REGULATION OF GOLGI-DERIVED VESICULAR TRANSPORT BY THE PHOSPHOLIPID TRANSFER PROTEIN, SEC14, AND AN OXYSTEROL BINDING PROTEIN HOMOLOGUE, OSH4/KES1

by

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Abstract

Phosphatidylcholine (PC) is the most abundant phospholipid within eukaryotes, comprising over 50% of the cellular phospholipid, and thus plays a crucial role in the formation of biological membranes. Conversely, phosphatidylinositol 4-phosphate (PI-4P) is a rare phospholipid in eukaryotes comprising <0.1% of the total phospholipid; however, PI-4P is also essential for eukaryotic growth and viability. In this thesis, I exploited the genetic tractability of the budding yeast *Saccharomyces cerevisiae* to explore the regulation of PC and PI-4P homeostasis in eukaryotic cells.

Sec14 is essential for growth of *Saccharomyces cerevisiae*, with loss of function altering both PC and PI-4P metabolism. Consequences resulting from loss of Sec14 function include a block in secretion from the *trans*-Golgi, a subsequent accumulation of aberrant membranous structures and vesicles, and ultimately death. Mutations in the enzymes in the CDP-choline pathway for PC biosynthesis and Kes1, an oxysterol binding protein homologue and PI-4P binding protein, bypass the essential requirement for Sec14. Using high-throughput genetic analysis I determined that *sec14Δ ckilΔ* and *sec14Δ kes1Δ* strains had additional genetic requirements for life, not seen with wild-type cells. The screens identified components of the Transport Protein Particle II (TRAPP II) and the Rab protein Ypt31 as being required for the viability of the *sec14 ckil* strain. A yeast strain with an enfeebled Golgi PI 4-kinase, Pik1, also displayed a heightened genetic requirement for TRAPP II and Ypt31. The results suggested that Kes1 attenuates PI-4P signaling at the Golgi, as ablation of *KES1* alleviates secretion defects and the lethality associated with loss of Sec14 or Pik1 function. *Saccharomyces cerevisiae* cells contain nearly as many Kes1 molecules as PI-4P molecules, suggesting Kes1 could attenuate PI-4P signaling simply by binding all of the available PI-4P. Analysis of the cellular phosphoinositides revealed that cells lacking a functional Kes1 also have an increase in PI-4P production. Microscopic analysis using a fluorescent PI-4P probe revealed that the increased PI-4P production occurs in the Golgi. Thus the genetic, cellular and biochemical evidence suggests that Kes1 negatively regulates Golgi-derived secretion by regulating the production and availability of PI-4P produced by Pik1.

List of Abbreviations Used

ARF	ADP-ribosylation factor
DAG	diacylglycerol
DGK	diacylglycerol kinase
ER	endoplasmic reticulum
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
HPLC	high performance liquid chromatography
NAT	nourseothricin N-acetyltransferase
NSF	*N*-ethlymaleimde sensitive fusion protein
ORP	OSBP related protein (human)
OSBP	oxysterol binding protein
OSH	OSBP homologue (yeast)
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PIP	PI phosphate
PITP	PI transfer protein
PLB	phospholipase B
PLC	phospholipase C

PLD	phospholipase D
SGA	synthetic genetic array
SNAP	souble NSF attachment protein
SNARE	SNAP receptor
TLC	thin layer chromatography
TRAPP	transport protein particle
VAMP	vesicle associated membrane protein
VAP	VAMP-associated protein

CHAPTER I. INTRODUCTION

The Golgi apparatus is a central organelle in the secretory pathway and plays an essential role in the intracellular trafficking of proteins and membranes. Indeed, the integrity of many cellular membranes and compartments relies upon regulated trafficking to and from the Golgi. Lipids comprise much of these membranes, and it is now appreciated that they themselves are not inert or passive constituents of compartments or vesicles, although our knowledge of specific processes and protein function regulated by lipids is still sparse. An essential role for lipids in Golgi-derived secretion was first demonstrated in *Saccharomyces cerevisiae* where it was discovered that Sec14, the major phosphatidylcholine (PC)/ phosphatidylinositol (PI) transfer protein in these cells, is essential for vesicular transport from the *trans*-Golgi network (Bankaitis et al., 1990; Bankaitis et al., 1989). This thesis investigates factors influencing phospholipid metabolism and their impact on vesicular trafficking at the Golgi.

1.1 PHOSPHATIDYLCHOLINE METABOLISM IN *Saccharomyces cerevisiae*

Phospholipids comprise much of the cellular membranes in all living cells. Due to their amphipathic nature, phospholipids can form bilayers to provide protective barriers from the environment. In eukaryotes, membrane bilayers also enable compartmentalization within cells. While most phospholipids have the same general structure, comprised of a glycerol backbone with two long-chain fatty acids esterified in the *sn*-1 and *sn*-2 positions, variability at the *sn*-3 position gives rise to the different types of phospholipids. Phosphatidylcholine (PC) is the most abundant phospholipid in most eukaryotic cells, and comprises on average 50% of the phospholipid mass (Gaspar et al., 2006; Raetz, 1986).

1.1.1. *de novo* SYNTHESIS

Eukaryotic cells synthesize PC by two major pathways, the CDP-choline (or Kennedy) pathway and the CDP-DAG (or methylation) pathway (Figure 1) (McMaster, 2001). The CDP-choline pathway is present in yeast and all mammalian cells, with the methylation pathway restricted to hepatocytes in mammals (Vance and Walkey, 1998). Choline is imported into the cell *via* the choline transporter, Hnm1. Subsequently, choline is phosphorylated by the enzyme choline kinase (Cki1) or to a lesser extent ethanolamine

