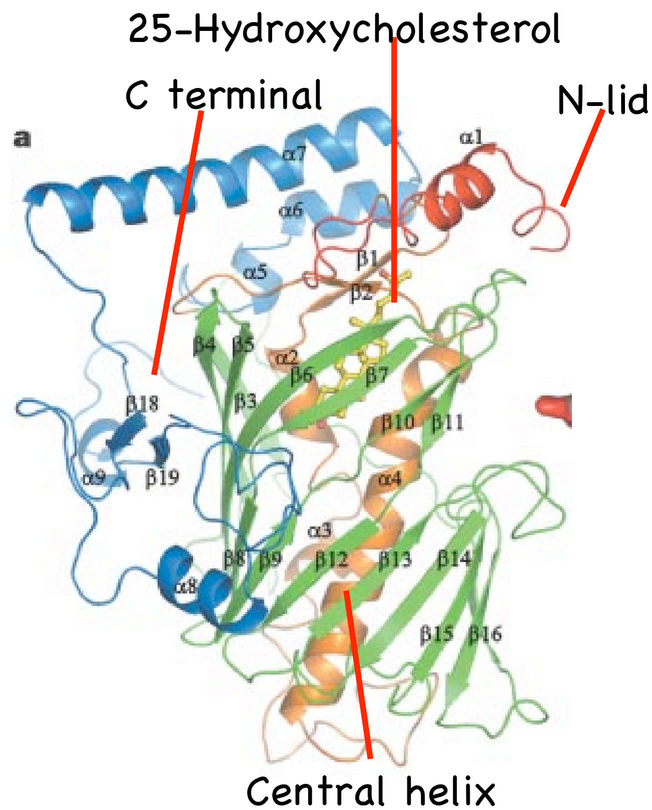
The crystal structure of Osh4/Kes1 revealed that it has a ß barrel shape with hydrophobic pocket large enough to accommodate a single cholesterol, ergosterol or oxysterol molecule with the 3ß hydroxyl group facing the bottom of the hydrophobic pocket. The bottom of the barrel is formed by alpha helices while the top end is occluded by a flexible lid domain (Fig 1-2). The lid protects the sterol molecule from the solvent in the sterol-bound condition. Sterol binding is attained indirectly by hydrogen bonding with the peptide chain through bridging

water molecules (Im *et al.*, 2005).

Genetic studies have suggested that Kes1p is a negative regulator of vesicle-mediated transport from the TGN. *kes1*Δ and loss of function *kes1* alleles suppress *sec14-ts* defects in budding vesicles from the TGN. Sec14 is a phosphatidylinositol/ phosphatidylcholine transfer protein that controls the lipid microenvironment of TGN by downregulating the synthesis of phosphatidylcholine and upregulating the PI-4P production. These regulatory events appear to produce a membrane environment condusive for vesicle-budding. However, over-expression of the other six yeast Osh proteins did not affect the Kes1-mediated bypass of *sec14* defects (Fang *et al.*, 1996). These observations suggest that Kes1 is specific regulator of Sec14. Moreover, alteration in bulk sterol composition and metabolic flux through the sterol/mevalonate pathway are not responsible for *sec14* bypass suppression by *kes1*. Kes1p is recruited to TGN membranes by PI 4-phosphate (PI4P) and may impart an inhibitory influence on vesicle budding by depressing PI4P levels or competing with other effectors for this lipid (Li *et al.*, 2002; Fairn *et al.*, 2007). Further, *kes1*Δ suppresses a mutation in *KRE11*, a component of the TRAPPII complex. TRAPPII is a nucleotide exchange complex for the Rab proteins (Ypt31 and Ypt32) that are involved in vesicle budding from the TGN and early endosome. Kes1 is by far the most abundant Osh protein, although it is unclear why Kes1 is so highly expressed. Hence Kes1 will influence the TGN vesicle mediated

transport or other cellular function in ways that the other Osh proteins cannot influence (Knodler and Mayinger, 2005).

Overexpression of Kes1/Osh4 or OSH family members suppressed the growth defect of temperature-sensitive alleles of the Rho GTPase family member *CDC42* (Kozminski *et al.*, 2006). Osh4/Kes1 was required for Cdc42 localization at polarized sites of growth in a cell cycle-dependent fashion. Transport of ß-1,3-glucanase (Bgl2), a marker protein of polarized exocytosis, and polarized localization of Rho1 and Sec4 (Rab GTPase) were disrupted in cells deleted for all *OSH* genes. These above findings suggest that Kes1 may integrate sterol trafficking and sterol dependent cell signaling. Finally, whether sterol, vesicle trafficking and cell signaling are separate functions or are connected to execute the essential function of OSBP family members remains to be resolved in this field.



Im YJ et al 2005, Nature 437(7055):154-8.

Fig 1-3 Crystal structure of Osh4.

Osh4 is built around a central antiparallel ß-sheet of 19 strands (residues 115–293) that form a near-complete ß-barrel (Green). Residues 1–29 form a lid that covers the tunnel opening and constitute the ALPS (ArfGAP lipid packing sensor) domain (Red). The central helices in orange (residues 30–117) runs the entire length of the barrel and the part of it fills the centre of the barrel, forming a plug at the far end of the tunnel. The large carboxy-terminal region in cyan (residues 308–434) continues after the barrel. The yellow structure is 25-hydroxycholesterol (Im *et al.*, 2005).

D. Evidence that Kes1 transports sterol

Kes1 has also been implicated in sterol trafficking (Raychaudhuri *et al.*, 2006). Yeast cells contain ~42, 000 Osh molecules per cell, of which Kes1 alone is 32,000 molecules. In a *osh*Δ *osh4-1* strain that has a temperature-sensitive allele of Kes1/Osh4 (*osh4-1*) and deletions of the other six *OSH* genes, (Beh and Rine, 2004) exogenously supplied sterol gets esterified at the ER at less than half the rate, at permissive temperature, compared to a strain that contains all Osh genes (Raychaudhuri *et al.*, 2006). At the nonpermissive temperature, the *osh*Δ *osh4-1* strain esterifies exogenous cholesterol seven times more slowly than the strain that contains all OSH genes. These observations suggest that Osh proteins mediate nonvesicular transport of sterol from the PM to the ER with Kes1/osh4 playing a primary role. Kes1/Osh4 can also transport sterol between liposomes. In addition, Kes1 transfers sterol more rapidly between membranes containing phosphoinositides (PIPs) (Raychaudhuri *et al.*, 2006). PIPs binding may stabilize the Kes1 at the membrane surface that in turn is helpful for sterol binding. Osh5 (which shows the highest similarity to Osh4/ Kes1), also binds and transports sterol in vivo and in vitro, although osh6 and osh7 are not capable of transporting the sterol. These results suggest that Osh4 and other Osh proteins may contribute to nonvesicular sterol transport. However, it is also possible that Kes1 is a lipid sensor that regulates other proteins that are actively involved in sterol transfer.

Prior to my work, no genetic interactions between Osh genes and P4-ATPase genes had been reported. The downstream target of repressive effect of Kes1 on Golgi vesicle budding was unknown. Moreover, the influence of Drs2 on the distribution of endogenous ergosterol between cellular membranes was not characterized. My research led to the discovery of a unique co-suppression phenotypes in *drs2*Δ*kes1*Δ double mutants, implicated Drs2p in the establishment of a membrane structure that restricts extraction of sterol, and identifies Drs2p as a critical downstream effector of the repressive effect of Kes1p on the TGN.

CHAPTER II

CONTROL OF PROTEIN AND STEROL TRAFFICKING BY ANTAGONISTIC ACTIVITIES OF A P4-ATPASE AND OXYSTEROL BINDING PROTEIN HOMOLOGUE

This Chapter was published on Molecular Biology of the cell, Vol 20(12):2920-31.

Introduction

A fundamental feature of the eukaryotic cell plasma membrane is the asymmetric distribution of phospholipid species between the inner and outer leaflets. Phosphatidylserine (PS) and phosphatidylethanolamine (PE) are highly concentrated in the inner, cytosolic leaflet with sphingolipids and phosphatidylcholine (PC) enriched in the extracellular leaflet (Balasubramanian and Schroit, 2003). The mechanism for establishing and maintaining phospholipid asymmetry is uncertain; however, type IV P-type ATPases (P4-ATPases) appear to facilitate this process by flipping PS and PE to the cytosolic leaflet (Graham, 2004; van Meer *et al.*, 2008). The budding yeast genome contains five P4-ATPase genes; *DRS2*, *DNF1, DNF2, DNF3* and *NEO1*, while mammalian genomes contain 14 members of this gene family (Graham, 2004). *NEO1* is an essential gene and the other four members (*DRS2* and the *DNF* genes) form an essential group with partially overlapping functions. Drs2p localizes to the TGN and is required for a flippase activity present in purified TGN membranes with specificity for PS and PE (Natarajan *et al.*, 2004; Alder-Baerens

et al., 2006). Dnf1p and Dnf2p appear to have a substrate preference for PE and PC and are primarily responsible for flippase activity in the yeast plasma membrane (Pomorski *et al.*, 2003).

Another well-conserved feature of the eukaryotic plasma membrane is its high concentration of sterol relative to internal organelles. Even though different species produce unique sterols, for example cholesterol in mammals and ergosterol in fungi, the majority of unesterified cellular sterol is localized to the plasma membrane (Daum *et al.*, 1998; Liscum and Munn, 1999). How sterols are concentrated in the plasma membrane is poorly understood. Surprisingly, sterols can move efficiently between the ER, the site of synthesis, and plasma membrane under conditions where vesicle-mediated protein transport through the secretory pathway is blocked (Kaplan and Simoni, 1985; Baumann *et al.*, 2005; Raychaudhuri *et al.*, 2006). Candidate proteins responsible for this nonvesicular, intracellular sterol transport include the oxysterol binding protein homologues (Osh proteins). These sterol binding proteins include 7 members in *S. cerevisiae* (Osh1p – Osh7p) and 16 human homologues (Beh *et al.*, 2001). The structure of Osh4p/Kes1p (hereafter referred to as Kes1p) has been solved with sterol present in a deep, hydrophobic binding pocket that is similar to that present in other lipid transfer proteins (Im *et al.*, 2005). Kes1p can transfer sterol between membranes *in vitro* and inactivation of Kes1p in a strain deficient for the other 6 Osh proteins perturbs nonvesicular sterol transport *in vivo* (Im *et al.*, 2005; Raychaudhuri *et al.*,

2006). However, whether Osh proteins mediate bulk sterol transport or function as signaling molecules in response to sterol binding is unresolved.

P4-ATPases and oxysterol binding proteins have also been implicated in vesicle-mediated protein transport (Graham, 2004; Mousley *et al.*, 2007). *DRS2* was recovered in a genetic screen for factors that function with ADP-ribosylation factor (ARF) in vesicle biogenesis from the Golgi (Chen *et al.*, 1999). This screen also recovered *CDC50* (Chen *et al.*, 2006), which encodes a membrane protein that chaperones Drs2p from the ER to the TGN (Saito *et al.*, 2004), the clathrin heavy chain gene (Chen and Graham, 1998) and an auxilin homologue required to uncoat clathrin-coated vesicles (Gall *et al.*, 2000). As implicated from this screen, Drs2p function is required for a subset of ARF and clathrin-dependent pathways mediating protein transport from the TGN to the cell surface, as well as between the TGN and early endosome (Gall *et al.*, 2002; Liu *et al.*, 2008a). The Dnf P4-ATPases cannot compensate for the loss of Drs2p in the exocytic and early endosome transport pathways. However, functional overlap between Drs2p and Dnf1p is observed in the transport of proteins from the TGN to late endosomes and the vacuole (Hua *et al.*, 2002).

Kes1p is a negative regulator of vesicle budding in the TGN-early endosomal system, although the mechanistic basis of this repression is not known (Mousley *et al.*, 2007). Loss of function *kes1* alleles were recovered in screens for

suppressors of the *kre11* and *sec14* protein trafficking mutants (Jiang *et al.*, 1994; Fang *et al.*, 1996). Kre11p is a component of the TRAPPII nucleotide exchange complex for the Rab proteins Ypt31p and Ypt32p, which regulate vesicle budding from the TGN and early endosomes (Chen *et al.*, 2005; Morozova *et al.*, 2006; Furuta *et al.*, 2007). Sec14p is a yeast phosphatidylinositol/phosphatidylcholine (PI/PC) transfer protein required for exocytic vesicle budding from the TGN (Novick *et al.*, 1980; Bankaitis *et al.*, 1990). Sec14p downregulates PC synthesis through the CDP-choline pathway (McGee *et al.*, 1994), and also stimulates phosphoinositide synthesis (Rivas *et al.*, 1999), perhaps by presenting substrate to PI 4-kinase (Schaaf *et al.*, 2008). Thus, Sec14p helps generate a membrane substrate that is permissive for vesicle budding. Disruption of *KES1* can bypass the essential requirement for Sec14p by a mechanism that is downstream of the CDP-choline pathway for PC synthesis (Fang *et al.*, 1996; Li *et al.*, 2002). Kes1p is recruited to TGN membranes by PI 4-phosphate (PI4P) and may impart an inhibitory influence on vesicle budding by depressing PI4P levels or competing with other effectors for this lipid (Li *et al.*, 2002; Fairn *et al.*, 2007).

Here we report that *kes1*Δ is also a suppressor of *drs2* alleles, although *kes1*Δ cannot bypass the essential function of the *DRS2/DNF* family of P4-ATPases. Genetically, Kes1p appears to repress Dnf and Drs2p function at the TGN and we provide biochemical evidence that Kes1p potently antagonizes Drs2p flippase activity in purified TGN membranes. Surprisingly, we also found

that Kes1p is hyperactive in *drs2*Δ cells and causes a significant increase in the rate of sterol transport to intracellular membranes. Thus, Drs2p also antagonizes the influence of Kes1p on intracellular sterol transport. These observations imply that a system of checks and balances between a P4-ATPase and oxysterol binding protein controls vesicular and nonvesicular transport processes essential for membrane biogenesis.

Materials and Methods

Filipin, Nystatin, SDS, calcofluor white and mevastatin were purchased from Sigma-Aldrich, (St. Louis, MO). Papuamide B and Ro09-0198 (Ro)-peptide were generous gifts from Raymond Andersen (University of British Columbia, Canada), and Masato Umeda (Kyota University, Japan), respectively. ^{35}S-Methionine was from PerkinElmer LAS (Waltham, Massachusetts, USA) and the yeast knockout collection was from Invitrogen (Carlsbad, CA).

A. Media and Strains

Yeast strains used in this study are listed in supplemental Table 1 and were grown in standard rich medium (YPD) or synthetic defined (Cummins and Mangelsdorf) minimal media containing the required nutritional supplements (Sherman, 1991). Yeast transformations were performed using a lithium acetate method (Gietz and Woods, 2006). *KES1* was disrupted by PCR-mediated replacement with *HIS3* from *Saccharomyces kluyveri* using primers forward (5'-

TCG AAA AAT TTA TAA GAT TTA GTC TCA AGA ATT TCA AGT CCG GAT CCC CGG GTT AAT TAA-3') and reverse (5'-ATT AGT GCA ACG GTA ACA AGT TGT TAC TTT ATC GTT CTC CGA ATT CGA GCT CGT TTA AAC-3') primers with the pFA6a-His3MX6 template as previously described (Longtine *et al.*, 1998). In other strains, *KES1* was replaced with *URA3* by transformation with pRE352 (Fang *et al.*, 1996) digested with EcoRI and BamHI. The BY4741 *kes1Δ drs2Δ* strain was constructed using pZH523 (Hua *et al.*, 2002) to disrupt *DRS2* in the BY4741 *kes1Δ* strain background. For strains used for the cholesterol transport assays, *UPC2* was replaced with *upc2-1* by homologous recombination to allow these strains to take up exogenous cholesterol during aerobic growth (Raychaudhuri *et al.*, 2006)

To test for growth inhibition, cells in early log phase were diluted to 0.1 OD/ml in rich media containing potential inhibitors at various concentrations and growth was monitored using an ELISA plate reader. The OD_{600} after 36 hours in rich media without inhibitor was defined as 100% growth for a particular strain and used to normalize data with inhibitor (+/- standard deviation, n=6). Tests for growth at high-pressure was done as previously described (Abe and Minegishi, 2008).

B. Screen for suppressors of *drs2*Δ and cloning of *KES1*

Individual colonies of *MATα* and *MATa drs2Δ* strains (SEY6210 *drs2Δ*::*TRP1* and SEY6211 *drs2Δ*::*LEU2*) were streaked on rich media and incubated at the non-permissive growth temperature (20C or 17C) to select for spontaneous bypass suppressors. Cold-resistant (CR) suppressor strains were backcrossed to parental strains to define recessive and dominant suppressor alleles. Recessive suppressor mutants were intercrossed to define two complementation groups *SDK1* and *SDK2* (suppressor of *drs2* knockout). To clone *SDK1,* strain *BMY1001 (drs2Δ sdk1-1)* was transformed with a genomic library (Goodson *et al.*, 1996) and approximately 50,000 Leu^+ transformants were replica-plated and incubated at 17C for 5-7 days to screen for cold-sensitive colonies. The genomic library plasmids pBMP1 and pBMP2 were rescued from two cold-sensitive (cs) colonies and were identical in restriction digestion pattern. pBMP1 restored cs growth to BMY1001 upon retransformation and DNA sequencing indicated that it carried an 8.6 Kbp fragment of chromosome XVI containing *KES1*.