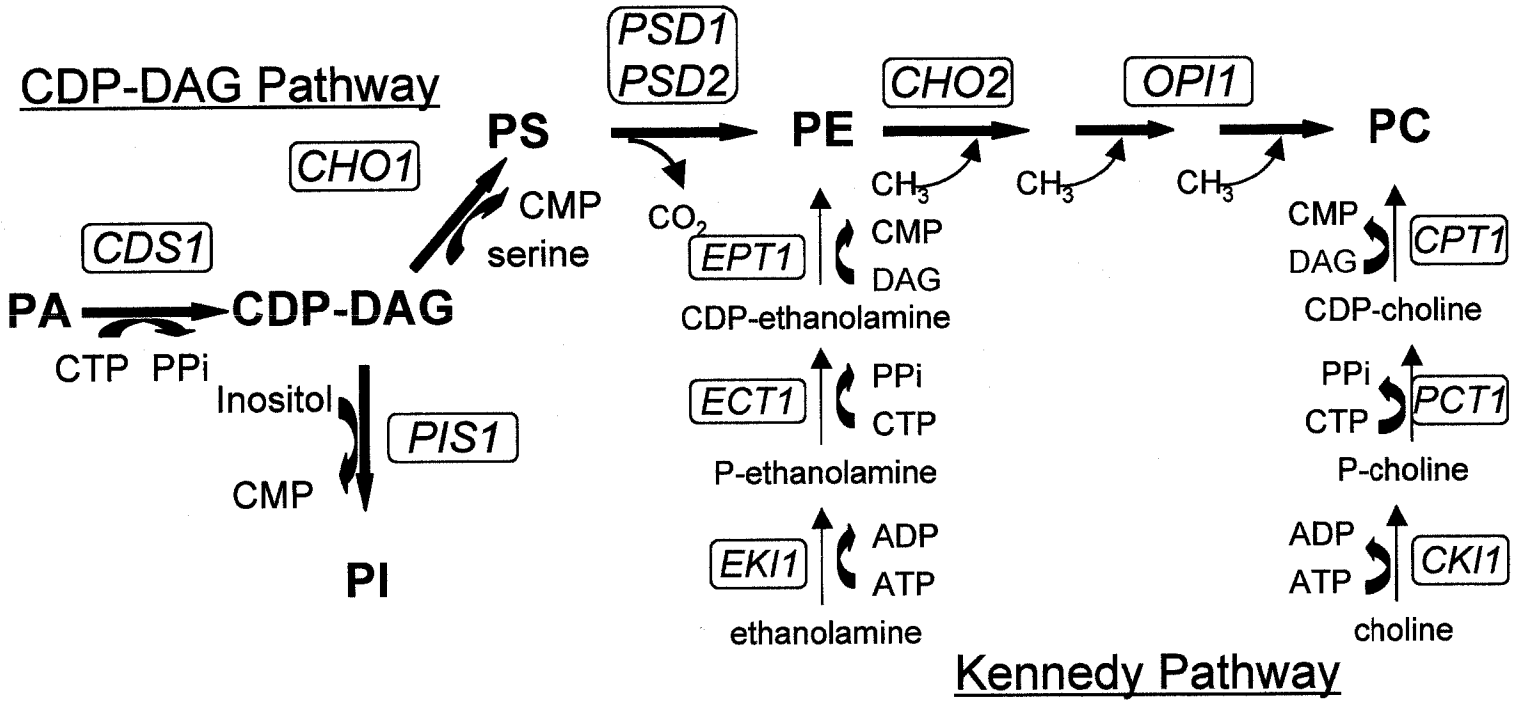


Figure 1. Pathways for the synthesis of phospholipids in yeast. The Kennedy and CDP-DAG pathways are illustrated with genes known to encode the enzyme catalyzing each step indicated in boxes. The insoluble lipid products and intermediates are indicated with blue font, with soluble metabolites in black. Note that a major difference between the two pathways is that the CDP-DAG pathway consumes phosphatidic acid (PA), while conversely the Kennedy pathway indirectly consumes PA.

kinase (Eki1) to yield phosphocholine (P-choline). Conversion of P-choline to CDP-choline is the rate-limiting step of the pathway and is catalyzed by CTP:phosphocholine cytidylyltransferase (Pct1). The final step in the pathway involves the transfer of P-choline from CDP-choline to diacylglycerol (DAG) to produce PC. This final reaction can be catalyzed by two enzymes in yeast, cholinephosphotransferases (Cpt1) or choline/ ethanolaminephosphotransferase (Ept1).

Choline kinase is a soluble enzyme, encoded by *CKI1* in *S. cerevisiae,* with a molecular weight of 66 kDa (Brostrom and Browning, 1973; Kim et al., 1998). While the preferred substrate for Cki1 is choline, ethanolamine can also serve as a substrate. Deletion of *CKI1* causes *S. cerevisiae* to lose nearly 90% of its choline kinase activity, as demonstrated by metabolic labeling studies *in vivo*. The remaining activity is thought to come from Eki1(Kim et al., 1999). Cki1 activity is stimulated by phosphorylation on specific serine residues by protein kinase A and protein kinase C (Carman and Kersting, 2004; Choi et al., 2005; Kim and Carman, 1999; Yu et al., 2002).

Pct1 catalyzes the rate-limiting step *in vivo* and *in vitro* in the *de novo* pathway for PC synthesis, and deletion of *PCT1* eliminates all CTP:phosphocholine cytidyldyltransferase activity, suggesting it is the sole enzyme capable of catalyzing the reaction. As Pct1 is the rate-determining enzyme for the pathway, its regulation appears to be complex. *PCT1* is predicted to encode a protein with an expected molecular weight of 49 kDa that forms homodimers and exists in both membrane-associated and soluble forms (Wilgram and Kennedy, 1963). Pct1 has been characterized as having four functional regions: a nuclear-localization signal; the catalytic domain; a membrane-binding domain; and a phosphorylation domain. The yeast and mammalian Pct1 protein homologues have similar amino acid sequences and the mammalian enzyme has been the subject of many studies. Less is known about the yeast protein. Translocation of the human protein to artificial membranes *in vitro* occurs preferentially to bilayers rich in the fatty acid oleic acid and anionic phospholipids. The limited work with the yeast protein suggests it is stimulated by fatty acids but not by diacylglycerol in PC vesicles (Johnson et al., 1992). The membrane-binding domain of human CCT1 appears to have a dual role, acting as an inhibitor in the absence of stimulatory signals and aiding in the targeting to membranes (Cornell, 1991a; Cornell, 1991b). The phosphorylation domain has a number of potential phosphorylation sites with

phosphorylation thought to negatively regulate the protein by decreasing activity and protein stability (Arnold et al., 1997). Although kinases and phosphatases that phosphorylate and dephosphorylate Pct1 have yet to be identified.

The last step of the CDP-choline pathway is catalyzed by Cpt1 with a small contributions from Ept1 *in vivo* (McMaster and Bell, 1994a; McMaster and Bell, 1994b). Cpt1 has a molecular weight of 46 kDa and is predicted to have multiple transmembrane domains. Consistent with this prediction, during cell fractionation experiments Cpt1 remains solely with the membrane fraction (McMaster and Bell, 1997). To date there have been no described post-translational events that regulate its activity.

The alternative pathway for PC synthesis in yeast, and in the hepatocytes of mammals, is the successive methylation of phosphatidylethanolamine (PE) to PC (Figure 1) (Carman and Han, 2006). Two PE N-methyltransferases, Pem1/Cho2 and Pem2/Opi3, catalyze these reactions in yeast, with both enzymes using *S*-adenosyl-methionine as the donor of the methyl group (Kodaki and Yamashita, 1987; McGraw and Henry, 1989).

1.1.2. CATABOLISM

In addition to its role as a bulk phospholipid for membrane composition, PC is also a participant in signaling pathways by conversion to additional molecules through catabolism by phospholipases (Figure 2) (Pedro Fernandez-Murray and McMaster, 2006). Deacylation at both the *sn-1 and sn-2* positions of PC produces two free fatty acids and glycerophosphocholine (GPC) through the action of phospholipase B (PLB). Three genes encoding PLB activity that is localized to the plasma membrane have been identified in *S.cerevisiae*, with the GPC produced being excreted into the extracellular medium (Fyrst et al., 1999; Lee et al., 1994; Merkel et al., 1999). Recently, an additional PLB activity has been characterized that produces GPC and free fatty acids in response to the presence of choline in the medium, or to growth at elevated temperatures, both conditions known to increase the rate of PC synthesis (Dowd et al., 2001). The gene responsible for this activity has been termed *NTE1* for neuropathy target esterase, as it shares high similarity and function with a similar human protein first identified by the ability to be inhibited by organophosphate ester pesticides, resulting in neuropathy (Murray and McMaster, 2005; Zaccheo et al., 2004).

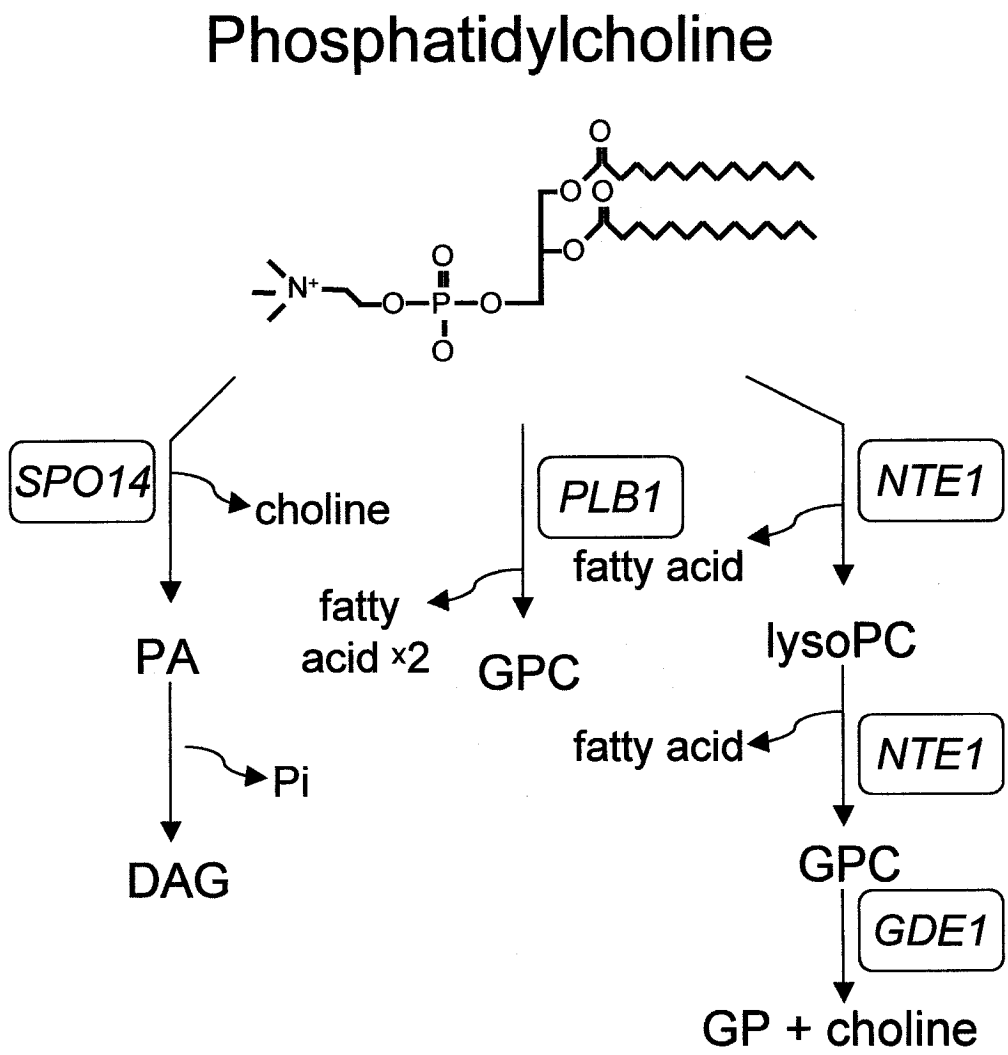


Figure 2. Phosphatidylcholine catabolism in *S. cerevisiae*. Two types of enzymes, phospholipase B (encoded by genes *PLB1* and *NTE1*) and phospholipase D (encoded by *SPO14*) ,can use PC as a substrate. The intermediates and end products generated by each are shown. GPC (glycerophosphocholine), LPC (lysophosphocholine), PA (phosphatidic acid), DAG (diacylglycerol), GP (glycerophosphate) . The enzymes that convert PA to DAG are referred to as PAP (phosphatidic acid phosphohydrolases).

The other enzyme activity known to degrade PC is a phospholipase D (PLD). PLD enzymes catalyze the hydrolysis of PC to phosphatidic acid (PA) and choline (Sreenivas et al., 1998; Waksman et al., 1996). In mammalian cells, PA is an important second messenger and is required for the structural integrity of the Golgi apparatus; however, its role in yeast is less clear (Sweeney et al., 2002). PA can be dephosphorylated to DAG through the action of PA phosphohydrolases. PLDs contain a highly conserved catalytic (His-Lys-Asp, HKD) motif and are stimulated by PI-4,5P_2 (Sciorra et al., 2002).