Table 3 Strains used in this study

BY4741	*MAT**a** his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Invitrogen
BY4742	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	Invitrogen
BY4741 YPL145C	BY4741 *kes1Δ*[a]	Invitrogen
BY4742 YPL145C	BY4742 *kes1Δ*	Invitrogen
ZHY615D1C	*MAT**a** his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 drs2Δ*	(Hua *et al.*, 2002)
ZHY615M2D	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 drs2Δ*	(Hua *et al.*, 2002)
ZHY2149D	ZHY615M2D *dnf1Δ*	(Hua *et al.*, 2002)
PFY3273A	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 dnf1Δ dnf2Δ dnf3Δ*	(Hua *et al.*, 2002)
ZHY704	PFY3273A *drs2Δ::LEU2 pRS416-DRS2*	(Hua *et al.*, 2002)
SEY6210	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1–Δ901 lys2-801 suc2-Δ9*	(Robinson *et al.*, 1988)
SEY6211	*MAT**a** leu2-3, 112 ura3-52 his3-Δ200 trp1–Δ901 ade2-101 suc2-Δ9*	(Robinson *et al.*, 1988)
SEY6210*drs2Δ*	SEY6210 *drs2Δ::TRP1*	(Chen and Graham, 1998)
SEY6211*drs2Δ*	SEY6211 *drs2Δ::LEU2*	(Chen and Graham, 1998)
BMY01	ZHY615M2D *kes1Δ::HIS3*	This study
BMY01B	ZHY615D1C *kes1Δ::HIS3*	This study
BMY02	BY4742 *kes1Δ::HIS3*	This study
BMY02B	BY4741 *kes1Δ::HIS3*	This study
BMY03	PFY3273A *kes1Δ::HIS3*	This study
BMY04	ZHY704 *kes1Δ::HIS3*	This study
BMY05	ZHY2149D *kes1Δ::HIS3*	This study

BMY44a	BY4741 *kes1Δ drs2Δ::LEU2*	This study
BMY45a	BY4742 *kes1Δ drs2Δ::LEU2*	This study
BMY1164	SEY6211 *drs2Δ sdk2-64*	This study
YLR133W*cki1Δ*	BY4742 *cki1Δ*	Invitrogen
KLY891	BY4742 *cki1Δ drs2Δ::LEU2*	This study
YKL212W*sac1Δ*	BY4742 *sac1Δ*	Invitrogen
KLY901	BY4742 *sac1Δ drs2Δ::LEU2*	This Study
BMY1001	SEY6210 *drs2Δ sdk1-1*	This study
ZHY708	*MAT**a** his3Δ1 leu2Δ0 ura3Δ 0 met15Δ0 dnf1Δ dnf3Δ drs2Δ::LEU2*	(Hua *et al.*, 2002)
BMY029	ZHY708 *kes1Δ::URA3*	This study
ZHY7282C	*MAT**a** his3Δ1 leu2Δ0 ura3Δ 0 met15Δ0 dnf2Δ dnf3Δ drs2Δ::LEU2*	(Hua *et al.*, 2002)
BMY039a	ZHY7282C *kes1Δ::URA3*	This study
ZHY409	*MAT**a** his3Δ1 leu2Δ0 ura3Δ 0 met15Δ0 dnf1Δ dnf2Δ dnf3Δ drs2Δ*::*LEU2 pRS313-DRS2*	(Hua *et al.*, 2002)
ZHY410–3A	*MAT**a** his3Δ1 leu2Δ0 ura3Δ 0 met15Δ0 dnf1Δ dnf2Δ dnf3Δ drs2Δ*::*LEU2 pRS313-drs2-31*	(Natarajan *et al.*, 2004)
BMY041	ZHY 410-3A *kes1Δ::URA3*	This study
ZHY6281-15B	BY4742 *neo1-1ts*	This study
BMY034	ZHY6281-15B *kes1Δ::URA3*	This study
TGY1912	SEY6210 *sla2/end4-1*	(Gall *et al.*, 2002)
CCY642	SEY6210 *sla2 drs2Δ::TRP1*	(Gall *et al.*, 2002)
BMY15	CCY642 *kes1Δ::HIS3*	This study
YML008C*erg6Δ*	BY4742 *erg6Δ*	Invitrogen
BMY33	BY4742 *erg6Δ drs2::LEU2 pRS416-DRS2*	This study
BMY20	BY4742 *erg6Δ drs2Δ::LEU2 kes1Δ::HIS3*	This study
BMY18	BY4742 *erg6Δ kes1Δ::HIS3*	This study
BMY031	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 cdc50Δ:: HIS3 kes1Δ::URA3*	This study

YJL099W*chs6Δ*	BY4742 *chs6Δ*	Invitrogen
KL023	BY4742 *drs2Δ chs6Δ*	(Liu *et al.*, 2008a)
BMY38a	KL023 *kes1Δ::URA3*	This study
BMY37a	BY4742 *chs6Δ kes1Δ::URA3*	This study
KLY691	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 gga1Δ gga2Δ*	(Liu *et al.*, 2008a)
BMY40a	KLY691 *kes1Δ::URA3*	This study
upc2-1	BY4742 *upc2-1::URA3*	This study
upc2-1drs2Δ	ZHY615M2D *upc2-1::URA3*	This study
upc2-1kes1Δ	BMY02 *upc2-1::URA3*	This study
upc2-1 drs2Δkes1Δ	BMY01 *upc2-1::URA3*	This study

Table 3 Plasmids used in this study

pBMP1	Genomic clone carrying *KES1* insert rescued from BMY1001 CS phenotype	This Study
pBMP2	Genomic clone carrying *KES1* insert rescued from BMY1001 CS phenotype	This Study
pGST/OSH4	Kes1p /Osh4p expression vector	(Im *et al.*, 2005)
pZH523	DRS2 disruption plasmid	(Hua *et al.*, 2002)
pCTY244	YCp(KES1)	(Li *et al.*, 2002)
pCB236 to pCB242	OSH genes in 2μ plasmids	(Kozminski *et al.*, 2006)
pRE352	YCp kes1*Δ::URA3*	(Fang *et al.*, 1996)

[a] Gene distruptions (Δ) are KanMX6 replacements unless otherwise indicated

C. Microscopy and Immunological Methods

Imaging of cells expressing green fluorescent Protein (GFP) fusion proteins and stained with filipin was done as previously described (Beh and Rine, 2004; Liu *et al.*, 2007). Samples for electron microscopy were prepared as described previously (Chen *et al.*, 1999). Sections (50–100 nm) were observed on a CM12 electron microscope (Philips, Eindhoven, Netherlands). 100nm diameter vesicles were counted in 50 cell sections for each strain. Error bars are standard deviation (n=2). Metabolic labeling, immunoprecipitation (Graham, 2001), and immunoblotting (Chen *et al.*, 1999) were performed as described previously. An Odyssey infrared fluorescence detector (LI-COR, Lincoln, NE) was used to quantify western blots and coomassie stained gels.

D. Phospholipid translocase assays

The methods for purifying TGN membranes and assaying Kex2p and ATP-dependent NBD-PS translocase activities have been previously described (Natarajan and Graham, 2006). Briefly, equal volumes of purified TGN membranes (0.5 mg/ml), NBD hospholipids (10 μM), and an ATP regenerating system (without ATP) were mixed on ice and then shifted to 37C to initiate the assay. After 2 hrs of incubation at 37C with no ATP, the samples were split and 3 μl of 100 mM ATP or buffer H (10 mM Hepes pH 7.5, 150 mM NaCl) was added per 100 μl of sample. The *kes1*Δ membrane samples were split again and received 1 μl of Kes1p or buffer H per 100 μl of sample. The samples were

incubated an additional 2 hours at 37C. Aliquots of membranes were removed at 0, 2 (prior to ATP addition) and 4 hours of incubation and NBD-PS in the cytosolic leaflet was extracted onto fatty-acid free BSA and quantified. The difference in the percentage of NBD-PS in the cytosolic leaflet of WT TGN membranes incubated with or without ATP at the 4-hour time point was defined as 100% NBD-PS flippase activity and was used to normalize data for other conditions assayed. Assays were performed in duplicate and averages from at least three independent experiments (+/- standard deviation) are reported.

Results

A. *kes1* suppresses *drs2Δ* cold-sensitive growth

Cells harboring knockout alleles of *DRS2* exhibit a striking cold-sensitive growth defect. Wild-type yeast can grow over a range of temperatures from 10 - 40C, whereas *drs2*Δ strains can only grow over a temperature range of 21 - 40C. To explore the mechanism underlying this extreme cold-sensitive (cs) growth defect, we carried out a selection for spontaneous extragenic suppressors that allowed growth of *drs2Δ* at 17C. Complementation tests indicated that the recessive *drs2Δ* suppressor mutants fell into two complementation groups, which we named *SDK1* and *SDK*2 for Suppressor of *drs2* knock-out (*SDK*). The *SDK1* and *SDK2* groups are comprised of twelve and four recessive extragenic bypass suppressors of *drs2*Δ respectively. Figure 2-1A shows the 30C and 17C growth phenotypes of representative *drs2Δ sdk1* and *drs2Δ sdk2* mutants relative to parental *drs2Δ* and

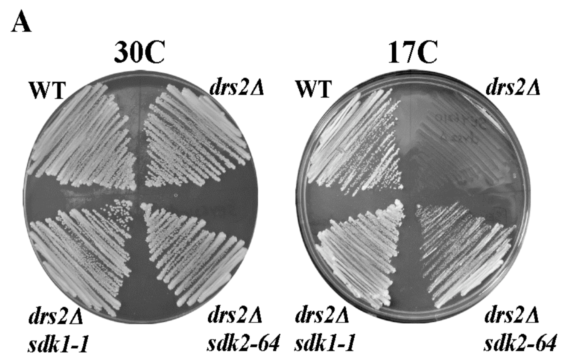
wild-type (WT) strains. Surprisingly, the *drs2*Δ *sdk1-1* double mutant formed larger colonies than even the WT strain at 17C (Fig. 2-1B).

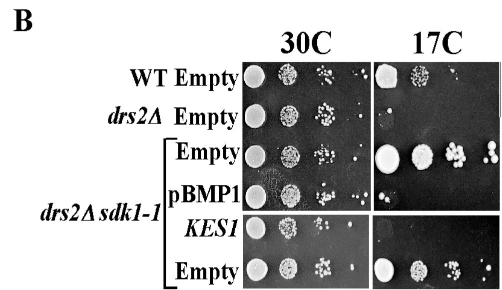
SDK1 was cloned by complementation of the cold-resistant growth phenotype of *drs2*Δ *sdk1-1*. A genomic library plasmid (p*BMP1*) was isolated that was able to complement *sdk1-1* upon retransformation of *drs2Δ sdk1-1* cells (Fig. 2-1B). Among the five ORFs in pBMP1, *KES1* was the most likely candidate for the *sdk1*-complementing gene and as expected, a *KES1* subclone complemented the cold-resistant phenotype of *drs2Δ sdk1-1* (Fig. 2-1B). Several of the *kes1* alleles isolated in the *sec14* suppressor screen contained nonsense mutations, leading to the expression of truncated, nonfunctional proteins (Fang *et al.*, 1996; Li *et al.*, 2002). We examined the expression of Kes1p in the *drs2*Δ *sdk* mutants by western blotting and found that seven of the 12 *sdk1* alleles failed to express full length Kes1p, suggesting that they would also carry nonsense mutations (Fig 2-2).

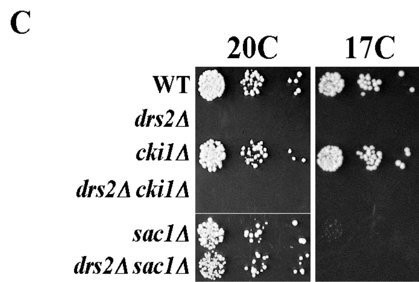
These data indicate that *sdk1* strains carry mutations in *KES1* (i.e. *SDK1* and *KES1* are the same gene) and that loss of function *kes1* mutations suppress *drs2Δ* cs growth. Kes1p is one of seven oxysterol binding protein homologues in yeast. None of the other six members was able to restore a cs growth phenotype to either *drs2Δ sdk1-1* or *drs2*Δ *sdk2,* even when overexpressed from multicopy plasmids (data not shown). These results indicate that among the

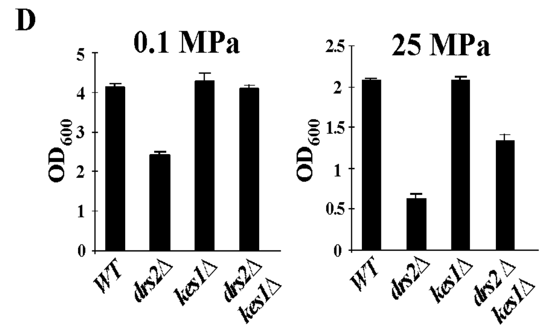
oxysterol binding protein homologues, Kes1p is uniquely capable of inhibiting growth of *drs2Δ* cells at cold-temperatures. We have not yet succeeded in cloning *SDK2* and so this report will focus on *SDK1/KES1*.

Fig 2-1 *kes1* is a suppressor of *drs2*Δ cold-sensitive and high-pressure growth defects. (A) Growth of WT (SEY6210), *drs2*Δ (SEY6210 *drs2*Δ), *drs2*Δ *sdk1-1* (BMY1001) and *drs2*Δ *sdk2-64* (BMY1164) strains streaked onto rich medium and incubated at 30C and 17C. (B) *KES1* confers cs growth to *drs2*Δ *sdk1-1*. The WT, *drs2*Δ and *drs2*Δ *sdk1-1* strains were transformed with empty plasmid (pRS315), pBMP1 (genomic library clone carrying *KES1*) or a *KES1* subclone (pCTY244) and tenfold serial dilutions were spotted on minimal medium and incubated at 17C. (C) Serial dilutions of WT (BY4742), *drs2*Δ (ZHY615M2D), *cki1*Δ (BY4742 YLR133W), *drs2*Δ *cki1*Δ (KLY891), *sac1*Δ (BY4742 YKL212W) and *drs2*Δ *sac1*Δ (KLY901) strains were incubated on rich medium at 20C and 17C. (D) Equal cell numbers of WT (BY4742), *drs2*Δ (ZHY615M2D), *kes1*Δ (BMY02) and *drs2*Δ *kes1*Δ (BMY01) strains were seeded in minimal medium and incubated at atmospheric (0.1 mPa) or high pressure (25MPa) for 10 hours at 30C.


A
30C
17C
WT
drs2Δ
WT
drs2Δ
drs2Δ
sdk1-1
drs2Δ
sdk2-64
drs2Δ
sdk1-1
drs2Δ
sdk2-64


B
30C
17C
WT Empty
drs2Δ Empty
Empty
pBMP1
drs2Δ sdk1-1
KES1
Empty


C
20C
17C
WT
drs2Δ
cki1Δ
drs2Δ cki1Δ
sac1Δ
drs2Δ sac1Δ


D
0.1 MPa
25 MPa
OD600
OD600
WT
drs2Δ
kes1Δ
drs2Δ kes1Δ
WT
drs2Δ
kes1Δ
drs2Δ kes1Δ

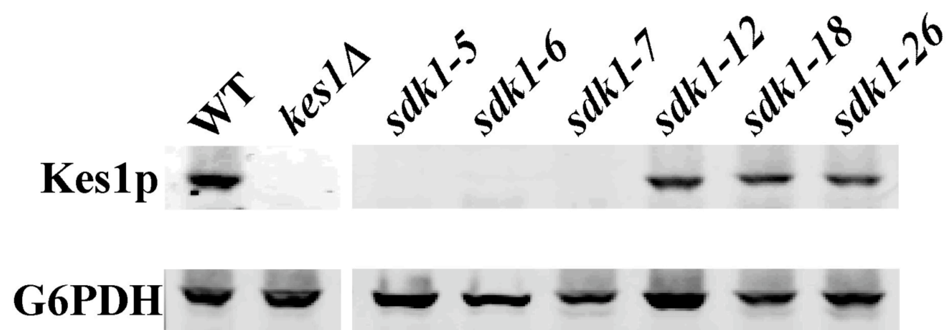


Fig 2-2. Whole cell lysates prepared from strains carrying the indicated *KES1/SDK1* alleles and immunoblotted to detect Kes1p and glucose-6-phosphate dehydrogenase (G6PDH)

Mutations in three genes (*CKI1, PCT1, CPT1*) encoding enzymes of the CDP-choline pathway involved in phosphatidylcholine biosynthesis can bypass the essential requirement of *SEC14*. However, we found that disruption of *CKI1* in the *drs2Δ* background failed to suppress cs growth of *drs2Δ* (Fig 2-1C). Hence, inactivation of phosphatidylcholine biosynthesis via the CDP-choline pathway does not exert bypass suppression of *drs2Δ*. Deletion of *SAC1,* a phosphoinositide phosphatase, increases PI4P levels and suppresses *sec14-ts* (Rivas *et al.*, 1999). *SAC1* deletion suppressed the cold sensitivity of *drs2Δ* at 20C but not at 17C (Fig. 2-1C). However, the *sac1*Δ single mutant also failed to grow at 17C and so the lack of *drs2*Δ suppression at this temperature may not be significant. Stt4p, a plasma membrane PI 4-kinase, synthesizes most of the PI4P that accumulates in *sac1*Δ mutants. Accumulation of PI4P at non-TGN sites causes partial mislocalization of Kes1p from the TGN, thereby relieving its repressive effect on this organelle (Li *et al.*, 2002). This influence on Kes1p appears to account for the suppression of *sec14-ts* by *sac1*Δ and can also explain the suppression of *drs2*Δ by *sac1*Δ at 20˚C.

A high percentage of *Saccharomyces* mutants that cannot grow at low temperature, including *drs2*Δ, also fail to grow at high hydrostatic pressure (Abe and Minegishi, 2008). This strong positive correlation suggests that the mutants have a defect in membrane order and fluidity. To determine if *kes1*Δ would also suppress the high-pressure growth defect of *drs2*Δ at 30C, we measured the

growth of WT, *drs2*Δ*, kes1*Δ and *drs2*Δ *kes1*Δ strains at atmospheric pressure (0.1 MPa) and high pressure (25 MPa) in liquid medium over a 10 hr hour period. Under these conditions, *drs2*Δ grew slower than WT or *kes1*Δ cells at 0.1 MPa and this growth defect was exacerbated at 25 MPa. At both pressure conditions, the *drs2*Δ *kes1*Δ cells showed improved growth, indicating that the *drs2Δ* high-pressure sensitivity was at least partially suppressed by *kes1Δ* (Fig. 2-1D).

***kes1Δ* suppression of *drs2Δ* requires *DNF* P4-ATPase genes**

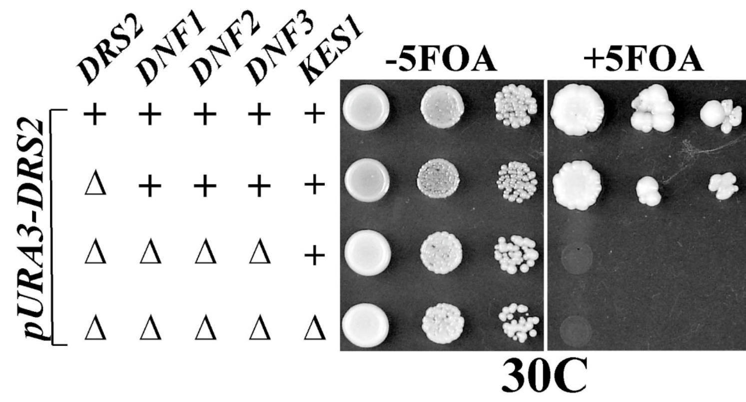
Drs2p is part of an essential group of P4-ATPases that also includes Dnf1p, Dnf2p and Dnf3p (Hua *et al.*, 2002). In the absence of Dnf P4-ATPases (*dnf1,2,3*Δ cells), Drs2p can support growth of yeast over the full temperature range by itself, but the *drs2*Δ *dnf1,2,3*Δ quadruple mutant is dead at any temperature (Hua *et al.*, 2002). To determine if *kes1Δ* could bypass the essential requirement for *DRS2/DNF* function, or if the mechanism of suppression required the presence of Dnf ATPases, we tested if deletion of *KES1* would rescue viability of a *drs2Δ dnf1,2,3Δ* strain. A strain was generated that carries null alleles of all four members of the *DRS2/DNF* group by first introducing a wild-type copy of *DRS2* on a *URA3*-based plasmid (p*URA3-DRS2*) (Hua *et al.*, 2002). This quadruple mutant strain (*drs2*Δ *dnf1,2,3*Δ) could not lose p*URA3-DRS2* and grow on 5-fluoro-orotic acid (5-FOA) (Fig 2A), a compound that kills cells retaining *URA3*-based plasmids. In contrast, WT and *drs2*Δ cells were able to lose the p*URA3-DRS2* plasmid and form colonies on 5-FOA plates. Importantly, the

drs2Δ dnf1,2,3Δ kes1Δ strain failed to grow on the 5-FOA plate (Fig. 2-3A). Therefore, *kes1*Δ suppression of *drs2Δ* requires one or more Dnf P4-ATPase.

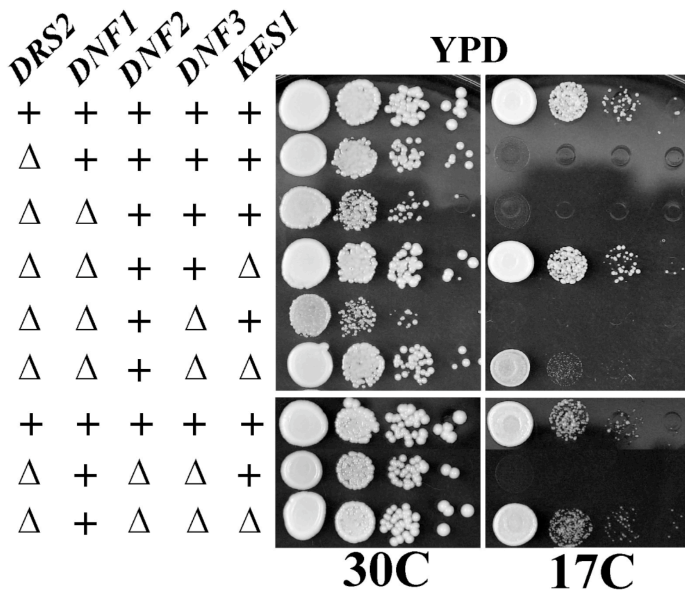
To determine which Dnf proteins are required to support suppression, we tested the ability of *kes1Δ* to suppress different combinations of *drs2Δ* and *dnfΔ* alleles (Fig. 2-3B). Growth defects of a *drs2Δ dnf1Δ* strain at 30C and 17C were efficiently suppressed by *kes1*Δ, indicating that *DNF2* and/or *DNF3* can support *kes1Δ* suppression. A strain expressing only *DNF2* was weakly suppressed whereas a strain carrying only *DNF1* exhibited a WT level of suppression. We were unable to recover a *drs2*Δ *dnf1,2*Δ *kes1*Δ strain expressing only *DNF3*. However, the strain with wild-type *DNF2* and *DNF3* was suppressed better than the strain carrying only *DNF2*, indicating some influence from *DNF3*. Therefore, all three Dnf ATPases contribute to the suppression of *drs2*Δ by *kes1*Δ. We also tested if *kes1*Δ could suppress a temperature-sensitive (ts) for function allele of *drs2* in the absence of the Dnf ATPases. Disruption of *KES1* restored a wild-type growth rate at 37C to a *drs2-ts dnf1,2,3*Δ strain (Fig 2-3C), and so loss of Kes1p can improve the function of a crippled Drs2p.

Fig 2-3. Specificity for *kes1*Δ suppression of P4-ATPase mutants. (A) *kes1*Δ cannot bypass the essential function of the *DRS2-DNF* gene family. Strains harboring the indicated combination of null (Δ) and WT (+) alleles for P4-ATPase and *KES1* genes, and carrying wild-type *DRS2* on a *URA3* plasmid, were spotted on synthetic complete media with and without 5-FOA. Strains used were BY4742, ZHY615M2D, ZHY704 and BMY04. (B) Strains harboring the indicated combination of null (Δ) and WT (+) alleles were spotted on rich medium and incubated at 30C and 17C. Strains used were BY4742, ZHY615M2D, ZHY2149D, BMY05, ZHY708, BMY029, ZHY7282C, and BMY039a. (C) *kes1*Δ suppresses the temperature-sensitive growth defect of a *drs2-ts dnf1,2,3*Δ strain at 37C. Strains were ZHY409, ZHY410-3A and BMY041.

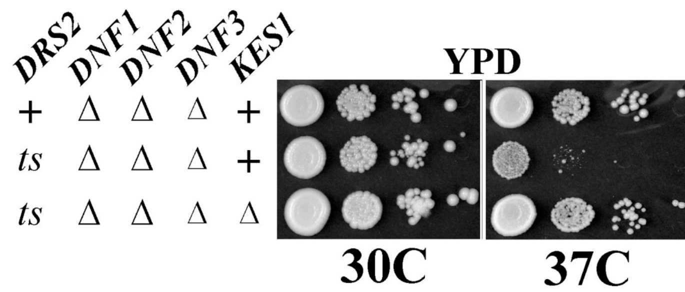
A



B



C



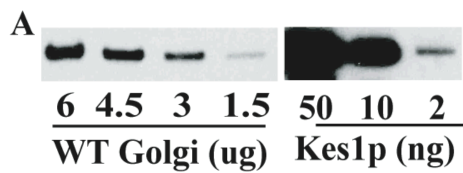
Kes1p attenuates Drs2p flippase activity at the TGN

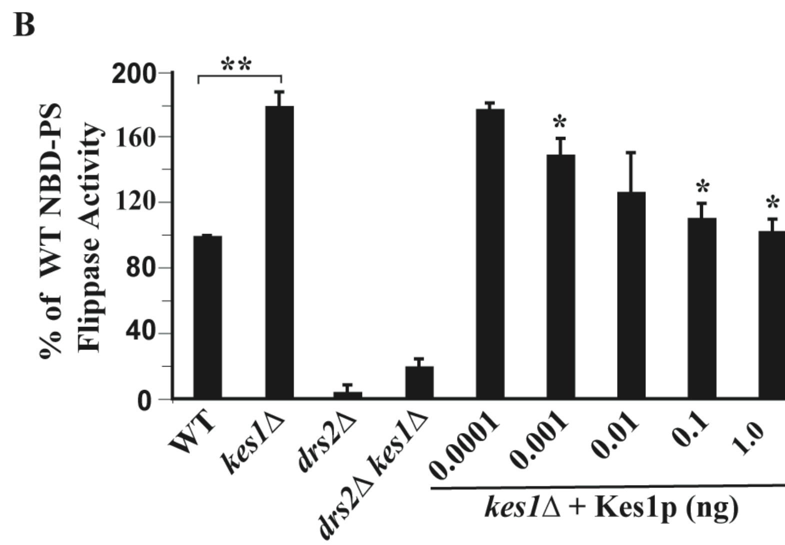
The ability of *kes1*Δ to suppress *drs2-ts* in the *dnf1,2,3*Δ background suggests that Kes1p antagonizes Drs2p activity at the Golgi complex. To test this possibility biochemically, TGN membranes were purified from WT, *drs2*Δ, *kes1*Δ and *drs2*Δ *kes1*Δ cells to assay for phospholipid translocase (flippase) activity. Equal amounts of protein from each TGN preparation were first probed for Kes1p by immunoblot to determine if Kes1p co-purifies with these membranes (data for WT membranes is shown in Fig 2-4A). Relative to the recombinant Kes1p standard, the TGN membranes from WT and *drs2*Δ cells carry approximately 2 ng (40 femtomoles) of Kes1p per 3 μg of total protein. From similar quantitative immunoblots, the WT and *kes1*Δ membranes contain approximately 0.6 ng of Drs2p (4 femtomoles) per 3 μg of total protein.

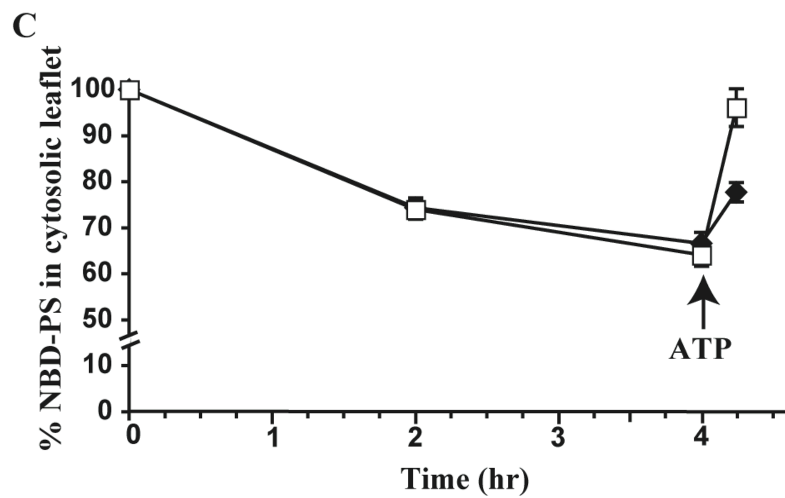
Each TGN membrane preparation was assayed for NBD-PS translocase activity as described under materials and methods. The ATP-dependent translocation (flip) of NBD-PS from the lumenal leaflet to the cytosolic leaflet was quantified and normalized to the WT membrane sample. The *kes1*Δ membranes displayed significantly greater NBD-PS flippase activity than WT membranes. This flippase activity was eliminated in the *drs2*Δ membrane sample and was substantially reduced in *drs2*Δ *kes1*Δ membranes (Fig 2-4B). Thus, Drs2p was primarily responsible for the enhanced NBD-PS flippase activity in *kes1*Δ membranes. The Dnf P4-ATPases may have contributed the small increase

in *drs2*Δ *kes1*Δ NBD-PS flippase activity relative to *drs2*Δ membranes, although these data are not statistically different.

Fig 2-4. Kes1p represses the flippase activity of Drs2p in TGN membranes. (A) TGN membranes were purified from WT (BY4742), *kes1*Δ (BMY02), *drs2*Δ (ZHY615M2D) and *drs2*Δ *kes1*Δ (BMY01) cells. The indicated amounts of WT TGN membranes and recombinant Kes1p were immunoblotted to quantify Kes1p in this membrane sample. (B) ATP-dependent translocation of NBD-PS from the lumenal leaflet to the cytosolic leaflet of the TGN membranes (flippase activity) was measured as described under Materials and Methods. NBD-PS flippase activity in WT membranes was defined as 100% activity and used to normalize data for other samples. Error bars are standard deviation (n=3; **P = 0.005; *P ≤ 0.0223 compared to *kes1*Δ membranes using student t test). (C) Kinetics of NBD-PS translocation by Drs2p. Golgi membranes from WT (closed symbols) and *kes1*Δ (open symbols) cells were incubated without ATP for 4 h to allow passive translocation of NBD-PS into the inner leaflet. ATP was then added and the amount of NBD-PS flipped to the outer (cytosolic) leaflet in 15 min was measured as described under materials and methods.


A
6 4.5 3 1.5
WT Golgi (ug)
50 10 2
Kes1p (ng)


B
% of WT NBD-PS Flippase Activity
200
160
120
80
40
0
**
*
*
*
WT
kes1Δ
drs2Δ
drs2Δ kes1Δ
0.0001
0.001
0.01
0.1
1.0
kes1Δ + Kes1p (ng)


C
% NBD-PS in cytosolic leaflet
100
90
80
70
60
50
10
0
ATP
0
1
2
3
4
Time (hr)

It was formally possible that the enhanced NBD-PS flippase activity in *kes1*Δ membranes was an indirect effect of the chronic Kes1p deficiency. Therefore, we tested if adding back recombinant Kes1p to the mutant membranes would repress the Drs2-dependent flippase activity. Addition of recombinant Kes1p to the *kes1*Δ TGN membranes attenuated NBD-PS flippase activity to wild-type levels (Fig 3B). Remarkably, the half-maximal inhibitory concentration was in the range of 1 to 10 picograms of Kes1p per 17 μg of total Golgi protein (0.02 pM to 0.2 pM Kes1p). For comparison, the WT membrane preparation contained approximately 10 ng of endogenous Kes1p and addition of up to 1 ug of recombinant Kes1p to the *kes1*Δ membranes conferred no additional inhibition of NBD-PS flippase activity (unpublished observation).

The NBD-PS flippase activity shown in fig 2-3B was determined after 2 h of incubation with ATP, at which time all the NBD-PS probe in the *kes1*Δ samples was flipped to the cytosolic leaflet, and so the flippase assay was saturated. To better assess the influence of Kes1p on the kinetics of NBD-PS translocation, we measured the amount of NBD-PS flipped in 15 min of incubation with ATP (Fig 2-3C). After initial incorporation of the NBD-PS probe in the cytosolic leaflet, WT and *kes1*Δ TGN membranes were incubated for 4 h without ATP to allow spontaneous redistribution of ~40% of the probe to the lumenal leaflet (NBD-PS that is resistant to back-extraction with fatty-acid free BSA). ATP was then added and the membranes were incubated at 37C for 15 min

before back-extraction of the probe with BSA. Again, nearly all of the NBD-PS was flipped to the cytosolic leaflet of the *kes1*Δ membranes in 15 min. Under these conditions, we estimate that ~345 NBD-PS molecules were flipped per Drs2p molecule per minute in the *kes1*Δ membranes, relative to ~160 NBD-PS molecules flipped/Drs2p/min in WT membranes.

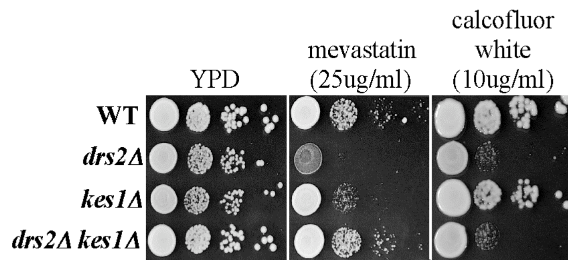
Drs2p antagonizes the influence of Kes1p on sterol trafficking and subcellular distribution

Because Kes1p binds ergosterol and is implicated the intracellular transport of this sterol, we examined the relationship of *drs2Δ* suppression by *kes1Δ* to ergosterol metabolism and localization. We found that *drs2Δ* cells are hypersensitive to mevastatin (Fig 2-5A) an inhibitor of 3-hydroxy–3-methylglutaryl-CoA reductase, the rate-limiting enzyme of ergosterol synthesis. *kes1Δ* cells are slightly hypersensitive to mevastatin but the *drs2Δ kes1Δ* strain exhibits WT resistance. Perturbation of TGN function in *drs2Δ* cells also alters the trafficking and/or function of cell wall biosynthetic enzymes. This causes hypersensitivity of *drs2Δ* to calcofluor white (CW), a chitin binding compound that can interfere with cell wall assembly. By contrast to the mevastatin hypersensitivity, *kes1Δ* does not suppress the CW hypersensitivity of *drs2Δ* (Fig 2-5A). *kes1Δ* also completely suppressed the hypersensitivity of *drs2Δ* to nystatin (Fig 2-5B), a polyene antifungal compound that binds ergosterol in the plasma membrane.

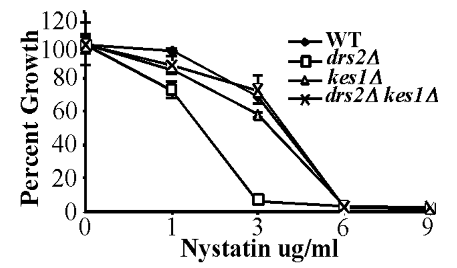
drs2Δ is synthetically lethal with *erg6Δ*, a gene encoding the Δ(24)-sterol C-methyltransferase that converts zymosterol to fecosterol in the ergosterol biosynthetic pathway (Kishimoto *et al.*, 2005). Viability of a *drs2Δ erg6Δ* strain can be sustained with WT *DRS2* carried on a *URA3* plasmid (*pURA3-DRS2*). This strain cannot lose *pURA3-DRS2* and therefore failed to form colonies on media containing 5-FOA, whereas the other single and double mutants grew well (Fig 2-5C). However, a *drs2Δ erg6Δ kes1Δ* strain readily lost the *pURA3-DRS2* plasmid and grew robustly on 5-FOA. Thus, *kes1Δ* suppresses the synthetic lethality between *drs2Δ* and *erg6Δ*. The data shown in Figure 2-5A-C suggests that *drs2Δ* cells might have a defect in ergosterol synthesis. However, a normal concentration of ergosterol in *drs2Δ* cells has been reported (Fei *et al.*, 2008) and we have confirmed this result (data not shown).

Fig 2-5. Relationship of ergosterol to *kes1*Δ suppression of *drs2*Δ. (A) *kes1*Δ suppresses *drs2*Δ hypersensitivity to mevastatin. WT (BY4742), *drs2*Δ (ZHY615M2D)*, kes1*Δ (BMY02), *drs2*Δ *kes1*Δ (BMY01) were incubated at 30C on rich media with or without mevastatin or calcofluor white. (B) *kes1*Δ suppresses *drs2*Δ hypersensitivity to nystatin. The same strains were incubated in liquid rich medium with the indicated concentration of nystatin for 36 hrs and the percent growth (OD_{600}) relative to samples without drug is shown. The error bar indicates standard deviation of three independent experiments. (C) *kes1*Δ suppresses the synthetic lethality of *drs2*Δ with *erg6*Δ. The indicated strains are BY4742 derivatives carrying *pURA3-DRS2* (PRS416-DRS2) spotted on minimal media with or without 5FOA and incubated at 30C. (D) *kes1*Δ suppresses *drs2*Δ defects in ergosterol localization. The same strains as in (A) were grown to mid-log phase and stained with filipin as described under material and methods.

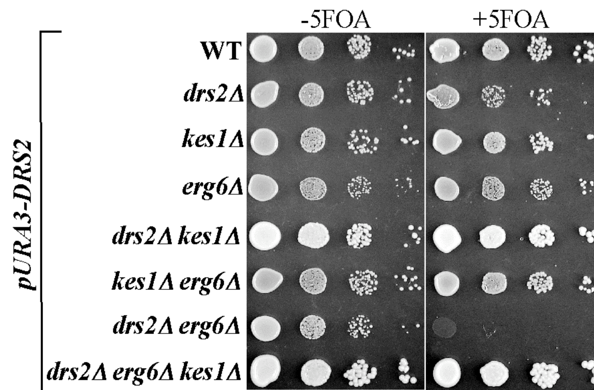
A



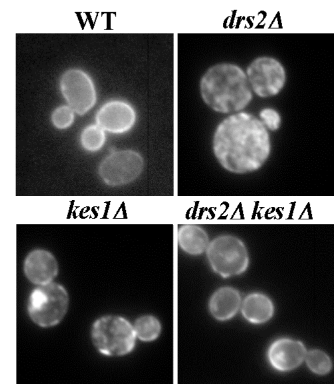
B



C



D



To determine if *drs2Δ* perturbs ergosterol subcellular distribution, we stained cells with filipin, a fluorescent polyene antifungal compound that also binds ergosterol. Filipin primarily stained the plasma membrane of WT cells (Fig 2-5D), where most cellular ergosterol is localized. In contrast, substantial intracellular filipin staining was observed with *drs2Δ* cells (Fig 2-5D). *kes1Δ* cells also exhibited more intracellular filipin staining than WT cells. However filipin primarily stained the plasma membrane of *drs2Δ kes1Δ* cells (Fig 2-5D). In this case, we observed co-suppression because the *drs2Δ kes1Δ* double mutant appeared more similar to WT than either single mutant.

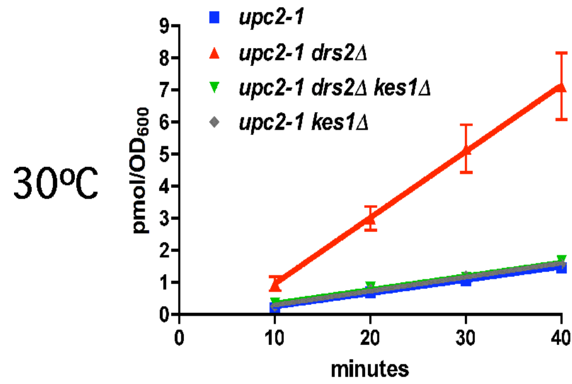
Previous studies indicated that inactivation of Kes1p/Osh4p perturbs transport of sterols from the plasma membrane to the endoplasmic reticulum (ER) (Raychaudhuri *et al.*, 2006). The toxicity of Kes1p to *drs2*Δ and the enhanced intracellular filipin staining in this mutant suggested the possibility that Kes1p is hyperactive in transporting sterol from the *drs2*Δ plasma membrane to internal organelles. To test this hypothesis, we compared the rate of esterification for exogenously supplied radiolabeled cholesterol in WT, *drs2Δ*, *kes1Δ* and *drs2Δ kes1Δ* cells. Esterification of cholesterol requires its transport from the plasma membrane, where it is taken up, to the ER, where the acyl-coenzyme A: cholesterol acyltransferases (ACATs) are localized. *S. cerevisiae* will not normally take up exogenous sterol in aerobic conditions. Therefore, we introduced an altered allele of a transcription factor (*upc2-1*) into our strains to

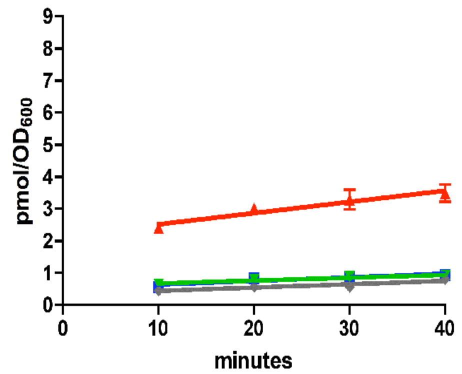
permit aerobic sterol uptake (Raychaudhuri *et al.*, 2006). After incubation with ^{14}C-cholesterol, the cells were harvested at the times indicated in Figure 2-6 and the amount of free cholesterol and cholesteryl ester were measured. The *drs2Δ* cells transported cholesterol from the plasma membrane to the ER five to six times faster than WT cells at 30C, and three times faster than WT at 20C (Fig 2-6, cholesteryl ester). The *drs2*Δ cells also took up significantly more cholesterol (free) than WT cells. In contrast, the *drs2*Δ *kes1*Δ cells took up cholesterol and transported it to the ER at rates indistinguishable from the WT cells. Therefore, Kes1p was responsible for the markedly enhanced rate of cholesterol transport in *drs2*Δ cells.