1.1.3. BIOLOGICAL SIGNIFIGANCE OF DAG

In mammalian cells diacylglycerol is not a prevalent lipid, comprising only 1 mol% of the glycerolipids in membranes (Goni and Alonso, 1999). In yeast cells, DAG composes 3 mol % of glycerolipids (Han et al., 2006). While the level of DAG varies in different species of eukaryotes, DAG is still an important intermediate in the metabolism of triglycerides and glycerophospholipids. Furthermore, localized changes in DAG concentration can alter the biophysical properties of the bilayer, which can ultimately affect signal-transduction pathways and cellular trafficking events like vesicle fission and fusion (reviewed by Goni and Alonso, 1999; Bard and Malhotra, 2006).

DAG can be produced through three main ways in the Golgi. The first is through the production of sphingolipids. In yeast, PI and ceramide are used by phosphatidylinositol: ceramide phosphoinositol transferase (IPC synthase) to produce inositolphosphoceramide and DAG (Nagiec et al., 1997). In the second pathway, PA is converted to DAG, largely by Pah1 (the PA is thought to be derived from either PLD activity or from the sequential acylation of glycerol 3-phosphate by glycerol 3-phospate acyltransferases and lysophosphatidic acid acyltransferase) (Han et al., 2006; Zaremberg and McMaster, 2002). The third source of DAG is from the conversion of PI (or PIPs) to DAG and free inositol (or inositol phosphates) catalyzed by Plc1 (Flick and Thorner, 1993). Each pathway is likely subjected to spatial regulation. Under some experimental conditions predicted to change DAG mass, the mol % of cellular DAG does not change in yeast (Kearns et al., 1997).

The biological effects of DAG are usually associated with altered physical properties of the bilayer such as modifying membrane fluidity, inducing lateral phase separation, and

altering the membrane surface potential (Goni and Alonso, 1999). Early studies involving the mixing of pure DAG with PC demonstrated that the two do not form an ideal mixture (Cunningham et al., 1989; Ortiz et al., 1988). In-plane demixing of PC and DAG results in the generation of domains either rich or poor in DAG. Domains rich in DAG favour a transition from lamellar to nonlamellar phases. Because of their small headgroup relative to their acyl chains, DAGs disturb the normally flat lamellar phase, and thus DAG is more favourably accommodated in membranes that have negative curvature. Thus, DAG on its own may drive vesicular transport through biophysical alterations.

DAG has been described in a number of reports to alter the enzyme activity of several proteins, such as ArfGAPs (discussed later) and the mammalian protein kinase D (PKD) (Antonny et al., 1997; Baron and Malhotra, 2002). PKD is essential for membrane fission from the *trans*-Golgi in mammalian cells (Liljedahl et al., 2001). DAG appears to recruit PKD and activate its kinase activity. One of the targets of PKD is a mammalian Golgi phosphatidylinositol 4-kinase (PI4K IIIβ), an enzyme essential for secretion (Hausser et al., 2005). However, yeast cells do not have a PKD enzyme, yet DAG still appears to be crucial for secretion from the *trans*-Golgi network in yeast. Yeast has a Golgi-resident phosphatidylinositol 4-kinase, Pik1, that contains potential phosphorylation sites similar to its mammalian homologue. It could be that DAG activates a different kinase in yeast, which in turn phosphorylates proteins required for secretion.

1.2. PHOSPHATIDYLINOSITOL METABOLISM IN *Saccharomyces cerevisiae*

Phosphatidylinositol (PI) is an essential phospholipid in all eukaryotes examined thus far. In addition to a role in the bulk composition of membranes, PI is used to produce phosphoinositides (PIPs), inositol polyphosphates (IPx), and, in yeast, complex sphingolipids (IPC) (Flick and Thorner, 1993; York et al., 1999). While PI is a significant portion of the total phospholipid composition in yeast (10-30%), PIPs are present at much lower concentrations and are turned over rapidly. PI and its metabolites have characterized roles in diverse cellular functions, including signal transduction, mRNA export, and vesicular transport (Odom et al., 2000; (Walch-Solimena and Novick, 1999). Even small alterations in PI or PIP metabolism can have a profound impact on the cell.

1.2.1. *de novo* SYNTHESIS OF PHOSPHATIDYLINOSITOL

In yeast, PI is synthesized *de novo* by the CDP-DAG pathway (Figure 1). Both human and yeast PI synthase, Pis1, are localized to the ER and this is the presumed site of PI synthesis . Pis1 levels appear to be unaffected by growth phase or by the presence of inositol or choline in the growth medium, an observation that is unique, as most enzymes involved in phospholipid synthesis are affected by both (Homann et al., 1987). When yeast are grown in the absence of inositol, PI composes 10-12% of the total phospholipid pool, but upon shift to inositol-containing medium PI rapidly expands the pool to 27-30% of the total phospholipid pool (Gaspar et al., 2006; Kelley et al., 1988). This change appears to be a direct result of the K_m of Pis1 for inositol, as cells grown in the absence or presence of inositol have an intracellular inositol concentration of 24 μM, while the K_m of Pis1 for inositol is nearly 10-fold higher at 210 μM (Fischl et al., 1986). However, cells grown in medium supplemented with inositol have >95% of the inositol taken from the medium incorporated into lipids (Angus & Lester, 1971). The observations are consistent with yeast cells being able to takeup inositol from the medium more quickly than produce it internally from glucose.

1.2.2. PRODUCTION OF PHOSPHORYLATED PI

Phosphorylated PIs, or phosphoinositides (PIPs), are derivatives of PI in which one or more hydroxyl group at positions 3,4 and/or 5 of the inositol ring is phosphorylated (Balla, 2005). Several kinases and phosphatases co-ordinate to maintain the appropriate levels of PIPs (Figure 3). A growing body of work has demonstrated that PIPs can be recognized and bound by several different modular protein domains and polybasic regions in proteins. The former include PH, PTB, FYVE, ENTH, PX and FERM domains, which in some cases bind to specific PIPs, while others are less selective (Balla, 2005). The yeast genome contains three genes whose products display PI 4-kinase activity, with *STT4* and *PIK1* being essential and *LSB6* being non-essential (Audhya et al., 2000; Cutler et al., 1997; Han et al., 2002; Walch-Solimena and Novick, 1999). While all three enzymes catalyze the production of PI-4P, they appear to produce largely non-overlapping pools of PI-4P (Audhya et al., 2000; Han et al., 2002).