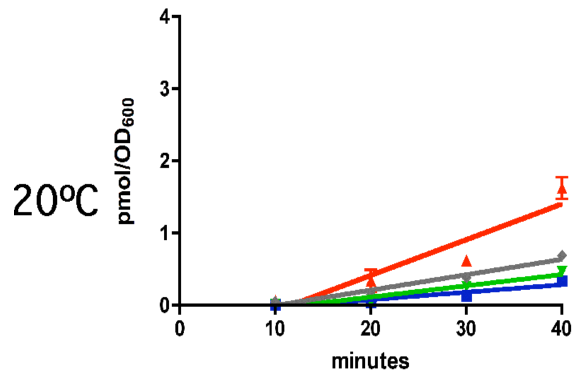
Fig 2-6. Enhanced rate of plasma membrane to ER sterol transport in *drs2*Δ cells is Kes1p-dependent. *upc2-1* derivatives of the strains described in Fig. 2-4A were grown at 30C and half of the cells were shifted to 20C for an hour and incubated with 2 μM ^{14}C-cholesterol. Cells were removed at the indicated times, lipids were extracted and the amount of cholesteryl ester and free cholesterol was quantified previously described (Li and Prinz, 2004). Error bars indicates standard deviation of two independent experiments.

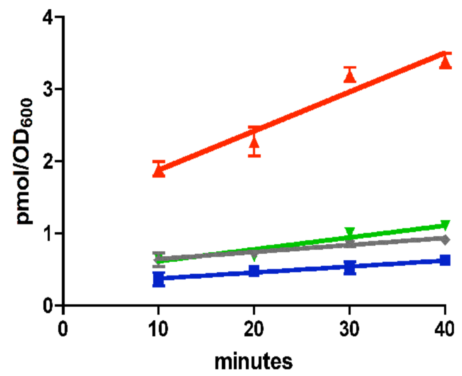
cholesteryl ester

free cholesterol









Phospholipid asymmetry of the plasma membrane is also perturbed in *drs2Δ* cells (Pomorski *et al.*, 2003; Chen *et al.*, 2006) and we tested if *kes1*Δ would suppress this defect. PS and PE, which are normally restricted to the inner, cytosolic leaflet, are aberrantly exposed on the outer leaflet of *drs2*Δ cells. This loss of asymmetry makes *drs2*Δ hypersensitive to papuamide B and Ro09-0198 (Ro), antifungal compounds that permeabilize cells exposing PS or PE, respectively. Disruption of membrane integrity can also make cells hypersensitive to membrane permeating agents, and so sensitivity to low concentrations of SDS was also examined to control for general effects of the mutations on membrane integrity. *kes1*Δ partially suppressed the SDS, papuamide B and Ro sensitivity of *drs2Δ,* but the double mutant remained significantly more sensitive to papuamide B and Ro than WT cells (Fig 2-7). We conclude that loss of Kes1p improves the plasma membrane integrity of *drs2*Δ cells, presumably by restoring sterol content, but does not restore the normal asymmetric distribution of PS and PE. Surprisingly, *kes1*Δ was also hypersensitive to papuamide B and Ro, yet resistant to SDS, indicating a partial loss of membrane asymmetry. This observation suggests that hyperactivity of P4-ATPases may also disrupt normal plasma membrane organization.

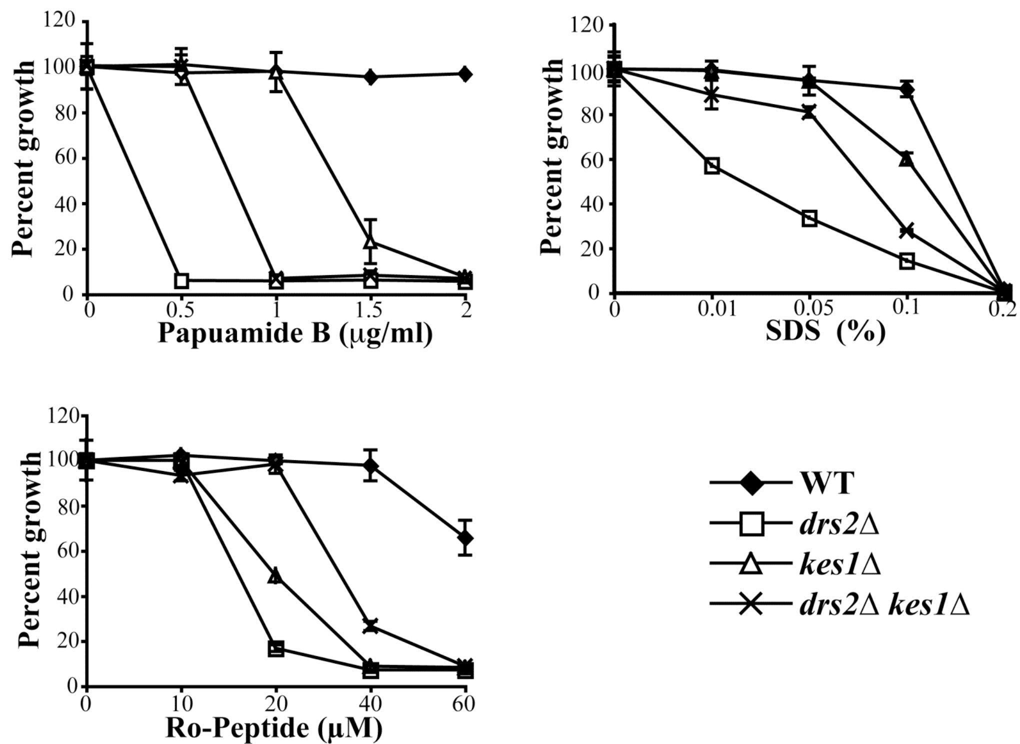


Fig 2-7. *kes1*Δ does not suppress *drs2*Δ loss of plasma membrane phospholipid asymmetry. WT, *drs2*Δ, *kes1*Δ and *drs2* Δ*kes1*Δ strains (same as in Fig. 2-4C) were subcultured to 0.1 OD600/ml in rich media with or without Papuamide B, Ro090198 (Ro peptide) or SDS and incubated at 30C for 36 hrs. Percent growth was determined as described in Fig. 2-5B. Error bar indicates standard deviation of three independent experiments.

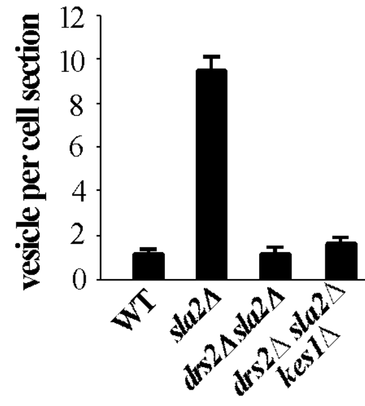
***kes1*Δ does not suppress *drs2*Δ defects in protein trafficking between the TGN, plasma membrane and early endosomes**

The suppression of *sec14-ts* secretion defects by *kes1*Δ suggested that the protein trafficking defects of *drs2*Δ would also be suppressed by *kes1*Δ. Therefore, we examined the ability of *kes1*Δ to suppress trafficking defects in several distinct pathways caused by *drs2*Δ. Loss of Drs2p function perturbs formation of one class of exocytic vesicles that rely on actin cables for efficient transport to the bud plasma membrane (Gall *et al.*, 2002). The *sla2*Δ mutation interferes with actin assembly and causes an accumulation of an average of 10 post-Golgi transport vesicles per cell section in electron micrographs. However, *sla2Δ drs2Δ* cells have only an average of 1 - 2 vesicles per cell section, comparable to WT cells, indicating that *drs2Δ* is epistatic to *sla2Δ* for the vesicle accumulation phenotype and is essential for budding these vesicles at 30C (Fig 2-8A and (Gall *et al.*, 2002). Surprisingly, *kes1*Δ did not suppress the *drs2*Δ defect in budding these post-Golgi exocytic vesicles as the *sla2Δ drs2Δ kes1Δ* cells contained about the same low number of vesicles as *sla2*Δ *drs2*Δ cells (Fig 2-8A).

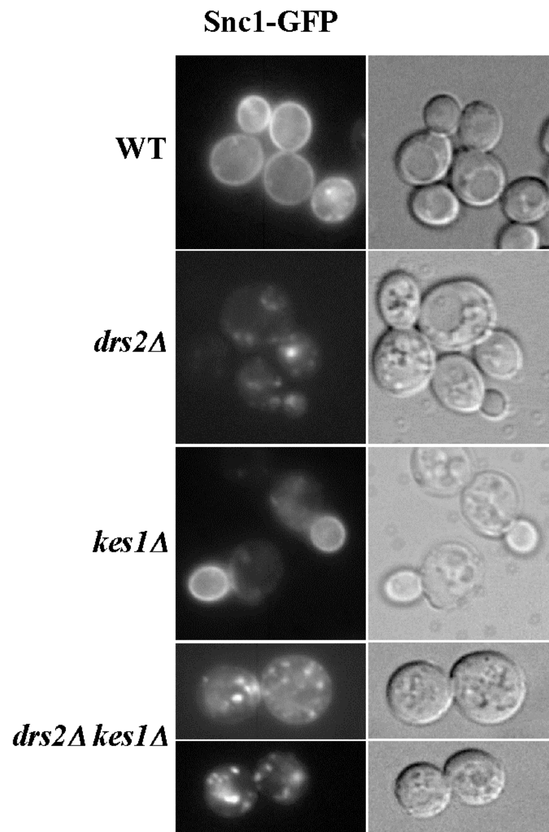
Fig 2-8. *kes1*Δ does not suppress *drs2*Δ defects in protein trafficking between the TGN, plasma membrane and early endosome.

(A) *kes1*Δ does not suppress the *drs2*Δ defect in budding post Golgi exocytic vesicles. WT (SEY6210), *sla2*Δ (TGY1912), *drs2*Δ *sla2*Δ (CCY642) and *drs2*Δ *sla2*Δ *kes1*Δ (BMY15) strains were grown to mid-log phase in rich media at 30C and processed for electron microscopy as previously described. 80-100 nm vesicles were counted in 50 sections for each strain. Error bars are standard deviation of two independent experiments (n=2). (B) *kes1*Δ does not suppress the Snc1p recycling defect of *drs2*Δ cells. WT, *drs2*Δ, *kes1*Δ and *drs2*Δ *kes1*Δ cells (same strains as Fig 2-6C) expressing GFP-Snc1p were grown to mid-log phase at 30C and viewed by fluorescence microscopy.

A

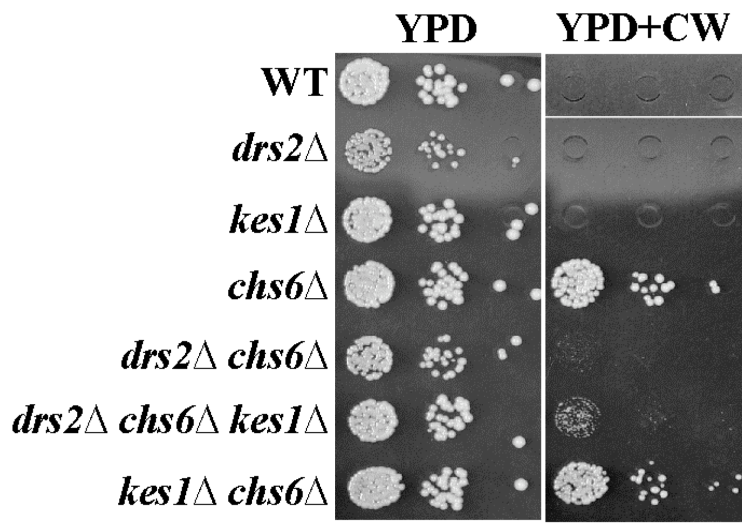


B



Loss of Drs2p function also perturbs trafficking of Snc1p, an exocytic SNARE, which normally cycles in a TGN → plasma membrane → early endosome → TGN loop. In wild-type cells, Snc1-GFP primarily localized to the bud plasma membrane whereas in *drs2Δ*, Snc1-GFP was mislocalized to internal punctate structures (Fig. 2-8B and (Hua *et al.*, 2002), reflecting a defect in the early endosome to TGN transport step (Saito *et al.*, 2004). We found no difference in the localization of Snc1-GFP to intracellular punctate structures in *drs2Δ* and *drs2Δ kes1Δ* cells **(**Fig. 2-8B). We also found no evidence for *kes1*Δ suppression of the *drs2*Δ defect in AP-1-dependent trafficking of chitin synthase between the TGN and early endosome (Fig 2-9).

Fig 2-9. *kes1*Δ does not suppress the *drs2*Δ defect in AP-1/clathrin-dependent trafficking of chitin synthase III (Chs3p) between the TGN and early endosomes. Chs3p is removed from the TGN-endosomal system and exported to the bud plasma membrane by the Chs5p and Chs6p coat complex. Thus, *chs6*Δ cells retain nearly all Chs3p intracellularly, have reduced chitin incorporation at the bud site, and are resistant to the chitin-binding compound calcofluor white (CW). In the *chs6*Δ background, deletion of AP-1 subunit genes or *DRS2* prevents intracellular Chs3p retention and allows cell surface transport of Chs3p in constitutive exocytic vesicles, restoring calcofluor white sensitivity to *chs6*Δ *drs2*Δ cells. Tenfold serial dilutions of WT (BY4742), *drs2*Δ (ZHY615M2D), *drs2*Δ *kes1*Δ (BMY01), *kes1*Δ (BMY02), *chs6*Δ (BY4742 YJL099W), *drs2*Δ *chs6*Δ (KLY023), *drs2*Δ *chs6*Δ *kes1*Δ (BMY38a) and *chs6*Δ *kes1*Δ (BMY37a) strains were spotted onto rich medium (YPD) with or without 100 µg/ml calcofluor white and incubated at 30°C. As expected, growth of WT, *drs2*Δ, and *kes1*Δ cells was inhibited by calcofluor white, but the *chs6*Δ strain was resistant. Sensitivity to calcofluor white was restored for the *chs6*Δ *drs2*Δ cells, while the *chs6*Δ *kes1*Δ cells remained resistant. Again, we failed to see significant suppression of *drs2*Δ by *kes1*Δ, as the *drs2*Δ *kes1*Δ *chs6*Δ cells grew only slightly better than *drs2*Δ *chs6*Δ cells in the presence of calcofluor white.


YPD
YPD+CW
WT
drs2Δ
kes1Δ
chs6Δ
drs2Δ chs6Δ
drs2Δ chs6Δ kes1Δ
kes1Δ chs6Δ

***kes1Δ* suppresses *drs2Δ* defects in TGN to vacuole protein transport**

The protein trafficking steps examined above are disrupted at high temperatures (30C – 37C) as well as low temperatures in *drs2*Δ cells. In contrast, transport of carboxypeptidase Y (CPY) from the Golgi to the vacuole is kinetically delayed in *drs2*Δ cells at low temperatures, but is normal at 30C (Chen *et al.*, 1999). We tested if *kes1*Δ would suppress this cold-sensitive defect in CPY transport kinetics using pulse-chase analyses. CPY is initially synthesized in the ER as a 67 kDa p1 precursor form that is further modified in the Golgi to the 69 kDa p2 precursor. p2 CPY is then sorted from secretory cargo at the TGN, transported to the late endosome and on to the vacuole where it is processed to the 61 kDa mature form (mCPY)(Stevens *et al.*, 1982). WT, *drs2Δ, kes1Δ* and *drs2Δ kes1Δ* strains were labeled with ^{35}S-amino acids and chased at 30C and 15C for the times indicated in Figure 7A. At 30C, no significant difference was found for CPY maturation kinetics in these four strains. In contrast, *drs2Δ* cells exhibited a 3 fold kinetic delay in CPY transport at 15C relative to WT and *kes1*Δ cells (Fig. 2-10A). This cold-sensitive CPY transport defect was completely suppressed in the *drs2*Δ *kes1*Δ cells.

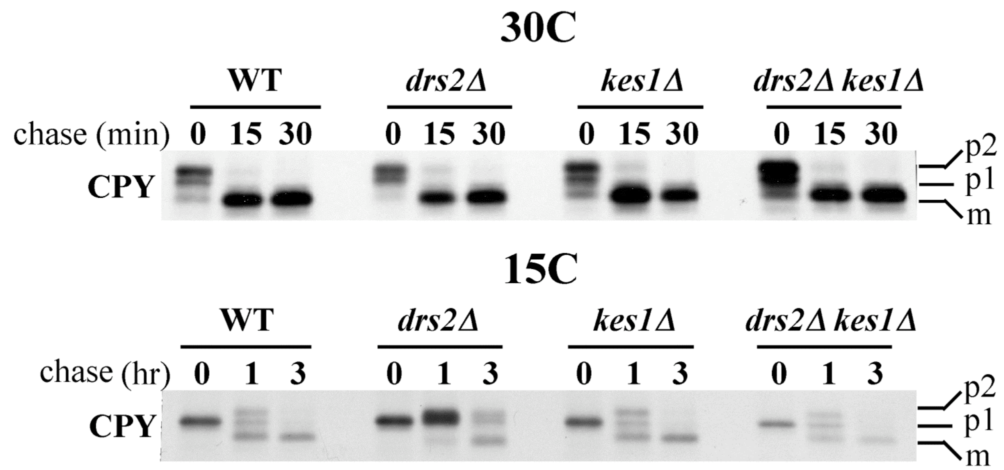
In addition, p2 CPY was not fully formed in *drs2*Δ cells at 15C (p1 and p2 CPY were not completely resolved by SDS-PAGE), indicating a partial defect in a terminal mannosylation event catalyzed within late Golgi compartments (Chen

et al., 1999). This cold-sensitive glycosylation defect was also completely suppressed by *kes1*Δ. We also examined the transport kinetics of carboxypeptidase S (CPS), another vacuolar protein that appears to follow the same route to the vacuole as CPY (Costaguta *et al.*, 2001). The *drs2*Δ cells also showed a cs, three-fold kinetic defect in the transport of CPS to the vacuole that was suppressed by *kes1*Δ (data not shown).

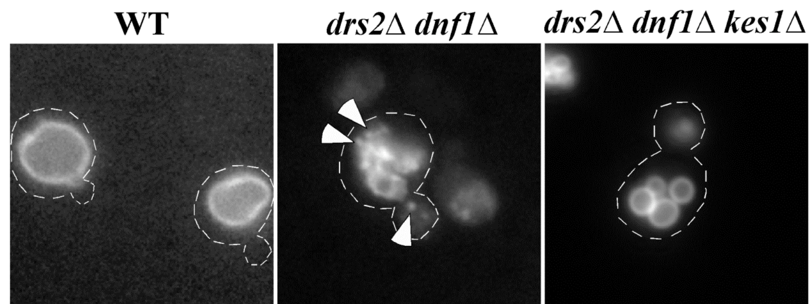
Fig 2-10. *kes1*Δ suppresses *drs2*Δ defects in TGN to vacuole protein trafficking.