Pik1 is a soluble enzyme with a predicted size of 199 kDa that localizes to both the Golgi and nucleus (Strahl et al., 2005). The isolation of temperature-sensitive alleles of *PIK1*

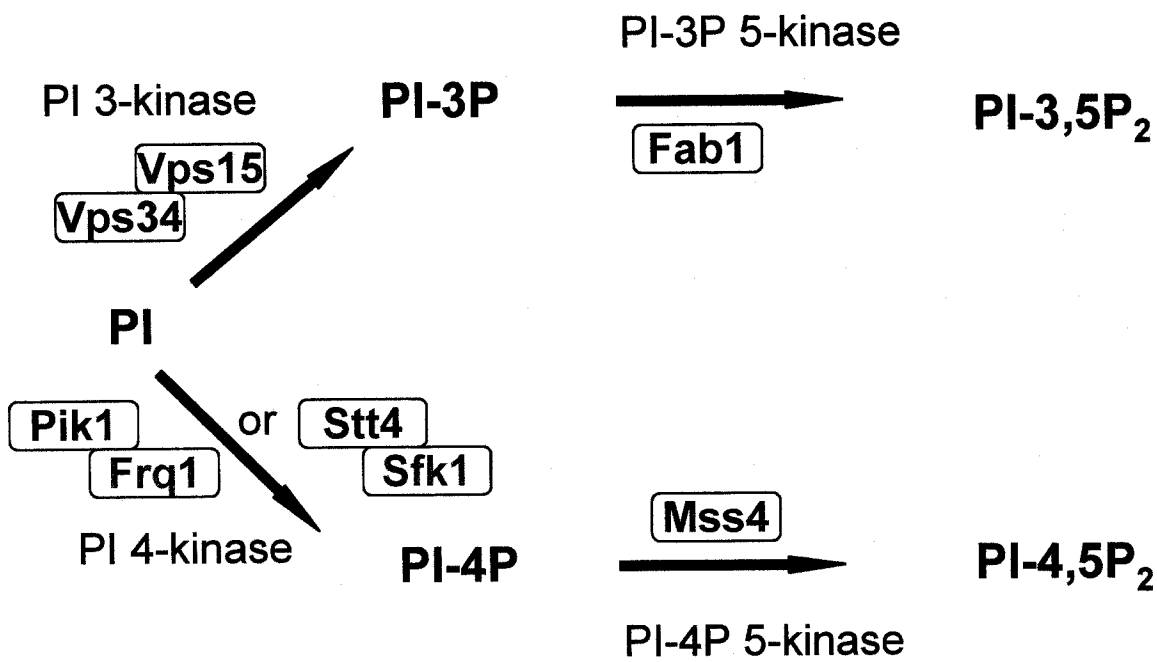


Figure 3. Pathways for the biosynthesis of phosphoinositides in *S. cerevisiae*. The synthetic pathways that mediate the synthesis of mono- and di-phosphorylated phosphatidylinositols are shown. Kinases responsible for the phosphorylation are shown in **Blue**, while regulatory proteins are shown in **Green.** *S. cerevisiae* contain a third PI 4-kinase, Lsb6, which is non-essential and produces ~5% of cellular PI-4P.

has demonstrated that Pik1 activity is required for structural integrity and secretion from the Golgi, as well as affecting other vesicular trafficking events (Walch-Solimena and Novick, 1999). Pik1 also has a role in the nucleus, as cells expressing a Pik1 lacking a nuclear-localization signal are nonviable (Strahl et al., 2005). The K_m of ATP and PI are 100 μM and 50 μM, respectively, for purified Pik1 (Flanagan and Thorner, 1992). *In vivo,* the localization of Pik1 to the Golgi is dependent upon direct protein-protein interactions with the small calcium-binding protein Frq1 (Strahl et al., 2005). Frq1 (the yeast homologue of the human frequenin also referred to as neuronal calcium sensor-1), in addition to recruiting Pik1 to the Golgi, also stimulates Pik1 activity *in vivo* and *in vitro* (Hendricks et al., 1999). Consistent with these observations cells containing a temperature-sensitive *FRQ1* allele display decreased secretion from the Golgi at non-permissive temperatures (Strahl et al., 2005).

Stt4 is a soluble protein with a predicted weight of 215 kDa that localizes to the plasma membrane (Audhya and Emr, 2002). While Stt4 has not been visualized in the ER, Stt4 activity is required for the non-vesicular transport of phosphatidylserine from the ER to the Golgi (Choi et al., 2006; Voelker, 2005). Stt4 (staurosporine and temperature sensitive) is an upstream component of the yeast Pkc1 pathway (Yoshida et al., 1994). Stt4 is localized to the plasma membrane by Sfk1 (suppressor of PI 4-kinase); while it is unknown if Sfk1 is an activator of Stt4 activity *in vitro,* cells over-expressing Sfk1 have increased levels of PI-4P (Audhya and Emr, 2002). Stt4 activity is required for cell-wall integrity and vacuole maintenance (Audhya et al., 2000). Stt4 activity is inhibited by wortmannin, whereas Pik1 is not (Cutler et al., 1997).

Lsb6 is a soluble 55-kDa protein that is localized to both the plasma membrane and the vacuole (Han et al., 2002). Increased expression of Lsb6 can restore growth to cells containing an $stt4^{ts}$ allele but not to $pik1^{ts}$ cells (Han et al., 2002). *LSB6* is not essential, with *lsb6* cells showing a minor (5%) reduction in cellular PI-4P levels. Lsb6 (Las Seventeen Binding partner 6) has a role in controlling endosomal trafficking; however, this is independent of its PI 4-kinase activity (Chang et al., 2005).

PI-4P produced by Stt4, Pik1 and Lsb6 can be used as a substrate by Mss4 to produce PI-4,5P_2. Mss4 is a soluble 90-kDa protein with PI-4P 5-kinase activity (Desrivieres et al., 1998). Mss4 is located in the plasma membrane as well as in the nucleus (Audhya and Emr, 2003). *MSS4* is essential, and cells containing a temperature-sensitive allele of *MSS4* have

defects in actin cytoskeleton organization and the cell integrity pathway at non-permissive temperatures. While Mss4 can be found in the nucleus, cells expressing an Mss4 that lacks its nuclear-localization signal are viable (Audhya and Emr, 2003).

PI can also be used as a substrate by Vps34, a PI 3-kinase, to produce PI-3P (Schu et al., 1993). Vps34 is a soluble protein with a molecular weight of 101 kDa. Vps34 is found in two complexes containing Vps15 and Vps30, with either Vps38 or Apg14 as the fourth component (Kihara et al., 2001). Vps34-produced PI-3P has roles in endosomal sorting, trafficking to the vacuole, and autophagy (Stack and Emr, 1994; Wurmser and Emr, 1998). Cells lacking *VPS34* are viable at 25°C, but display defects in vacuolar protein targeting. GFP-tagged Vps34 is located mainly in endosomes, consistent with the major site of PI-3P localization (Gillooly et al., 2000).

PI-3P can be used by Fab1, a PI-3P 5-kinase, to produce PI-3,5P. Fab1 is a soluble 257-kDa protein that localizes to vacuolar membranes (Yamamoto et al., 1995). *FAB1* is not essential; however *fab1* cells display a temperature-sensitive growth phenotype and have an enlarged vacuole. PI3,5P is found exclusively on vacuolar membranes and appears to play a role in multivesicular-body formation and sorting (Rudge et al., 2004a).

Two additional PIPs (PI-3,4P and PI-3,4,5P) can be synthesized by some eukaryotic cells, but have not been detected in *S. cerevisiae* (Odorizzi et al., 2000). Consistent with this observation, *S. cerevisiae* cell extracts contain no PI-4,5P 3-kinase activity. Recently, PI-3,4,5P was observed in *S. pombe,* a fission yeast, and the production appears to be *via* SpVps34, the *S. pombe* version of Vps34 (Mitra et al., 2004).

1.2.3. CATABOLISM OF PHOSPHORYLATED PI

Phosphoinositides are short-lived molecules within the cell and serve as substrates for two types of enzymes, phospholipase C (Plc1) and PIP phosphatases. Plc1 is essential in stressful environments (Flick and Thorner, 1993; York et al., 1999). Plc1 uses PI, PI-4P and PI-4,5P as substrates, leading to the production of inositol, inositol 4-phosphate or inositol 4,5-phosphate, respectively, as well as DAG. Plc1 may have regulatory roles in vacuole fusion through the generation of DAG, as well as in mRNA production and export through the generation of inositol phosphates (York et al., 1999).

Within the last ten years, enzymes referred to as PIP phosphatases have been identified and characterized in *S. cerevisiae* (Rudge et al., 2004a). Seven PIP phosphatases are encoded in the yeast genome: three contain a promiscuous Sac1 domain that can hydrolyze PI-3P, PI-4P, PI-4,5P and PI3,5P (Sac1, Sjl2 and Sjl3) (Foti et al., 2001); two phosphatases are specific for the D-5 position of PI-4,5P (Sjl1 and Inp54) (Wiradjaja et al., 2001); one is specific for PI-3,5P (Fig4) (Rudge et al., 2004a); and one is specific for PI-3P (Ymr1) (Parrish et al., 2004). In addition, Sjl2 and Sjl3 contain a separate PI 5-phosphatase domain that renders them uniquely capable of converting all major PIPs to PI (Hughes et al., 2000). Several of the phosphatases appear to have over-lapping functions, and for this thesis the important observation is that Sac1, Sjl2 and Sjl3 form an essential triad, mutants lacking all three have a 25-fold increase in PI-4P. These mutants appear to have a delay in secretion and trafficking of invertase and CPY, respectively, causing both proteins to be hypoglycosylated (Foti et al., 2001).

1.3 VESICULAR TRAFFICKING IN *Saccharomyces cerevisiae*

Vesicular transport involves the co-ordination and action of hundreds of proteins that must function in a spatial and temporal fashion to ensure proper trafficking. While the exact mechanisms are being elucidated with respect to specific steps and locations of transport, the process of vesicular transport can be considered to comprise 7 general steps: 1) initiation; 2) budding; 3) scission; 4) uncoating; 5) tethering; 6) docking; and 7) fusion (Bonifacino and Glick, 2004). During initiation, "early" coat proteins are recruited to the donor membrane by binding to GTPases or phosphoinositides. At this point, it is thought that SNARE (soluble NSF attachment receptor) proteins and cargo are recruited to this site. During the course of budding, additional coat proteins are recruited to the growing vesicle, cargo becomes more concentrated and the membrane curvature increases (possibly due to the presence of DAG). Scission is the process by which the membrane is pinched off from the donor membrane. Coat proteins or accessory factors are thought to facilitate the act of scission. For the vesicle to be able to recognize or fuse with a target membrane it must lose its coat. This is accomplished through the inactivation of the small GTPases, protein uncoating factors, and/or through phosphoinositide metabolism. The uncoated vesicle is then tethered to the target membrane through the action of a GTP- bound Rab protein. Docking occurs when the

t-SNARE (on the target membrane) and v-SNARE (on the vesicle) proteins form a four-helix bundle (Fasshauer et al., 1997; Sutton et al., 1998). This SNARE complex promotes fusion of vesicle and acceptor membrane lipids.

1.3.1. ANTEROGRADE TRANSPORT – ER TO GOLGI

Initiation of secretion occurs at the endoplasmic reticulum (ER), where ribosomes associate with the membrane and synthesize protein that becomes translocated into the ER lumen or ER membrane. Within the ER, proteins reside that perform protein quality control and folding, including protein chaperones and disulfide isomerases (Schuldiner et al., 2005). Proteins that have a final destination other than the ER, once folded and properly modified, are sorted into vesicles.

The transmembrane protein Sec12 initiates vesicular transport from the ER (Barlowe and Schekman, 1993). Sec12 is a guanine-nucleotide exchange factor (GEF) for the small GTPase Sar1. GTP-bound Sar1 is activated and this leads to the recruitment of the vesicle coat. The components of this vesicle coat are Sec23/24, the early (or initial) coat proteins, followed by the addition of Sec13/31 (Barlowe et al., 1994). This protein coat is referred to as the COPII protein coat. Once the vesicle is pinched off from the ER, Sec23, a GTPase-activating protein (GAP), stimulates GTP hydrolysis to GDP by Sar1, which in turn initiates uncoating (Yoshihisa et al., 1993).

The cargo proteins are concentrated into COPII vesicles during budding and prior to scission, making vesicular transport a more efficient process (Balch et al., 1994). Transmembrane cargo can bind directly to some of the COPII coat proteins through ER export signals to facilitate their trafficking (Kuehn et al., 1998). These export signals are located on the cytosolic side of the transmembrane protein and are quite diverse, with di-acidic and short hydrophobic patches being two examples. Soluble protein cargo is unable to do bind COPII vesicles directly. To facilitate their transport they bind indirectly to COPII coat proteins by binding to transmembrane export receptors such as Erv14 (Powers and Barlowe, 2002). Clearly, for efficient vesicular transport from the ER to Golgi, proteins that participate in this process (e.g. Sec12 and Erv14) must be recycled from the Golgi back to the ER for subsequent rounds of anterograde transport.