(A) *kes1*Δ suppresses a *drs2*Δ cs defect in the kinetics of CPY transport to the vacuole. WT, *drs2*Δ*, kes1*Δ and *drs2*Δ *kes1*Δ cells were grown to mid-log phase in minimal medium at 30°C, half of the culture was shifted to 15C for 15 min and the cells were labeled with [^{35}S]methione/cysteine for 10 min. Cells were chased with cold methionine/cysteine for the indicated times. CPY was recovered from each sample by immunoprecipitation and subjected to SDS-PAGE and autoradiography. (B) *kes1*Δ suppresses *drs2*Δ *dnf1*Δ defects in ALP localization. WT (BY4742) *drs2*Δ *dnf1*Δ (ZHY2149D) and *drs2*Δ *dnf1*Δ *kes1*Δ (BMY03) cells harboring pGO41 (GFP-ALP) were grown at 30°C to mid-log phase and examined by fluorescence microscopy. (C) Steady-state distribution of CPY and ALP precursor and mature forms. WT, *drs2*Δ *dnf1*Δ*, drs2*Δ *dnf1*Δ *kes1*Δ (same strains as in (B)), *gga1*Δ *gga2*Δ (KLY691) and *gga1*Δ *gga2*Δ *kes1*Δ (BMY40a) cells were grown in rich media at 30°C to mid-log phase. Whole cell lysates were prepared and immunoblotted to detect precursor (van Meer *et al.*) and mature (m) forms of CPY and ALP.

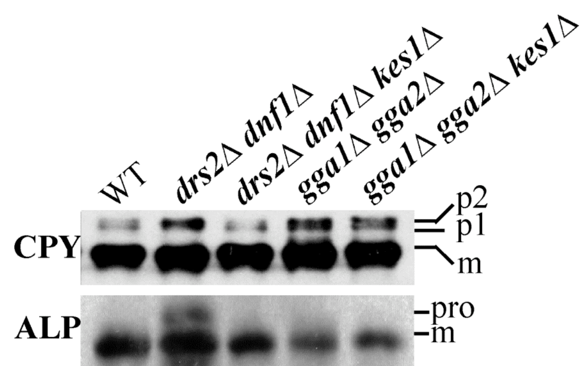
A



B



C



Drs2p and Dnf P4-ATPases have a redundant function in the transport of CPY and alkaline phosphatase (Alphey *et al.*) to the vacuole (Hua *et al.*, 2002). In contrast to CPY and CPS, ALP appears to be transported directly from the Golgi to the vacuole without passing through an endosomal intermediate. ALP vacuolar transport is weakly perturbed by *drs2*Δ, but a strong defect is observed in *drs2*Δ *dnf1*Δ double mutants. To test for *kes1*Δ suppression of this transport defect, we examined the localization of ALP-GFP in WT, *drs2*Δ *dnf1*Δ and *drs2*Δ *dnf1*Δ *kes1*Δ cells. ALP-GFP was mislocalized to punctate structures outside the vacuolar membrane in *drs2Δ dnf1Δ* (Fig 2-10B, arrowheads). In contrast, ALP-GFP was properly targeted to the *drs2Δ dnf1Δ kes1Δ* vacuolar membrane (Fig 2-10B). The steady-state levels of CPY and ALP precursor forms, which are normally transient and hard to detect by western blot of WT cell lysates, were readily apparent in the *drs2Δ dnf1Δ* cells because of the trafficking defect. However, only the mature form of ALP and CPY were detected in *drs2Δ dnf1Δ kes1Δ* (Fig. 2-10C), indicating that *kes1*Δ suppressed the trafficking defect. The Gga1p and Gga2p clathrin adaptors are also implicated in the CPY and CPS transport pathway (Costaguta *et al.*, 2001). By comparison, *gga1*Δ *gga2*Δ showed a weaker accumulation of p2 CPY than *drs2*Δ *dnf1*Δ*,* which was not suppressed in a *gga1*Δ *gga2*Δ *kes1*Δ strain. Thus, *kes1*Δ cells retain their dependence on GGAs for CPY transport, but become less discriminate for the P4-ATPase requirement.

Discussion

Here we report evidence that P4-ATPases, in particular Drs2p, are critical downstream targets of the repressive effect of Kes1p on vesicle formation from late Golgi cisternae. Genetic data indicate that Kes1p is hyperactive in *drs2*Δ and inhibits growth of these cells at low temperature through inhibition of Dnf P4-ATPases at the Golgi and their function in TGN to late endosome/vacuole protein transport. Moreover, Kes1p regulates the phospholipid flippase activity of Drs2p in purified TGN membranes. Drs2p flippase activity is significantly enhanced in TGN membranes lacking Kes1p and remarkably, flippase activity is attenuated to wild-type levels by addition of pM concentrations of recombinant Kes1p.

In addition, Kes1p hyperactivity in *drs2*Δ cells causes a substantial increase in the flux of exogenously applied sterol from the plasma membrane to the ER and an alteration in the distribution of endogenous ergosterol. Elimination of Kes1p restored a wild-type rate of transport in spite of the presence of six other Osh proteins in the *drs2*Δ *kes1*Δ cells. Thus, Kes1p is the only oxysterol binding protein homolog that is hyperactive in *drs2*Δ cells. These data support the proposed sterol transport function for Kes1p (Raychaudhuri *et al.*, 2006), although we cannot rule out the possibility that Kes1p regulates the activity of other proteins that directly mediate nonvesicular sterol transport. We conclude that mutual repression between Kes1p and Drs2p provides a critical homeostatic mechanism for controlling the intracellular trafficking of both protein and sterol.

Drs2p plays a critical role in vesicle budding from the TGN and early endosomes by a mechanism that is independent of coat recruitment. We previously proposed that the physical displacement of phospholipid from the lumenal to cytosolic leaflet by Drs2p induces curvature in the membrane that is captured and molded by coat proteins into vesicles (Chen *et al.*, 1999; Graham, 2004; Liu *et al.*, 2008a). It is logical that an appropriately balanced and coordinated activity between Drs2p and the vesicle budding machinery would be essential for the orderly segregation of protein and lipids into different transport pathways. Kes1p is well suited to regulate the activity of P4-ATPases in generating membrane curvature because it contains an ArfGAP lipid packing sensor (ALPS) domain that binds highly curved membranes (Drin *et al.*, 2007). Here we propose that Drs2p imparts a degree of curvature to the TGN membrane that has an ideal set point for vesicle formation. As Drs2p flippase activity drives membrane curvature beyond this set point, Kes1p would sense the stress on the membrane and inhibit Drs2p activity until the membrane relaxes and inhibition is relieved. This model is consistent with the observed mode of Kes1p inhibition of Drs2p flippase activity: Kes1p potently prevents hyperactivity of Drs2p but will not inhibit Drs2p activity below a basal level, even at very high concentrations of Kes1p.

We also propose that the influence of Drs2p on membrane curvature (or lateral tension) and phospholipid asymmetry at the TGN has an ideal set point for

establishing sterol rich raft-like membrane structures for export to the plasma membrane. The membrane structure at this set point would restrict the ability of sterol transfer proteins to extract ergosterol from the membrane. The set point model would explain why *drs2*Δ (reduced TGN flippase activity) and *kes1*Δ (enhanced TGN flippase activity) single mutants both display reduced filipin staining of the plasma membrane while the double mutant appears more similar to wild-type cells. We also suggest that hyperactivity of Kes1p in *drs2*Δ is a function of membrane disorganization and the escape tendency of sterol from the membrane. Sterol biosynthetic intermediates are known to have a lower affinity for raft lipids and a higher escape tendency (Li and Prinz, 2004; Lange and Steck, 2008). *drs2*Δ is synthetically lethal with *erg* mutations that accumulate late biosynthetic intermediates to ergosterol (Kishimoto *et al.*, 2005) and strikingly, this synthetic lethality is completely suppressed by deletion of *KES1*. We suggest that the *drs2*Δ and *erg* perturbations additively increase occupancy of Kes1p with sterol and increase its ability to inhibit the remaining P4-ATPases at the Golgi. It does not appear to be the asymmetric distribution of PS and PE to the inner leaflet that is the critical structural feature of the plasma membrane needed to retain ergosterol. *dnf1,2,3*Δ cells exhibit a similar loss of PE and PS asymmetry as *drs2*Δ, and yet the plasma membrane of these cells stains normally with filipin (data not shown). This suggests a unique and undefined influence of Drs2p on membrane organization that specifically restricts Kes1p activity.

The role of Drs2-related P4-ATPases in establishing a membrane structure that resists sterol extraction appears to be conserved through evolution and is medically relevant. The human disease progressive familial intrahepatic cholestasis type I is caused by mutations in the Atp8b1 P4-ATPase (also called FIC1), which has 41% amino acid sequence identity to Drs2p. Defects in the flux of bile through the liver in these patients leads to liver damage that is fatal unless a transplant can be obtained (Bull *et al.*, 1998). Normally, cholesterol excretion into bile requires the action of the Abcg5/8 transporter, but surprisingly, mice deficient for both Atp8b1 and Abcg8 excrete wild-type levels of cholesterol into bile (Groen *et al.*, 2008). This observation suggests that the disorganized structure of the Atp8b1$^{(-/-)}$ bile canalicular membrane fails to retain cholesterol leading to its nonspecific extraction into the bile.

The requirement of Drs2p for growth at low temperature is also conserved. Depletion of *ALA1*, an Arabidopsis homologue of *DRS2*, causes a cs growth phenotype of the plant that is analogous to the cs growth of *drs2*Δ cells (Gomes *et al.*, 2000). It is reasonable to propose that this could be due to conservation of the feedback regulatory mechanism between plant P4-ATPases and oxysterol binding protein homologues. We previously suggested that the inability of *drs2*Δ cells to grow below 23C was due to a failure of the Dnf P4-ATPases to support an essential Drs2p function at colder temperatures (Hua *et al.*, 2002). The current studies support this hypothesis because suppression of *drs2*Δ growth defects by

*kes1*Δ requires the presence of the Dnf P4-ATPases. Importantly, *kes1*Δ cannot suppress *drs2*Δ defects in protein transport pathways where Dnf P4-ATPases cannot compensate for loss of Drs2p. In contrast, *drs2*Δ defects in protein transport from the TGN to the vacuole, pathways supported by either Drs2p or Dnf P4-ATPases (Hua *et al.*, 2002), are suppressed by *kes1*Δ.

The ability of *kes1*Δ to restore growth of *drs2*Δ cells at low temperature correlates best with the restoration of a wild-type rate of protein transport in the TGN → late endosome → vacuole route, a GGA/clathrin pathway. The GGA and AP-1 clathrin adaptors appear to mediate parallel transport pathways emanating from the TGN and simultaneous loss of both pathways severely abrogates growth (Costaguta *et al.*, 2001). We had previously shown that *drs2*Δ cells exhibit a temperature-independent loss of the AP-1 pathway and that *drs2*Δ *gga1*Δ *gga2*Δ triple mutants are severely compromised for growth (Liu *et al.*, 2008a). Here we propose that the cold-sensitive defect in growth of *drs2*Δ cells is caused by a temperature-conditional defect in the GGA/clathrin pathway elicited only at low temperatures, combined with a constitutive defect in AP-1/clathrin function. It is possible that a TGN membrane phase transition to a more liquid-ordered state combined with the repressive effect of Kes1p underlies the cs growth defect of *drs2*Δ.

The half-maximal inhibitory effect of Kes1p on Drs2p was attained at a stoichiometry of approximately 1 to 10 Kes1p molecules per 1000 Drs2p molecules. A 1000-fold increase in Kes1p concentration had no further inhibitory effect on flippase activity. Thus, the inhibitory influence of Kes1p on Drs2 activity is unlikely to be mediated by a direct protein-protein interaction between Kes1p and Drs2p. It also seems unlikely that these low levels of Kes1p could effectively compete with other effectors for PI4P binding. The potency of the Kes1p attenuation of flippase activity suggests it is acting through an enzymatic intermediate, perhaps comparable to the signal transducing function of oxysterol binding protein (OSBP), a mammalian homolog of Kes1p. When bound to cholesterol, OSBP forms a complex with protein phosphatase 2A and a tyrosine phosphatase that attenuates signaling through the ERK pathway. In response to 25-hydroxycholesterol binding, the OSBP phosphatase complex dissociates and OSBP translocates onto the Golgi (Wang *et al.*, 2005). Thus, it is possible that Kes1p attenuates Drs2p activity by regulating protein phosphorylation at the TGN. Alternatively, Kes1p may regulate the activity of PI 4-kinases or 4-phosphatases that could secondarily regulate flippase activity. Deletion of *KES1* increases PI4P levels or availability at the TGN and suppresses growth defects caused by *pik1-ts* (Fairn *et al.*, 2007). The *in vitro* assay for Drs2p activity in purified TGN membranes should be amenable to testing these potential regulatory

mechanisms of Kes1p. Regardless of the mode of regulation, P4-ATPases appear to be critical downstream targets for the repressive effect of Kes1p on vesicle formation from the TGN.

CHAPTER III

SUMMARY

Drs2 is a P-type ATPase and a phospholipid translocase that translocates phosphatidylserine (PS) and to a lesser extent phosphatidylethanalamine (PE) across the TGN (Natarajan *et al.*, 2004) and post-Golgi secretory vesicle (Alder-Baerens *et al.*, 2006) membranes. *drs2*Δ exhibits defects in phospholipid translocation, protein trafficking and cold tolerance (Chen *et al.*, 1999; Gall *et al.*, 2002; Hua *et al.*, 2002; Liu *et al.*, 2008b). Moreover, Drs2 displays extensive genetic and physical interactions with the protein trafficking machinery functioning in transport between the TGN, plasma membrane, endosomes and vacuole (Fig 3-1). Although our lab has extensively studied the role of Drs2 in phospholipid translocation and protein trafficking, the essential function of Drs2 at low temperature was still unknown. Similarly, depletion of Ala1 (*ALA1* is homologue of *DRS2*) also caused chilling sensitivity in *Arabidopsis*, but the mechanism by which lipid translocation might relate to chilling tolerance was not investigated (Gome` s et al., 2000). *ALA1* complements the cold-sensitivity of *drs2*Δ in yeast, suggesting that the Drs2 requirement for growth at low temperature is conserved from yeast to more complex eukaryotes. My research focused on providing a understanding of why Drs2 is essential at low temperature.

In this study, I performed a bypass suppressor screen to search for extragenic suppressors that would allow growth of *drs2*Δ cells at low temperature. We hypothesized that identification of suppressors of the cold-sensitivity (cs) growth defect of Drs2p would provide insight into the essential function of Drs2 at low temperatures. This screen identified a number of spontaneous recessive suppressor mutations that fell into two complementation groups that I named *SDK1* and *SDK2*. A few dominant suppressor mutations were also recovered.

Cloning of SDK

Cloning of *SDK2* was not successful because of the high rate of spontaneous reversion of the *drs2*Δ*sdk2* suppressor phenotype. To try to resolve this problem, an undergraduate student Rohini Khatri generated a *drs2*Δ*sdk2/drs2*Δ*sdk2* homozygous diploid, which reduced the rate of spontaneous phenotypic reversion significantly. However, a 20% rate of reversion was still observed in the homozygous diploid and so it was not possible to clone *SDK2* by complementation with this strain. Jason Wen also crossed the *sdk2* mutation into the strongly cold-sensitive *drs2*Δ*dnf1*Δ in order to reduce the reversion frequency, but the *sdk2* mutation was not strong enough to suppress the cs growth defect in the *drs2*Δ*dnf1*Δ*sdk2* background. Moreover, I moved the *sdk2* mutation to another strain background (from SEY6211 to SEY6210) that was used for *SDK1* cloning, but was still unsuccessful in cloning *SDK2*. The only possible way to clone *SDK2* is to genetically map the location of *SDK2* using

markers for specific chromosomes. We had hoped that identifying the *sdk2* gene would enlighten our understanding of how *kes1* suppresses the cold-sensitive growth defect of *drs2*Δ. Identifying *SDK2* would allow us to characterize the mechanisms of suppression for *sdk2* as we have done for *sdk1/kes1*.

Based on tetrad analyses, the dominant suppressor mutations are single gene mutations. To identify the mutated dominant gene, we would have to generate a genomic library from these dominant mutants. This library would be transformed into *drs2*Δ*,* screened for clones that show cold resistance, and then the genomic insert would be identified through sequencing. In yeast, dominant mutants are less attractive because of the cumbersome work required to analyse them. The genetic relationship of the dominant mutations and the *SDK* genes was not defined in this study.

SDK1 is allelic to *KES1* (KrE11-1 Suppressor)*,* an oxysterol binding protein proposed to be a negative regulator of vesicle-mediated protein trafficking from TGN. Mutations in *kes1* suppress the TGN vesicle budding defect of *sec14*^{ts}. Mutations in genes lying in the Kennedy pathway for phosphatidylcholine biosynthesis also suppress the *sec14*^{ts} growth and secretion defects. This finding led to the discovery that Sec14p downregulates PC synthesis through the Kennedy pathway, and the proposal that either excess PC, or a decrease in diacylglycerol (or phosphatidic acid) levels disrupts vesicle budding from the TGN (Fang *et al.*,

1996). However, a Kennedy pathway mutation could not suppress the cs growth defect of *drs2*Δ. Therefore, the cs growth defect of *drs2*Δ is unlikely to be caused by enhanced phosphatidylcholine synthesis. Similarly, the mechanism for *kes1* suppression of *sec14*^{ts} appears to be independent of PC synthesis.

Loss of function *KES1* alleles were also recovered in another screen for suppressors of *kre11* (Jiang *et al.*, 1994). Kre11/Trs65 is a part of the ten-subunit TRAPPII complex that serve as nucleotide exchange factor for Golgi Rab proteins that serve as nucleotide exchange Ypt1, Ypt31p and Ypt32p. Ypt31/32 regulates vesicle budding from the TGN and early endosomes (Morozova *et al.*, 2006; Furuta *et al.*, 2007). Rcy1 is an effector of Ypt31/32 at the early endosome and Rcy1 interacts with the Cdc50-Drs2 complex. It also appears that Cdc50-Drs2 is recycled to the TGN in a pathway that requires Rcy1 and Drs2 function. The functional significance of the interaction between Rcy1 and Cdc50-Drs2 is unknown. It is possible that Rcy1 is positively regulating Drs2 activity, thus placing Drs2 as a downstream effector of Ypt31/32 and Rcy1. If this is the case, *kes1*Δ may suppress the TRAPPII defects by increasing Drs2 activity at the early endosome.

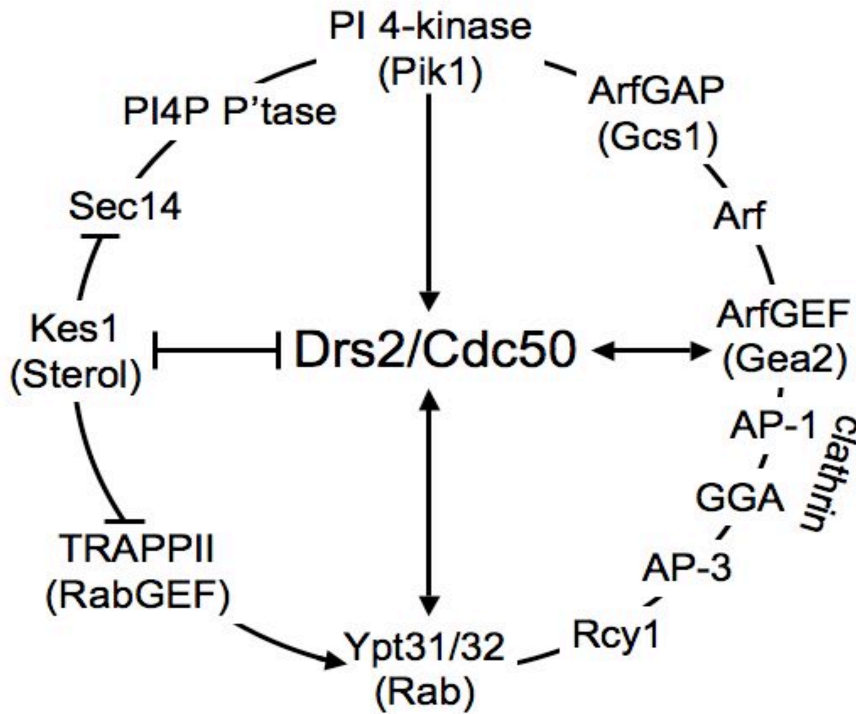


Fig 3-1. Physical and Genetic interaction of Drs2 with traffic machinery proteins. Upstream effectors of Drs2 may include PI4P (kinase and phosphatase), Kes1, TRAPPII and Ypt31/32. Among upstream effectors, PI4P, TRAPPII and Ypt31/32 influence the Drs2 positively for vesicle biogenesis from TGN whereas Kes1 influences protein trafficking negatively. The downstream effectors of Drs2 are the coat protein clathrin, adaptor and accessory proteins including Arf, Arf GAP and ArfGEF.

Kes1 acts upstream to Drs2 in protein trafficking

The ARFGAP Gcs1 and its activity is required for *kes1*-mediated bypass of Sec14p (Li *et al.*, 2002). Mutations in *arf1* and *gcs1* both abrogate *kes1*-mediated bypass *sec14*^{ts} suggesting that mutated Kes1p enhances ArfGAP function, whereas elevated function of Kes1p would reduce ArfGAP function. However, Kes1 did not inhibit the ArfGAP activity of Gcs1 in vitro (Li *et al.*, 2002). *DRS2* and *CDC50* mutant alleles are synthetically lethal with *arf, gcs1* and ArfGEF mutations. In addition, the ArfGEFs Gea1 and Gea2 bind directly to the C-terminal tail of Drs2 and stimulate the flippase activity of Drs2 (Natarajan *et al.*, 2009). These data argue that Drs2 and Sec14 execute their roles in vesicle biogenesis in pathways that converge on Arf function. There is a possibility that the negative role of Kes1 in vesicle budding from TGN functions downstream of Arf, Drs2 and Sec14. However, the observation that Drs2 flippase activity is hyperactive in *kes1*Δ TGN membranes, and repressed in vitro by addition of recombinant Kes1p, argues that Kes1 acts upstream of Drs2.

PI4P in kes1Δ

Phosphatidylinositol-4-phosphate (PI4P) also plays a critical role in vesicle budding from the TGN (Walch-Solimena and Novick, 1999). PI4P is generated at the TGN by the PI 4-kinase Pik1 and is degraded by the Sac1, Inp51, Inp52 and Inp53 phosphatases. Kes1 binds PI4P and is mislocalized from Golgi membranes in *sac1* mutants due to accumulation of PI4P in unusual (non-TGN)

compartments (Li *et al.*, 2002). Moreover, deletion of *KES1* suppresses the growth defect of *pik1-ts* suggesting Kes1 may inhibit the PI 4-kinase or activate the PI 4-phosphatase. Sec14 is also proposed to stimulate PI4P synthesis. The downstream effectors of PI4P in yeast have been mysterious, although the GGA adaptors appear to require PI4P for TGN recruitment. However, a synthetic lethal genetic interaction between *drs2* and *pik1-ts* (Sciorra *et al.*, 2005) suggested a potential biochemical relationship between PI4P, Kes1p and flippase activity at the TGN. Recently, our lab has found that Drs2 flippase activity in the TGN requires PI4P synthesized by Pik1p. The carboxyl-terminal tail of Drs2p contains a regulatory domain that binds phosphoinositides and overlaps with the site that binds the ArfGEF Gea2p. These Pik1 and Gea2 interactions with the C-terminal tail stimulate flippase activity and are required for Drs2p function *in vivo* (Natarajan *et al.*, 2009). Further, deletion of *KES1* is reported to increase PI4P levels or availability at the TGN (Fairn *et al.*, 2007). Preliminary data suggests that there may be an increased level of PI4P production in TGN membranes from *kes1*Δ cells incubated with γ^{32}P ATP than those of wild-type (Fig 3-2). These data need to be confirmed by better quantitative methods like mass spectrometry or HPLC. The above findings strengthen the view that Kes1 repression of Drs2 is mediated through reduction of PI4P levels at the TGN.

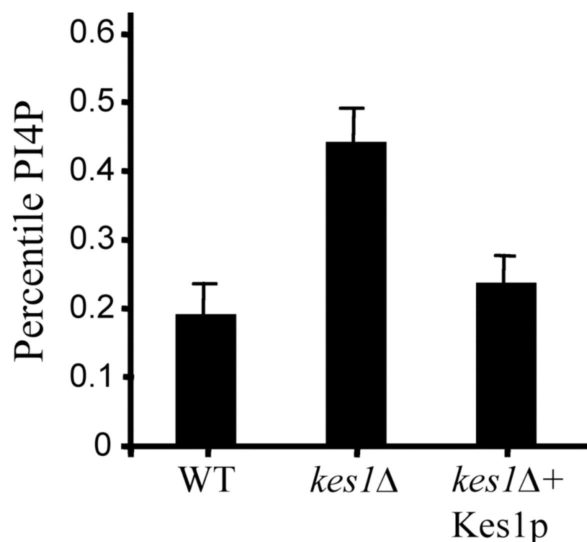


Fig 3-2. PI4P production in WT and *kes1*Δ.

TGN membranes (0.5mg protein/ml) from WT and *kes1*Δ were incubated at 37°C for 5 minutes in 75 μl of kinase assay buffer (20mM HEPES, pH7.5, 10mM $MgCl_2$, 100μM ATP and 2μCi [γ-^{32}P] ATP). Samples were incubated with (γ-^{32}P) ATP for 10 minutes at 35°C before phosphoinositides were extracted from the membranes and separated on TLC plates using the borate system (Walsh *et al.*, 1991). Error bar indicates standard deviation of two independent experiments.

Even though the negative regulation of Kes1 in vesicle budding from TGN has been studied in an extensive manner, a positive role of Kes1 in raft-mediated proteins transport out of the TGN has also been suggested (Reiner *et al.*, 2006). It could be possible that the positive role merges with sterol extraction or loading, or the curvature sensor domain function of Kes1p.

Removal of Kes1 may improve the activity of Dnfs at the TGN

Although Drs2 acts in several protein trafficking pathways from TGN (Fig 3-3), *kes1*Δ cannot suppress all protein trafficking defects of *drs2*Δ cells. Among the P4-ATPases, Drs2 is alone required to form dense exocytic vesicles in a TGN to PM pathway (Gall *et al.*, 2002), and also vesicle formation in AP-1/Rcy1 pathways between TGN and early endosomes (Liu *et al.*, 2008b). Even with Kes1 absent, these pathways are still nonfunctional in *drs2*Δ cells. We previously hypothesized that Dnfs can compensate for the loss of Drs2 at higher temperature but not at low temperature (Hua *et al.*, 2002). Moreover, *kes1*Δ requires Dnfs to suppress the growth defects of *drs2*Δ, and *kes1*Δ can only suppress the protein transport defects in pathways that are carried out by both Dnfs and Drs2. Drs2/ Dnfs contribute additively for protein trafficking from TGN to late endosome and vacuole, and my data suggests that *kes1*Δ improves Dnf activity in these pathways. From my findings, we proposed that the cold-sensitive growth defect of *drs2*Δ is caused by a defect in GGA/clathrin pathway at low temperatures, combined with a constitutive defect in the AP-1/clathrin pathway. Loss of Kes1p

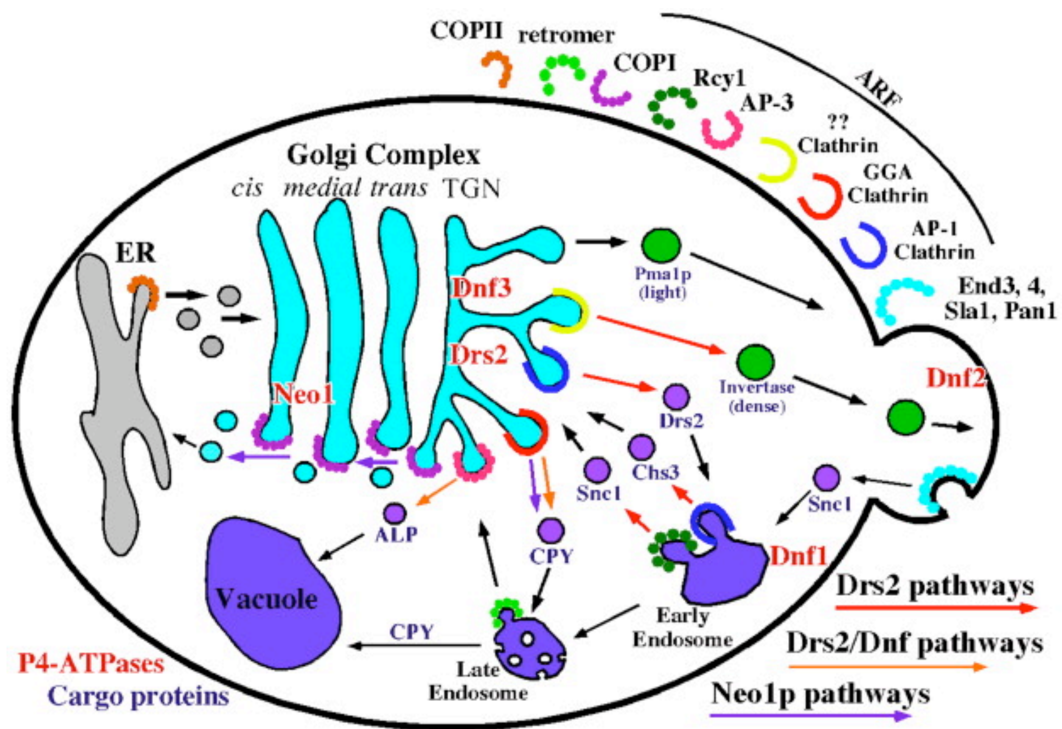


Fig. 3-3 P4-ATPase requirements for vesicle-mediated protein transport in the secretory and endocytic pathways. Transport pathways are defined by cargo protein (purple) traveling the route and the vesicle coat protein (labeled in the upper right quadrant) required for sorting and transport of the cargo. The coat requirement for early endosome to TGN recycling pathway traveled by Snc1 is indicated by Rcy1, although it is not known if Rcy1 is a vesicle coat constituent. Pathways with known P4-ATPase requirements are indicated with colored arrows.

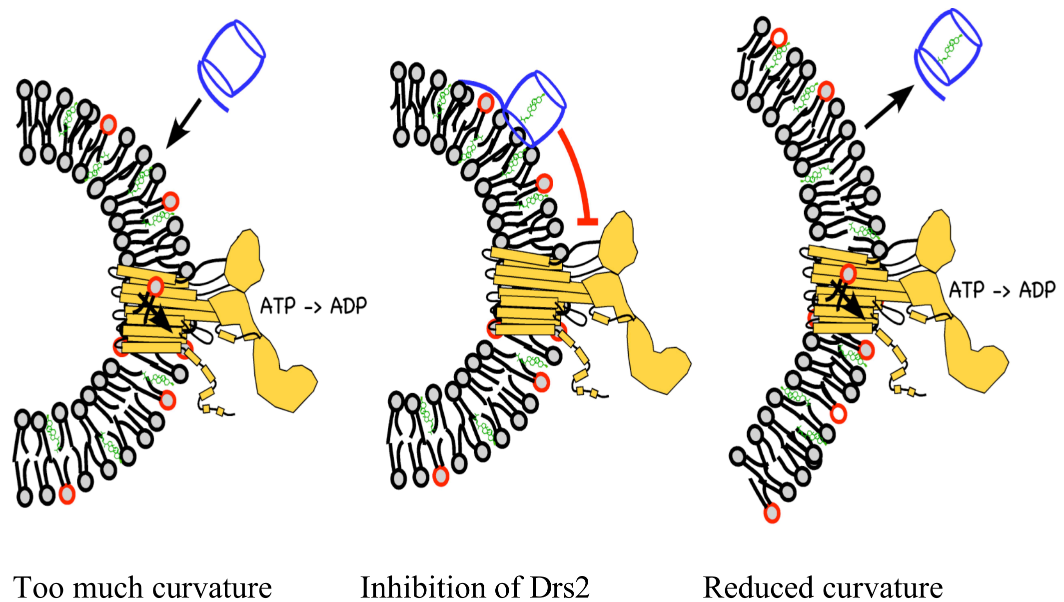
appears to improve the ability of Dnf proteins to support the GGA/clathrin pathway in the absence of Drs2.

Kes1 may influence the sterol content of the TGN

Kes1 appears to be hyperactive in *drs2*Δ cells, which may lead to more ergosterol in TGN membranes in *drs2*Δ cells relative to WT. We would also expect that deletion of *kes1*Δ would lead to normal levels of sterol in the *drs2*Δ*kes1*Δ TGN. To address this issue, we quantified the sterol content from WT and mutant membranes. The initial findings showed an increased ergosterol content in the *drs2*Δ TGN membranes relative to WT, and the predicted reduction in *drs2*Δ *kes1*Δ membranes (data not shown). However, a second set of membrane preps failed to show this difference. Further experiments are needed to determine whether or not the loss of Drs2 causes a Kes1-dependent increase in TGN sterol content. A higher ergosterol content along with reduced temperature could lead to a liquid ordered phase or rigid membrane that affects the performance of Dnfs at low temperature, thus perturbing the GGA pathway. We considered the possibility that the recombinant Kes1p was attenuating Drs2p activity by extracting ergosterol from the TGN membrane. However, the *kes1*Δ TGN membranes used for these assays contained 60 +/- 15 mg of ergosterol per mg of protein. At 0.1 ng of Kes1p, the Kes1p to ergosterol stoichiometry would be 1:10,000 and so it is unlikely that this concentration of Kes1p would significantly alter the ergosterol

content of the TGN membrane. Increasing membrane fluidity by growing *drs2*Δ cells on oleic acid failed to suppress the cs growth defect (data not shown), suggesting that the cs defect in the GGA pathway is not caused by a simple phase transition.

Drs2 flips phospholipid from the luminal to cytosolic leaflet of the TGN and we propose that this activity induces positive curvature in the membrane, which is captured and molded by coat proteins to bud vesicles (Chen *et al.*, 1999; Graham, 2004; Liu *et al.*, 2008a). To produce a proper curvature that would be essential for the orderly segregation of protein and lipids into different transport pathways, Kes1 may regulate the activity of P4-ATPases in generating membrane curvature by its ArfGAP lipid packing sensor (ALPS) domain that binds highly curved membranes (Drin *et al.*, 2007). When the Drs2 flippase activity drives membrane curvature beyond an ideal curvature, Kes1p would sense the curvature on the membrane and inhibit Drs2p activity until the attainment of an ideal curvature. This model is suited with the observed mode of Kes1p inhibition of Drs2p Flippase activity. Kes1 potently prevents hyperactivity of Drs2p but will not inhibit Drs2p activity below a basal level, even at very high concentrations of Kes1p (Fig 3-4).



Too much curvature Inhibition of Drs2 Reduced curvature

Fig 3-4 Potential role of Drs2 and Kes1 in estabilishing membrane curvature

Drs2 flips phospholipids from the lumenal leaflet to the cytosolic leaflet of TGN to create positive curvature on the cytosolic side of the membrane. This curvature would then be stabilized by coat, accessory and adaptor proteins to bud vesicles. As Drs2 flips continuously, a highly curved membrane would be produced. This may place too much tension on the membrane to accommodate enough sterol and raft associated proteins in the membrane. In order to allow appropriate curvature, Kes1 senses the level of curvature using its ALPS domain. When the specific degree of curvature is reached, then Kes1 would inhibit the flippase activity of Drs2 to allow proper vesicle budding.

The N-terminal amino acids 1 to 29 of Kes1p constitute the ALPS domain. When I introduced the *1-29Δkes1* into the WT and mutant strains, the truncated form of Kes1p appeared to be more toxic to *drs2*Δ cells than to the WT, *kes1*Δ or *drs2*Δ*kes1*Δ. Initially, I failed to obtain any *drs2*Δ transformants. Even after I obtained transformants, they died quickly on the plates. These observations suggest that Kes1p lacking the ALPS domain is very toxic to *drs2*Δ cells, perhaps because it constitutively represses P4-ATPase activity.

Recombinant mutant proteins (1-29Δ*kes1*, Y97F*kes* and HH143/144LL*kes1*) represent non-functional mutants that are defective in ergosterol binding. These mutant forms of Kes1 should help us determine if sterol-binding by Kes1 is needed for inhibition of Drs2 activity. Initial experiments suggested that the recombinant mutant protein inhibited Drs2 flippase activity similarly to wild-type Kes1. However, the mutant Kes1 proteins were fused to GST (glutathione *S*-transferase) and tested at 1ng concentration. The WT Kes1 used in these experiments was not fused to GST, therefore it would be necessary to cleave GST from the mutant proteins for an appropriate comparison. In addition it is possible that the mutant protein will be less potent of an inhibitor, and therefore it would be important to perform a titration experiment to determine the half maximal inhibitory concentration for each Kes1 variant.

There is also a possibility that Kes1 extracts a rare lipid (ergosterol intermediate or oxysterol) from the TGN membrane that is a potent activator of Drs2. If so, the defective sterol binding kes1p would fail to extract the rare lipids and there would be no inhibition of the increased flippase activity of Drs2.

Not only does Kes1 repress the flippase activity of Drs2, but Drs2 also represses the sterol transport function of Kes1p (Muthusamy *et al.*, 2009b). Disorganization of the membrane and an increased escape tendency of sterol in *drs2*Δ cells could contribute to the hyperactivity of Kes1. Sterol biosynthetic intermediates are known to have a lower affinity for rafts and a higher escape tendency (Li and Prinz, 2004; Lange and Steck, 2008). The synthetic lethality of *drs2*Δ *erg6*Δ could be explained by increased occupancy of Kes1p with sterol in double mutants relative to either single mutant, thus enhancing Kes1 inhibition of the remaining P4-ATPases at the Golgi. Even though *dnf1,2,3*Δ cells exhibit a similar loss of PE and PS asymmetry as *drs2*Δ, *kes1*Δ did not suppress any defects associated with *dnf1,2,3*Δ. This suggests a unique and undefined influence of Drs2p on membrane organization that specifically restricts Kes1p activity.

Kes1 may influence the flippase activity by regulating a protein phosphatase or kinase at the TGN

Oxysterol binding proteins are involved in signaling pathways and this may also be relevant to Kes1 inhibition of Drs2. Kes1 inhibits Drs2 flippase activity at approximately 1 to 10 Kes1p molecules per 1000 Drs2p molecules. This suggests that the mode of inhibition of Kes1p on flippase activity occurs through an enzymatic intermediate. A previous report suggests that OSBP, the mammalian homologue, acts in the MAP kinase pathway by regulating dephosphorylation of ERK (Wang *et al.*, 2005). Thus, it is possible that Kes1p attenuates Drs2 activity by regulating the phosphorylation of proteins at the TGN. There is evidence that P4-ATPases, particularly Dnf PATPases, are regulated by FPK1 and FPK2 (Flippase Protein Kinase) (Nakano *et al.*, 2008). However, it is not known whether Drs2 is regulated by these or other protein kinases. It will be interesting to see whether these protein kinases are regulated by Kes1 or increased level of oxysterol content in the cell. I have been able to identify a protein kinase activity in WT TGN membranes incubated with γ^{32}P ATP. My attempts to determine if Drs2 is phosphorylated and if this phosphorylation is regulated by Kes1 yielded negative results that are hard to interpret. Much more work will be needed to determine if Kes1 regulates protein phosphorylation at the TGN.

It is possible that the activating ligand for Kes1 is an oxysterol rather than ergosterol or an ergosterol biosynthetic intermediate. Yeast lack enzymes for oxysterol biosynthesis, but low levels of oxygenated sterol derivatives can form

nonenzymatically. It would be useful to determine if *drs2*Δ displays a cold-sensitive growth defect in anaerobic conditions, and if so, determine if *kes1*Δ would suppress this defect.