1.3.2. RETROGRADE TRANSPORT – GOLGI TO ER

Transport from the Golgi to the ER, called retrograde transport, is accomplished in a similar manner as anterograde transport to the Golgi. Transport is mediated by COPI coated vesicles with the components being Ret1, Ret2, Ret3, Sec21, Sec26, Sec27, and Sec28 (Letourneur et al., 1994). The formation of the vesicles relies on different small GTPases than Sar1, Arf1/2 (ADP-ribosylation factor) for their formation (Duden et al., 1994; Liang and Kornfeld, 1997). Similar to Sar1, Arf1/2 is activated by GEFs (Gea1, Gea2, Sec7, and Syt1) while inactivated by GAPs, (Gcs1 and Glo3) (Jones et al., 1999; Peyroche et al., 1996; Poon et al., 1999).

Mechanisms exist for enriching cargo into forming COP I vesicles to facilitate transport. Ror instance Rer1 serves as a receptor for returning Sec12 to the ER (Boehm et al., 1997; Sato et al., 1997; Schroder et al., 1995). Ret1 and Sec26 bind to transmembrane proteins containing a di-lysine, KKXX or KXKXX, motif on the cytoplasmic tail for selection into retrograde vesicles. Soluble cargo containing His-Asp-Glu-Leu (HDEL) in their C-terminus are returned to the ER by binding to the transmembrane protein Erd2, also referred to as the HDEL receptor (Lewis et al., 1990; Semenza et al., 1990).

1.3.3. TRAFFIC WITHIN THE GOLGI

The Golgi apparatus is responsible for modifying newly synthesized proteins destined for secretion or transport to the vacuole. Newly made proteins arrive from the ER at the *cis*-Golgi, then transit through the *medial*-Golgi and exit at the *trans*-Golgi. Two models have been developed to explain transport through the Golgi, the cisternal-maturation and vesicle-shuttle models (Glick and Malhotra, 1998). Recent evidence suggests that (in *S. cerevisiae* at least), the cisternal-maturation model appears to be correct.

The cisternal-maturation model postulates that vesicles from the ER fuse to form the *cis*-Golgi. The cisternae then mature into *medial*-Golgi and finally into the *trans*-Golgi. The *trans*-Golgi is then consumed in the formation of transport vesicle to the vacuole and plasma membrane. COPI vesicles retrieve and recycle Golgi-resident proteins at each step, assuring newly formed *cis*-Golgi have the proteins required to mature. In this model, sorting of protein cargo would occur in the *trans*-Golgi just before vesicle formation. In the vesicle-shuttling

model, the *cis-*, *medial-* and *trans*-Golgi are static and remain in place. COPI vesicles deliver cargo between the different cisternae and vesicles bud from the *trans*-Golgi.

The debate has been fueled by the role of COPI vesicles: in which direction are they going and what are they carrying? There are examples in cell biology of certain complexes being too large for COPI vesicles, and which therefore support the maturation model. For example, procollagen bundles and sugar protein conjugates can range from 5-20 times larger than the 50-60 nm size of COPI vesicles (Becker and Melkonian, 1996; Volchuk et al., 2000). Vesicle-shuttling advocates simply argue that the kinetics of budding controls the size of the vesicle formed. However, single-cell microscopy data favour the maturation model (Losev et al., 2006; Matsuura-Tokita et al., 2006). In *S. cerevisae* the Golgi cisternae are not stacked and this situation makes it possible to visualize the maturation dynamics of single cisternae, which is difficult in other organisms. A *cis*-Golgi protein (Vrg4) was tagged with green fluorescent protein and a *trans*-Golgi protein (Sec7) with DsRed fluorescent protein. Over time there was a progression in the single cisternae, from green to yellow to red (Losev et al., 2006). In addition, evidence suggests different cargoes that arrive at the *cis*-Golgi at the same time also exit the *trans*-Golgi at the same time (Matsuura-Tokita et al., 2006). These observations support the cisternal-maturation model.

1.3.4. *TRANS*-GOLGI NETWORK

The *trans*-Golgi network is the location of sorting and packaging of cargo for trafficking to endosomes, the vacuole (or lysosomes) or the plasma membrane. Protein sorting and secretion from the Golgi is essential for eukaryotic cells, and in metazoans allows the specialization of/and signaling between cells.

In addition to COPI-mediated retrograde transport described above, there are at least three described pathways of anterograde traffic from the *trans*-Golgi network (reviewed by Bard and Malhotra, 2006). First, the "Pma1 pathway" used by Pma1 (plasma membrane ATPase) and Bgl2 (an endoglucanase) appear to traffic directly from the Golgi to the plasma membrane (Harsay and Bretscher, 1995). Second, the "ALP pathway" is used by the vacuolar alkaline phosphatase to get to the vacuole (Vowels and Payne, 1998). Third, the "CPY pathway" is used by the carboxypeptidases S and Y and invertase to transit from the *trans*-Golgi network to the endosomes. Cells containing a temperature-sensitive *SEC6* allele, which

is defective an essential component of the multivalent exocyst complex required for fusion at the plasma membrane (TerBush et al., 1996), when shifted to the non-permissive temperature accumulates two types of vesicles (Harsay and Bretscher, 1995; Harsay and Schekman, 2002). One of these has a low buoyant density and contains Pma1 and Bgl2, and appears to come directly from the *trans*-Golgi network. The other is a clathrin-coated high-density vesicle and contains invertase and Gas1, and appears transit through endosomes on its path from the Golgi to the plasma-membrane. Clathrin was the first vesicle protein coat identified and assembles with three distinct adaptor (AP) complexes (Odorizzi et al., 1998). AP complexes are heterotetramers, with AP-1 and AP-3 involved in TGN/endosomal vesicle formation and AP-2 involved in endocytosis at the plasma membrane. Recent studies in cell-free systems have demonstrated that clathrin coat proteins and Vps4 (a dynamin homologue) are required for transport from the *trans*-Golgi network to the late endosomes. It appears CPY pathway require clathrin as its coat, while the ALP pathway uses the AP-3 adaptor complex (Vowels and Payne, 1998). To date, no coat has been identified for the Pma1 pathway and perhaps it does not need one. A vesicle high in DAG may not need a coat to deform the membrane and perform the act of scission (Bard and Malhotra, 2006).