Future Directions

Despite of all these findings, many questions still remain us to how Kes1 represses the flippase activity of Drs2. Several different approaches should be taken in the future to define the mechanisms of Kes1 repression of P4-ATPase activity.

1. Determine the relationship of the Kes1 ALPS domain to inhibition of Drs2 activity

2. Determine if Kes1 inhibits Drs2 by reducing PI4P levels in TGN membranes,

3. Determine the influence of ergosterol levels and its intermediate on the activity of Drs2 and other P4-ATPases.

4. Define the Kes1 influence on phosphorylation of Drs2 or other P4-ATPases

5. Determine if *kes1*Δ suppresses $neo1^{ts}$ defect.

The overall conclusion from my research is that P4-ATPases are downstream targets of the repressive effect of Kes1 on vesicle budding from the TGN. In addition, cold sensitivity of *drs2*Δ appears to be caused by a simultaneous defect in protein trafficking pathways from the TGN to early and late endosomes in AP1/GGA pathway.

REFERENCES

Abe, F., and Minegishi, H. (2008). Global screening of genes essential for growth in high-pressure and cold environments: searching for basic adaptive strategies using a yeast deletion library. Genetics *178*, 851-872.

Alder-Baerens, N., Lisman, Q., Luong, L., Pomorski, T., and Holthuis, J.C. (2006). Loss of P4 ATPases Drs2p and Dnf3p disrupts aminophospholipid transport and asymmetry in yeast post-Golgi secretory vesicles. Mol Biol Cell *17*, 1632-1642.

Alphey, L., Jimenez, J., and Glover, D. (1998). A Drosophila homologue of oxysterol binding protein (OSBP)--implications for the role of OSBP. Biochim Biophys Acta *1395*, 159-164.

Amigo, L., Mendoza, H., Zanlungo, S., Miquel, J.F., Rigotti, A., Gonzalez, S., and Nervi, F. (1999). Enrichment of canalicular membrane with cholesterol and sphingomyelin prevents bile salt-induced hepatic damage. J Lipid Res *40*, 533-542.

Andersen, J.P. (1995). Dissection of the functional domains of the sarcoplasmic reticulum Ca(2+)-ATPase by site-directed mutagenesis. Biosci Rep *15*, 243-261.

Andrew Nesbit, M., Bowl, M.R., Harding, B., Schlessinger, D., Whyte, M.P., and Thakker, R.V. (2004). X-linked hypoparathyroidism region on Xq27 is evolutionarily conserved with regions on 3q26 and 13q34 and contains a novel P-type ATPase. Genomics *84*, 1060-1070.

Anniss, A.M., Apostolopoulos, J., Dworkin, S., Purton, L.E., and Sparrow, R.L. (2002). An oxysterol-binding protein family identified in the mouse. DNA Cell Biol *21*, 571-580.

Axelsen, K.B., and Palmgren, M.G. (1998). Evolution of substrate specificities in the P-type ATPase superfamily. J Mol Evol *46*, 84-101.

Balasubramanian, K., and Schroit, A.J. (2003). Aminophospholipid asymmetry: A matter of life and death. Annu Rev Physiol *65*, 701-734.

Balhadere, P.V., and Talbot, N.J. (2001). PDE1 encodes a P-type ATPase involved in appressorium-mediated plant infection by the rice blast fungus Magnaporthe grisea. Plant Cell *13*, 1987-2004.

Bankaitis, V.A., Aitken, J.R., Cleves, A.E., and Dowhan, W. (1990). An essential role for a phospholipid transfer protein in yeast Golgi function. Nature *347*, 561-562.

Baumann, N.A., Sullivan, D.P., Ohvo-Rekila, H., Simonot, C., Pottekat, A., Klaassen, Z., Beh, C.T., and Menon, A.K. (2005). Transport of newly synthesized sterol to the sterol-enriched plasma membrane occurs via nonvesicular equilibration. Biochemistry *44*, 5816-5826.

Beh, C.T., Cool, L., Phillips, J., and Rine, J. (2001). Overlapping functions of the yeast oxysterol-binding protein homologues. Genetics *157*, 1117-1140.

Beh, C.T., and Rine, J. (2004). A role for yeast oxysterol-binding protein homologs in endocytosis and in the maintenance of intracellular sterol-lipid distribution. J Cell Sci *117*, 2983-2996.

Black, M.W., and Pelham, H.R. (2000). A selective transport route from Golgi to late endosomes that requires the yeast GGA proteins. J Cell Biol *151*, 587-600.

Bull, L.N., Carlton, V.E., Stricker, N.L., Baharloo, S., DeYoung, J.A., Freimer, N.B., Magid, M.S., Kahn, E., Markowitz, J., DiCarlo, F.J., McLoughlin, L., Boyle, J.T., Dahms, B.B., Faught, P.R., Fitzgerald, J.F., Piccoli, D.A., Witzleben, C.L., O'Connell, N.C., Setchell, K.D., Agostini, R.M., Jr., Kocoshis, S.A., Reyes, J., and Knisely, A.S. (1997). Genetic and morphological findings in progressive familial intrahepatic cholestasis (Byler disease [PFIC-1] and Byler syndrome): evidence for heterogeneity. Hepatology *26*, 155-164.

Bull, L.N., van Eijk, M.J., Pawlikowska, L., DeYoung, J.A., Juijn, J.A., Liao, M., Klomp, L.W., Lomri, N., Berger, R., Scharschmidt, B.F., Knisely, A.S., Houwen, R.H., and Freimer, N.B. (1998). A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis. Nat Genet *18*, 219-224.

Chen, C.Y., and Graham, T.R. (1998). An arf1Delta synthetic lethal screen identifies a new clathrin heavy chain conditional allele that perturbs vacuolar protein transport in Saccharomyces cerevisiae. Genetics *150*, 577-589.

Chen, C.Y., Ingram, M.F., Rosal, P.H., and Graham, T.R. (1999). Role for Drs2p, a P-type ATPase and potential aminophospholipid translocase, in yeast late Golgi function. J Cell Biol *147*, 1223-1236.

Chen, S., Wang, J., Muthusamy, B.P., Liu, K., Zare, S., Andersen, R.J., and Graham, T.R. (2006). Roles for the Drs2p-Cdc50p complex in protein transport and phosphatidylserine asymmetry of the yeast plasma membrane. Traffic *7*, 1503-1517.

Chen, S.H., Chen, S., Tokarev, A.A., Liu, F., Jedd, G., and Segev, N. (2005). Ypt31/32 GTPases and their novel F-box effector protein Rcy1 regulate protein recycling. Mol Biol Cell *16*, 178-192.

Clarke, D.M., Maruyama, K., Loo, T.W., Leberer, E., Inesi, G., and MacLennan, D.H. (1989). Functional consequences of glutamate, aspartate, glutamine, and asparagine mutations in the stalk sector of the Ca2+-ATPase of sarcoplasmic reticulum. J Biol Chem *264*, 11246-11251.

Copic, A., Starr, T.L., and Schekman, R. (2007). Ent3p and Ent5p exhibit cargo-specific functions in trafficking proteins between the trans-Golgi network and the endosomes in yeast. Mol Biol Cell *18*, 1803-1815.

Cornelius, F. (1995). Cholesterol modulation of molecular activity of reconstituted shark Na+,K(+)-ATPase. Biochim Biophys Acta *1235*, 205-212.

Costaguta, G., Stefan, C.J., Bensen, E.S., Emr, S.D., and Payne, G.S. (2001). Yeast Gga coat proteins function with clathrin in Golgi to endosome transport. Mol Biol Cell *12*, 1885-1896.

Cummins, C.L., and Mangelsdorf, D.J. (2006). Liver X receptors and cholesterol homoeostasis: spotlight on the adrenal gland. Biochem Soc Trans *34*, 1110-1113.

Daleke, D.L., and Huestis, W.H. (1985). Incorporation and translocation of aminophospholipids in human erythrocytes. Biochemistry *24*, 5406-5416.

Daleke, D.L., and Huestis, W.H. (1989). Erythrocyte morphology reflects the transbilayer distribution of incorporated phospholipids. J Cell Biol *108*, 1375-1385.

Darland-Ransom, M., Wang, X., Sun, C.L., Mapes, J., Gengyo-Ando, K., Mitani, S., and Xue, D. (2008). Role of C. elegans TAT-1 protein in maintaining plasma membrane phosphatidylserine asymmetry. Science *320*, 528-531.

Daum, G., Lees, N.D., Bard, M., and Dickson, R. (1998). Biochemistry, cell biology and molecular biology of lipids of Saccharomyces cerevisiae. Yeast *14*, 1471-1510.

Dekkers, D.W., Comfurius, P., Bevers, E.M., and Zwaal, R.F. (2002). Comparison between Ca2+-induced scrambling of various fluorescently labelled lipid analogues in red blood cells. Biochem J *362*, 741-747.

Dhar, M.S., Yuan, J.S., Elliott, S.B., and Sommardahl, C. (2006). A type IV P-type ATPase affects insulin-mediated glucose uptake in adipose tissue and skeletal muscle in mice. J Nutr Biochem *17*, 811-820.

Drin, G., Casella, J.F., Gautier, R., Boehmer, T., Schwartz, T.U., and Antonny, B. (2007). A general amphipathic alpha-helical motif for sensing membrane curvature. Nat Struct Mol Biol *14*, 138-146.

Edqvist, J., and Blomqvist, K. (2006). Fusion and fission, the evolution of sterol carrier protein-2. J Mol Evol *62*, 292-306.

Fairn, G.D., Curwin, A.J., Stefan, C.J., and McMaster, C.R. (2007). The oxysterol binding protein Kes1p regulates Golgi apparatus phosphatidylinositol-4-phosphate function. Proc Natl Acad Sci U S A *104*, 15352-15357.

Fang, M., Kearns, B.G., Gedvilaite, A., Kagiwada, S., Kearns, M., Fung, M.K., and Bankaitis, V.A. (1996). Kes1p shares homology with human oxysterol binding protein and participates in a novel regulatory pathway for yeast Golgi-derived transport vesicle biogenesis. Embo J *15*, 6447-6459.

Fei, W., Alfaro, G., Muthusamy, B.P., Klaassen, Z., Graham, T.R., Yang, H., and Beh, C.T. (2008). Genome-wide analysis of sterol-lipid storage and trafficking in Saccharomyces cerevisiae. Eukaryot Cell *7*, 401-414.

Folmer, D.E., Elferink, R.P., and Paulusma, C.C. (2009). P4 ATPases - Lipid flippases and their role in disease. Biochim Biophys Acta *1791*, 628-635.

Furuta, N., Fujimura-Kamada, K., Saito, K., Yamamoto, T., and Tanaka, K. (2007). Endocytic recycling in yeast is regulated by putative phospholipid translocases and the Ypt31p/32p-Rcy1p pathway. Mol Biol Cell *18*, 295-312.

Gadella, B.M., Tsai, P.S., Boerke, A., and Brewis, I.A. (2008). Sperm head membrane reorganisation during capacitation. Int J Dev Biol *52*, 473-480.

Gall, W.E., Geething, N.C., Hua, Z., Ingram, M.F., Liu, K., Chen, S.I., and Graham, T.R. (2002). Drs2p-dependent formation of exocytic clathrin-coated vesicles in vivo. Curr Biol *12*, 1623-1627.

Gall, W.E., Higginbotham, M.A., Chen, C., Ingram, M.F., Cyr, D.M., and Graham, T.R. (2000). The auxilin-like phosphoprotein Swa2p is required for clathrin function in yeast. Curr Biol *10*, 1349-1358.

Gietz, R.D., and Woods, R.A. (2006). Yeast transformation by the LiAc/SS Carrier DNA/PEG method. Methods Mol Biol *313*, 107-120.

Gilbert, M.J., Thornton, C.R., Wakley, G.E., and Talbot, N.J. (2006). A P-type ATPase required for rice blast disease and induction of host resistance. Nature *440*, 535-539.

Gomes, E., Jakobsen, M.K., Axelsen, K.B., Geisler, M., and Palmgren, M.G. (2000). Chilling tolerance in Arabidopsis involves ALA1, a member of a new family of putative aminophospholipid translocases. Plant Cell *12*, 2441-2454.

Goodson, H.V., Anderson, B.L., Warrick, H.M., Pon, L.A., and Spudich, J.A. (1996). Synthetic lethality screen identifies a novel yeast myosin I gene (MYO5): myosin I proteins are required for polarization of the actin cytoskeleton. J Cell Biol *133*, 1277-1291.

Graham, T.R. (2001). Metabolic labeling and immunoprecipitation of yeast proteins. Curr Protoc Cell Biol *Chapter 7*, Unit 7 6.

Graham, T.R. (2004). Flippases and vesicle-mediated protein transport. Trends Cell Biol *14*, 670-677.

Groen, A., Kunne, C., Jongsma, G., van den Oever, K., Mok, K.S., Petruzzelli, M., Vrins, C.L., Bull, L., Paulusma, C.C., and Oude Elferink, R.P. (2008). Abcg5/8 independent biliary cholesterol excretion in Atp8b1-deficient mice. Gastroenterology *134*, 2091-2100.

Groen, A., Kunne, C., Paulusma, C.C., Kramer, W., Agellon, L.B., Bull, L.N., and Oude Elferink, R.P. (2007). Intestinal bile salt absorption in Atp8b1 deficient mice. J Hepatol *47*, 114-122.

Hanson, P.K., Malone, L., Birchmore, J.L., and Nichols, J.W. (2003). Lem3p is essential for the uptake and potency of alkylphosphocholine drugs, edelfosine and miltefosine. J Biol Chem *278*, 36041-36050.

Hua, Z., Fatheddin, P., and Graham, T.R. (2002). An essential subfamily of Drs2p-related P-type ATPases is required for protein trafficking between Golgi complex and endosomal/vacuolar system. Mol Biol Cell *13*, 3162-3177.

Hua, Z., and Graham, T.R. (2003). Requirement for neo1p in retrograde transport from the Golgi complex to the endoplasmic reticulum. Mol Biol Cell *14*, 4971-4983.

Im, Y.J., Raychaudhuri, S., Prinz, W.A., and Hurley, J.H. (2005). Structural mechanism for sterol sensing and transport by OSBP-related proteins. Nature *437*, 154-158.

Itoh, T., and De Camilli, P. (2006). BAR, F-BAR (EFC) and ENTH/ANTH domains in the regulation of membrane-cytosol interfaces and membrane curvature. Biochim Biophys Acta *1761*, 897-912.

Jiang, B., Brown, J.L., Sheraton, J., Fortin, N., and Bussey, H. (1994). A new family of yeast genes implicated in ergosterol synthesis is related to the human oxysterol binding protein. Yeast *10*, 341-353.

Kaplan, M.R., and Simoni, R.D. (1985). Transport of cholesterol from the endoplasmic reticulum to the plasma membrane. J Cell Biol *101*, 446-453.

Kato, U., Emoto, K., Fredriksson, C., Nakamura, H., Ohta, A., Kobayashi, T., Murakami-Murofushi, K., Kobayashi, T., and Umeda, M. (2002). A novel membrane protein, Ros3p, is required for phospholipid translocation across the plasma membrane in Saccharomyces cerevisiae. J Biol Chem *277*, 37855-37862.

Kawasaki, M., Nakayama, K., and Wakatsuki, S. (2005). Membrane recruitment of effector proteins by Arf and Rab GTPases. Curr Opin Struct Biol *15*, 681-689.

Kishimoto, T., Yamamoto, T., and Tanaka, K. (2005). Defects in structural integrity of ergosterol and the Cdc50p-Drs2p putative phospholipid translocase cause accumulation of endocytic membranes, onto which actin patches are assembled in yeast. Mol Biol Cell *16*, 5592-5609.

Knisely, A.S. (2000). Progressive familial intrahepatic cholestasis: a personal perspective. Pediatr Dev Pathol *3*, 113-125.

Knodler, A., and Mayinger, P. (2005). Analysis of phosphoinositide-binding proteins using liposomes as an affinity matrix. Biotechniques *38*, 858, 860, 862.

Kozminski, K.G., Alfaro, G., Dighe, S., and Beh, C.T. (2006). Homologues of oxysterol-binding proteins affect Cdc42p- and Rho1p-mediated cell polarization in Saccharomyces cerevisiae. Traffic *7*, 1224-1242.

Kuhlbrandt, W. (2004). Biology, structure and mechanism of P-type ATPases. Nat Rev Mol Cell Biol *5*, 282-295.

Kurbegov, A.C., Setchell, K.D., Haas, J.E., Mierau, G.W., Narkewicz, M., Bancroft, J.D., Karrer, F., and Sokol, R.J. (2003). Biliary diversion for progressive familial intrahepatic cholestasis: improved liver morphology and bile acid profile. Gastroenterology *125*, 1227-1234.

Lange, Y., and Steck, T. (2008). Cholesterol homeostasis and the escape tendency (activity) of plasma membrane cholesterol. Prog Lipid Res *47*, 319-332.

Lehto, M., Hynynen, R., Karjalainen, K., Kuismanen, E., Hyvarinen, K., and Olkkonen, V.M. (2005). Targeting of OSBP-related protein 3 (ORP3) to endoplasmic reticulum and plasma membrane is controlled by multiple determinants. Exp Cell Res *310*, 445-462.

Lehto, M., Laitinen, S., Chinetti, G., Johansson, M., Ehnholm, C., Staels, B., Ikonen, E., and Olkkonen, V.M. (2001). The OSBP-related protein family in humans. J Lipid Res *42*, 1203-1213.

Lenoir, G., Williamson, P., Puts, C.F., and Holthuis, J.C. (2009). Cdc50p plays a vital role in the ATPase reaction cycle of the putative aminophospholipid transporter drs2p. J Biol Chem *284*, 17956-17967.

Lessmann, E., Ngo, M., Leitges, M., Minguet, S., Ridgway, N.D., and Huber, M. (2007). Oxysterol-binding protein-related protein (ORP) 9 is a PDK-2 substrate and regulates Akt phosphorylation. Cell Signal *19*, 384-392.

Levanon, D., Hsieh, C.L., Francke, U., Dawson, P.A., Ridgway, N.D., Brown, M.S., and Goldstein, J.L. (1990). cDNA cloning of human oxysterol-binding protein and localization of the gene to human chromosome 11 and mouse chromosome 19. Genomics *7*, 65-74.

Levine, T.P., and Munro, S. (1998). The pleckstrin homology domain of oxysterol-binding protein recognises a determinant specific to Golgi membranes. Curr Biol *8*, 729-739.

Lewis, M.J., Nichols, B.J., Prescianotto-Baschong, C., Riezman, H., and Pelham, H.R. (2000). Specific retrieval of the exocytic SNARE Snc1p from early yeast endosomes. Mol Biol Cell *11*, 23-38.

Li, X., Rivas, M.P., Fang, M., Marchena, J., Mehrotra, B., Chaudhary, A., Feng, L., Prestwich, G.D., and Bankaitis, V.A. (2002). Analysis of oxysterol binding protein homologue Kes1p function in regulation of Sec14p-dependent protein transport from the yeast Golgi complex. J Cell Biol *157*, 63-77.

Li, Y., and Prinz, W.A. (2004). ATP-binding cassette (ABC) transporters mediate nonvesicular, raft-modulated sterol movement from the plasma membrane to the endoplasmic reticulum. J Biol Chem *279*, 45226-45234.

Liscum, L., and Munn, N.J. (1999). Intracellular cholesterol transport. Biochim Biophys Acta *1438*, 19-37.

Liu, K., Hua, Z., Nepute, J.A., and Graham, T.R. (2007). Yeast P4-ATPases Drs2p and Dnf1p are essential cargos of the NPFXD/Sla1p endocytic pathway. Mol Biol Cell *18*, 487-500.

Liu, K., Surendhran, K., Nothwehr, S.F., and Graham, T.R. (2008a). P4-ATPase Requirement for AP-1/Clathrin Function in Protein Transport from the trans-Golgi Network and Early Endosomes. Mol Biol Cell.

Liu, K., Surendhran, K., Nothwehr, S.F., and Graham, T.R. (2008b). P4-ATPase requirement for AP-1/clathrin function in protein transport

from the trans-Golgi network and early endosomes. Mol Biol Cell *19*, 3526-3535.

Loewen, C.J., and Levine, T.P. (2005). A highly conserved binding site in vesicle-associated membrane protein-associated protein (VAP) for the FFAT motif of lipid-binding proteins. J Biol Chem *280*, 14097-14104.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953-961.

Lyssenko, N.N., Miteva, Y., Gilroy, S., Hanna-Rose, W., and Schlegel, R.A. (2008). An unexpectedly high degree of specialization and a widespread involvement in sterol metabolism among the C. elegans putative aminophospholipid translocases. BMC Dev Biol *8*, 96.

McGee, T.P., Skinner, H.B., Whitters, E.A., Henry, S.A., and Bankaitis, V.A. (1994). A phosphatidylinositol transfer protein controls the phosphatidylcholine content of yeast Golgi membranes. J Cell Biol *124*, 273-287.

Moir, D., Stewart, S.E., Osmond, B.C., and Botstein, D. (1982). Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. Genetics *100*, 547-563.

Moller, J.V., Juul, B., and le Maire, M. (1996). Structural organization, ion transport, and energy transduction of P-type ATPases. Biochim Biophys Acta *1286*, 1-51.

Moller, J.V., Olesen, C., Jensen, A.M., and Nissen, P. (2005). The structural basis for coupling of Ca2+ transport to ATP hydrolysis by the sarcoplasmic reticulum Ca2+-ATPase. J Bioenerg Biomembr *37*, 359-364.

Morozova, N., Liang, Y., Tokarev, A.A., Chen, S.H., Cox, R., Andrejic, J., Lipatova, Z., Sciorra, V.A., Emr, S.D., and Segev, N. (2006). TRAPPII subunits are required for the specificity switch of a Ypt-Rab GEF. Nat Cell Biol *8*, 1263-1269.

Morth, J.P., Pedersen, B.P., Toustrup-Jensen, M.S., Sorensen, T.L., Petersen, J., Andersen, J.P., Vilsen, B., and Nissen, P. (2007). Crystal structure of the sodium-potassium pump. Nature *450*, 1043-1049.

Mousley, C.J., Tyeryar, K.R., Vincent-Pope, P., and Bankaitis, V.A. (2007). The Sec14-superfamily and the regulatory interface between phospholipid metabolism and membrane trafficking. Biochim Biophys Acta *1771*, 727-736.

Mukherjee, S., and Maxfield, F.R. (2004). Lipid and cholesterol trafficking in NPC. Biochim Biophys Acta *1685*, 28-37.

Munn, A.L., Heese-Peck, A., Stevenson, B.J., Pichler, H., and Riezman, H. (1999). Specific sterols required for the internalization step of endocytosis in yeast. Mol Biol Cell *10*, 3943-3957.

Muthusamy, B.P., Natarajan, P., Zhou, X., and Graham, T.R. (2009a). Linking phospholipid flippases to vesicle-mediated protein transport. Biochim Biophys Acta *1791*, 612-619.

Muthusamy, B.P., Raychaudhuri, S., Natarajan, P., Abe, F., Liu, K., Prinz, W.A., and Graham, T.R. (2009b). Control of protein and sterol trafficking by antagonistic activities of a type IV P-type ATPase and oxysterol binding protein homologue. Mol Biol Cell *20*, 2920-2931.

Nakano, K., Yamamoto, T., Kishimoto, T., Noji, T., and Tanaka, K. (2008). Protein kinases Fpk1p and Fpk2p are novel regulators of phospholipid asymmetry. Mol Biol Cell *19*, 1783-1797.

Natarajan, P., and Graham, T.R. (2006). Measuring translocation of fluorescent lipid derivatives across yeast Golgi membranes. Methods *39*, 163-168.

Natarajan, P., Liu, K., Patil, D.V., Sciorra, V.A., Jackson, C.L., and Graham, T.R. (2009). Regulation of a Golgi flippase by phosphoinositides and an ArfGEF. Nat Cell Biol.

Natarajan, P., Wang, J., Hua, Z., and Graham, T.R. (2004). Drs2p-coupled aminophospholipid translocase activity in yeast Golgi membranes and relationship to in vivo function. Proc Natl Acad Sci U S A *101*, 10614-10619.

Noji, T., Yamamoto, T., Saito, K., Fujimura-Kamada, K., Kondo, S., and Tanaka, K. (2006). Mutational analysis of the Lem3p-Dnf1p putative phospholipid-translocating P-type ATPase reveals novel regulatory roles for Lem3p and a carboxyl-terminal region of Dnf1p independent of the phospholipid-translocating activity of Dnf1p in yeast. Biochem Biophys Res Commun *344*, 323-331.

Nossal, R. (2001). Energetics of clathrin basket assembly. Traffic *2*, 138-147.

Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell *21*, 205-215.

Obara, K., Miyashita, N., Xu, C., Toyoshima, I., Sugita, Y., Inesi, G., and Toyoshima, C. (2005). Structural role of countertransport revealed in Ca(2+) pump crystal structure in the absence of Ca(2+). Proc Natl Acad Sci U S A *102*, 14489-14496.

Odorizzi, G., Cowles, C.R., and Emr, S.D. (1998). The AP-3 complex: a coat of many colours. Trends Cell Biol *8*, 282-288.

Okamura, H., Yasuhara, J.C., Fambrough, D.M., and Takeyasu, K. (2003). P-type ATPases in Caenorhabditis and Drosophila: implications for evolution of the P-type ATPase subunit families with special reference to the Na,K-ATPase and H,K-ATPase subgroup. J Membr Biol *191*, 13-24.

Olkkonen, V.M., Johansson, M., Suchanek, M., Yan, D., Hynynen, R., Ehnholm, C., Jauhiainen, M., Thiele, C., and Lehto, M. (2006). The OSBP-related proteins (ORPs): global sterol sensors for co-ordination of cellular lipid metabolism, membrane trafficking and signalling processes? Biochem Soc Trans *34*, 389-391.

Olkkonen, V.M., and Lehto, M. (2004). Oxysterols and oxysterol binding proteins: role in lipid metabolism and atherosclerosis. Ann Med *36*, 562-572.

Owen, D.J., Collins, B.M., and Evans, P.R. (2004). Adaptors for clathrin coats: structure and function. Annu Rev Cell Dev Biol *20*, 153-191.

Parks, L.W., and Casey, W.M. (1995). Physiological implications of sterol biosynthesis in yeast. Annu Rev Microbiol *49*, 95-116.

Paulusma, C.C., de Waart, D.R., Kunne, C., Mok, K.S., and Elferink, R.P. (2009). Activity of the bile salt export pump (ABCB11) is critically dependent on canalicular membrane cholesterol content. J Biol Chem *284*, 9947-9954.

Paulusma, C.C., Folmer, D.E., Ho-Mok, K.S., de Waart, D.R., Hilarius, P.M., Verhoeven, A.J., and Oude Elferink, R.P. (2008). ATP8B1 requires an

accessory protein for endoplasmic reticulum exit and plasma membrane lipid flippase activity. Hepatology *47*, 268-278.

Paulusma, C.C., Groen, A., Kunne, C., Ho-Mok, K.S., Spijkerboer, A.L., Rudi de Waart, D., Hoek, F.J., Vreeling, H., Hoeben, K.A., van Marle, J., Pawlikowska, L., Bull, L.N., Hofmann, A.F., Knisely, A.S., and Oude Elferink, R.P. (2006). Atp8b1 deficiency in mice reduces resistance of the canalicular membrane to hydrophobic bile salts and impairs bile salt transport. Hepatology *44*, 195-204.

Pawlikowska, L., Groen, A., Eppens, E.F., Kunne, C., Ottenhoff, R., Looije, N., Knisely, A.S., Killeen, N.P., Bull, L.N., Elferink, R.P., and Freimer, N.B. (2004). A mouse genetic model for familial cholestasis caused by ATP8B1 mutations reveals perturbed bile salt homeostasis but no impairment in bile secretion. Hum Mol Genet *13*, 881-892.

Pedersen, B.P., Buch-Pedersen, M.J., Morth, J.P., Palmgren, M.G., and Nissen, P. (2007). Crystal structure of the plasma membrane proton pump. Nature *450*, 1111-1114.

Pomorski, T., Lombardi, R., Riezman, H., Devaux, P.F., van Meer, G., and Holthuis, J.C. (2003). Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. Mol Biol Cell *14*, 1240-1254.

Poulsen, L.R., Lopez-Marques, R.L., McDowell, S.C., Okkeri, J., Licht, D., Schulz, A., Pomorski, T., Harper, J.F., and Palmgren, M.G. (2008). The Arabidopsis P4-ATPase ALA3 localizes to the golgi and requires a beta-subunit to function in lipid translocation and secretory vesicle formation. Plant Cell *20*, 658-676.

Raychaudhuri, S., Im, Y.J., Hurley, J.H., and Prinz, W.A. (2006). Nonvesicular sterol movement from plasma membrane to ER requires oxysterol-binding protein-related proteins and phosphoinositides. J Cell Biol *173*, 107-119.

Reiner, S., Micolod, D., Zellnig, G., and Schneiter, R. (2006). A genomewide screen reveals a role of mitochondria in anaerobic uptake of sterols in yeast. Mol Biol Cell *17*, 90-103.

Ridgway, N.D., Dawson, P.A., Ho, Y.K., Brown, M.S., and Goldstein, J.L. (1992). Translocation of oxysterol binding protein to Golgi apparatus triggered by ligand binding. J Cell Biol *116*, 307-319.

Riekhof, W.R., Wu, J., Gijon, M.A., Zarini, S., Murphy, R.C., and Voelker, D.R. (2007). Lysophosphatidylcholine metabolism in Saccharomyces cerevisiae: the role of P-type ATPases in transport and a broad specificity acyltransferase in acylation. J Biol Chem *282*, 36853-36861.

Ripmaster, T.L., Vaughn, G.P., and Woolford, J.L., Jr. (1993). DRS1 to DRS7, novel genes required for ribosome assembly and function in Saccharomyces cerevisiae. Mol Cell Biol *13*, 7901-7912.

Rivas, M.P., Kearns, B.G., Xie, Z., Guo, S., Sekar, M.C., Hosaka, K., Kagiwada, S., York, J.D., and Bankaitis, V.A. (1999). Pleiotropic alterations in lipid metabolism in yeast sac1 mutants: relationship to "bypass Sec14p" and inositol auxotrophy. Mol Biol Cell *10*, 2235-2250.

Robinson, J.S., Klionsky, D.J., Banta, L.M., and Emr, S.D. (1988). Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol Cell Biol *8*, 4936-4948.

Robinson, M., Poon, P.P., Schindler, C., Murray, L.E., Kama, R., Gabriely, G., Singer, R.A., Spang, A., Johnston, G.C., and Gerst, J.E. (2006). The Gcs1 Arf-GAP mediates Snc1,2 v-SNARE retrieval to the Golgi in yeast. Mol Biol Cell *17*, 1845-1858.

Robinson, M.S. (2004). Adaptable adaptors for coated vesicles. Trends Cell Biol *14*, 167-174.

Saito, K., Fujimura-Kamada, K., Furuta, N., Kato, U., Umeda, M., and Tanaka, K. (2004). Cdc50p, a protein required for polarized growth, associates with the Drs2p P-type ATPase implicated in phospholipid translocation in Saccharomyces cerevisiae. Mol Biol Cell *15*, 3418-3432.

Schaaf, G., Ortlund, E.A., Tyeryar, K.R., Mousley, C.J., Ile, K.E., Garrett, T.A., Ren, J., Woolls, M.J., Raetz, C.R., Redinbo, M.R., and Bankaitis, V.A. (2008). Functional anatomy of phospholipid binding and regulation of phosphoinositide homeostasis by proteins of the sec14 superfamily. Mol Cell *29*, 191-206.

Schmid, S.L. (1997). Clathrin-coated vesicle formation and protein sorting: an integrated process. Annu Rev Biochem *66*, 511-548.

Schnabl, M., Daum, G., and Pichler, H. (2005). Multiple lipid transport pathways to the plasma membrane in yeast. Biochim Biophys Acta *1687*, 130-140.

Schroepfer, G.J., Jr. (2000). Oxysterols: modulators of cholesterol metabolism and other processes. Physiol Rev *80*, 361-554.

Schulz, T.A., and Prinz, W.A. (2007). Sterol transport in yeast and the oxysterol binding protein homologue (OSH) family. Biochim Biophys Acta *1771*, 769-780.

Sciorra, V.A., Audhya, A., Parsons, A.B., Segev, N., Boone, C., and Emr, S.D. (2005). Synthetic genetic array analysis of the PtdIns 4-kinase Pik1p identifies components in a Golgi-specific Ypt31/rab-GTPase signaling pathway. Mol Biol Cell *16*, 776-793.

Seigneuret, M., and Devaux, P.F. (1984). ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. Proc Natl Acad Sci U S A *81*, 3751-3755.

Seigneuret, M., Zachowski, A., Hermann, A., and Devaux, P.F. (1984). Asymmetric lipid fluidity in human erythrocyte membrane: new spin-label evidence. Biochemistry *23*, 4271-4275.

Sheetz, M.P., Painter, R.G., and Singer, S.J. (1976). Biological membranes as bilayer couples. III. Compensatory shape changes induced in membranes. J Cell Biol *70*, 193-203.

Sherman, F. (1991). Getting started with yeast. Methods Enzymol *194*, 3-21.

Sitcheran, R., Emter, R., Kralli, A., and Yamamoto, K.R. (2000). A genetic analysis of glucocorticoid receptor signaling. Identification and characterization of ligand-effect modulators in Saccharomyces cerevisiae. Genetics *156*, 963-972.

Skirpan, A.L., Dowd, P.E., Sijacic, P., Jaworski, C.J., Gilroy, S., and Kao, T.H. (2006). Identification and characterization of PiORP1, a Petunia oxysterol-binding-protein related protein involved in receptor-kinase mediated signaling in pollen, and analysis of the ORP gene family in Arabidopsis. Plant Mol Biol *61*, 553-565.

Stevens, H.C., Malone, L., and Nichols, J.W. (2008). The putative aminophospholipid translocases, DNF1 and DNF2, are not required for 7-nitrobenz-2-oxa-1,3-diazol-4-yl-phosphatidylserine flip across the plasma membrane of Saccharomyces cerevisiae. J Biol Chem *283*, 35060-35069.

Stevens, T., Esmon, B., and Schekman, R. (1982). Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. Cell *30*, 439-448.

Strauss, J.F., 3rd, Liu, P., Christenson, L.K., and Watari, H. (2002). Sterols and intracellular vesicular trafficking: lessons from the study of NPC1. Steroids *67*, 947-951.

Sugawara, K., Morita, K., Ueno, N., and Shibuya, H. (2001). BIP, a BRAM-interacting protein involved in TGF-beta signalling, regulates body length in Caenorhabditis elegans. Genes Cells *6*, 599-606.

Sugita, Y., Miyashita, N., Ikeguchi, M., Kidera, A., and Toyoshima, C. (2005). Protonation of the acidic residues in the transmembrane cation-binding sites of the ca(2+) pump. J Am Chem Soc *127*, 6150-6151.

Sun, X.L., Li, D., Fang, J., Noyes, I., Casto, B., Theil, K., Shuler, C., and Milo, G.E. (1999). Changes in levels of normal ML-1 gene transcripts associated with the conversion of human nontumorigenic to tumorigenic phenotypes. Gene Expr *8*, 129-139.

Taylor, F.R., and Kandutsch, A.A. (1985). Oxysterol binding protein. Chem Phys Lipids *38*, 187-194.

Toyoshima, C., and Inesi, G. (2004). Structural basis of ion pumping by Ca2+-ATPase of the sarcoplasmic reticulum. Annu Rev Biochem *73*, 269-292.

Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000). Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. Nature *405*, 647-655.

Toyoshima, C., and Nomura, H. (2002). Structural changes in the calcium pump accompanying the dissociation of calcium. Nature *418*, 605-611.

Ujhazy, P., Ortiz, D., Misra, S., Li, S., Moseley, J., Jones, H., and Arias, I.M. (2001). Familial intrahepatic cholestasis 1: studies of localization and function. Hepatology *34*, 768-775.

Umebayashi, K., and Nakano, A. (2003). Ergosterol is required for targeting of tryptophan permease to the yeast plasma membrane. J Cell Biol *161*, 1117-1131.

van Meer, G., Halter, D., Sprong, H., Somerharju, P., and Egmond, M.R. (2006). ABC lipid transporters: extruders, flippases, or flopless activators? FEBS Lett *580*, 1171-1177.

van Meer, G., Voelker, D.R., and Feigenson, G.W. (2008). Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol *9*, 112-124.

van Mil, S.W., Klomp, L.W., Bull, L.N., and Houwen, R.H. (2001). FIC1 disease: a spectrum of intrahepatic cholestatic disorders. Semin Liver Dis *21*, 535-544.

Walch-Solimena, C., and Novick, P. (1999). The yeast phosphatidylinositol-4-OH kinase pik1 regulates secretion at the Golgi. Nat Cell Biol *1*, 523-525.

Walsh, J.P., Caldwell, K.K., and Majerus, P.W. (1991). Formation of phosphatidylinositol 3-phosphate by isomerization from phosphatidylinositol 4-phosphate. Proc Natl Acad Sci U S A *88*, 9184-9187.

Wang, C.W., Hamamoto, S., Orci, L., and Schekman, R. (2006). Exomer: A coat complex for transport of select membrane proteins from the trans-Golgi network to the plasma membrane in yeast. J Cell Biol *174*, 973-983.

Wang, L., Beserra, C., and Garbers, D.L. (2004). A novel aminophospholipid transporter exclusively expressed in spermatozoa is required for membrane lipid asymmetry and normal fertilization. Dev Biol *267*, 203-215.

Wang, P.Y., Weng, J., and Anderson, R.G. (2005). OSBP is a cholesterol-regulated scaffolding protein in control of ERK 1/2 activation. Science *307*, 1472-1476.

Wicky, S., Schwarz, H., and Singer-Kruger, B. (2004). Molecular interactions of yeast Neo1p, an essential member of the Drs2 family of

aminophospholipid translocases, and its role in membrane trafficking within the endomembrane system. Mol Cell Biol *24*, 7402-7418.

Wilcox, L.J., Balderes, D.A., Wharton, B., Tinkelenberg, A.H., Rao, G., and Sturley, S.L. (2002). Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast. J Biol Chem *277*, 32466-32472.

Yeung, B.G., Phan, H.L., and Payne, G.S. (1999). Adaptor complex-independent clathrin function in yeast. Mol Biol Cell *10*, 3643-3659.

Zachowski, A. (1993). Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem J *294 (Pt 1)*, 1-14.

Zachowski, A., Henry, J.P., and Devaux, P.F. (1989). Control of transmembrane lipid asymmetry in chromaffin granules by an ATP-dependent protein. Nature *340*, 75-76.

Zhang, B., Groffen, J., and Heisterkamp, N. (2005). Resistance to farnesyltransferase inhibitors in Bcr/Abl-positive lymphoblastic leukemia by increased expression of a novel ABC transporter homolog ATP11a. Blood *106*, 1355-1361.

Zhou, X., and Graham, T.R. (2009). Reconstitution of phospholipid translocase activity with purified Drs2p, a type-IV P-type ATPase from budding yeast. Proc Natl Acad Sci U S A *106*, 16586-16591.

Zhu, Y., Drake, M.T., and Kornfeld, S. (1999). ADP-ribosylation factor 1 dependent clathrin-coat assembly on synthetic liposomes. Proc Natl Acad Sci U S A *96*, 5013-5018.