1.4 SECRETION (*sec*) MUTANTS AND Sec14

1.4.1. IDENTIFICATION OF 23 GENE PRODUCTS REQUIRED FOR SECRETION

Initial observations from the lab of Susan Henry suggested that cells could uncouple phospholipid and protein synthesis (Henry et al., 1977). Cells starved for inositol continued to produce macromolecular complexes, yet failed to grow bigger. This observation was extended, and it was predicted that cells that continue to take up nutrients from the medium (but fail to secrete or grow larger) should theoretically have increased density. These mutants of interest could be isolated from a wild-type population by density gradient centrifugation. To this end, *S. cerevisiae* cells were mutagenized and incubated at a non-permissive temperature, and the densest ~2% of the cells were isolated (Novick et al., 1980; Novick and Schekman, 1979). From these isolated mutants, only those that demonstrated a temperature-sensitive (ts) phenotype and were found to be defective in secretion were examined further. Secretion ability or efficiency was measured by the determining the ratio of the activites of

secreted invertase to intracellular invertase. Growth in medium containing low levels of glucose results in the derepression of *SUC2* (invertase) expression. Invertase is translated on the ER and glycosylated, transported to the Golgi for further glycosylation and finally transported to the plasma membrane were it is secreted into the extracellular space (Esmon et al., 1981). The secreted invertase can hydrolyze sucrose into fructose and glucose, the preferred carbon source for yeast. In yeast defective in secretion the synthesized invertase remains within the cell, accumulating in the organelle or vesicles depending upon where the secretion block is located (Novick et al., 1981). The secretion of an acid phosphatase that is induced in the presence of low phosphate medium was also examined.

The screen identified 23 complementation groups containing mutations that decrease secretion at the non-permissive temperatures (Novick et al., 1980). During the course of this experiment it was noted that upon shift to the non-permissive temperature bud emergence quickly stopped, and cells stopped growing with arrest present in all stages of the cell cycle. Visualization of the yeast ultrastructure by electron microscopy revealed that the same organelle did not accumulate in all strains. One class of mutants accumulated 80-100 nm vesicles (secretory vesicles destined for the plasma membrane). Another class of mutants displayed an extensive expansion of the ER with the accumulation of small (50-60 nm) vesicles. The gene products mutated in this case were found to control the traffic from the ER to the Golgi. The third class accumulated unique organelles, referred to as Berkeley bodies (Bbs), which appear to be two curved membranes containing a lumen. Bbs may be analogous to Golgi complexes that have been distorted by continuous vesicular traffic to the Golgi without the initiation of vesicular traffic from the Golgi. The *sec14-1*^{ts} mutant accumulated both Bbs and secretory vesicles (80-100 nm). This findings suggests that Sec14 functions at the Golgi and possibly the plasma membrane, while Sec14 appears to be enriched at the Golgi and ER, it has been visualized throughout the cell (Huh et al., 2003). Conversely, Sec14 functions at the Golgi, and vesicles that are produced in *sec14* cells have altered composition that renders them less competent for fusion with target membranes. Therefore, these vesicles accumulate.

Cell extracts of *S. cerevisiae* contain proteins that are able to facilitate the monomeric exchange of phospholipids between membranes. This activity is not an enzyme or catalyst in the traditional sense, considering that the energy required to remove a single acyl chain from

a membrane is 55 kcal/mol (Zimmerberg, 2006). Sec14 is a protein that has the ability to remove a phospholipid (containing two acyl chains) from a low energy state and transport it to another low energy state. In this sense, Sec14 facilitates a process with a huge energy barrier. Sec14 was found to transfer PI and PC between membranes *in vitro* and was classified as a PI transfer protein (PITP) (Bankaitis et al., 1990). PITPs were originally thought to be involved in transfer of phospholipids between organellar membranes *in vivo*. While this ability may be a component of their function, a growing consensus supports the idea that PITPs act as sensors of membrane composition (Ile et al., 2006). The isolation of spontaneously occuring bypass mutants of Sec14 aided in the deciphering of the *in vivo* role of Sec14.

1.4.2. SEC14-BYPASS SCREEN

The observation that the *sec14*^{ts} cells accumulate spontaneous mutations that allowed growth at the non-permissive temperature resulted in the isolation of 107 spontaneous suppressors of the *sec14*^{ts} phenotype (Cleves et al., 1991). Isolated strains were not simply revertants or secondary mutations within the *sec14-1*^{ts} allele, and therefore the mutations were second-site mutations affecting other genes. The isolated mutants could grow normally at 37°C and their secretion efficiencies were significantly increased compared to *sec14*^{ts} cells. Further analysis identified two dominant complementation groups comprising 81 of the 107 spontaneous suppressors, and five recessive complementation groups. Further analysis determined that all the suppressors were true bypasser suppressors, as these mutations allowed cells lacking the *SEC14* gene to grow and secrete. The "Sec14 bypassers" only suppressed defects associated with *SEC14* and not any of the other 22 known *sec* mutants (Cleves et al., 1991).

Subsequent gene cloning identified the recessive bypass-suppression genes as mutations *SAC1, KES1, CKI1, PCT1* and *CPT1* (Cleves et al., 1989; Fang et al., 1996). The identities of the two dominant bypassers are still unknown, and as a result they are referred to as *BSD1* and *BSD2* (for bypass of Sec14 dominant).

1.4.3 PC METABOLISM

Cki1, Pct1 and Cpt1 are the three enzymes comprising the CDP-choline pathway for PC biosynthesis (Cleves et al., 1991). Inactivation of these enzymes leads to Sec14 bypass, while over-expression of Sec14 inhibits the rate of synthesis of PC through this pathway (Skinner et al., 1995). *In vitro* enzyme assays suggest that Pct1 is the target of Sec14 inhibition of PC synthesis. Furthermore, inhibition of Pct1 activity by Sec14 was dependent upon the phospholipid bound to Sec14, as only Sec14 with PC bound could inhibit Pct1 enzyme activity (Skinner et al., 1995). The CDP-choline pathway consumes DAG and produces PC. Sec14 acts as a diffusible sensor of PC levels to regulate its production, which in turn regulates the consumption of DAG. Thus Sec14, through its ability to alter the biophysical state of membranes or by activating proteins, is a crucial molecule required for secretion from the Golgi.

To examine this further, upon the crystallization and structural determination of the Sec14 protein, the mutant, $Sec14^{(K66,239A)}$ was engineered that no longer binds PI with high affinity (Phillips et al., 1999; Sha et al., 1998). This PC-only Sec14 protein was characterized *in vivo* and was able to support life of otherwise Sec14-lacking cells. As well, this PC-only Sec14 could inhibit PC synthesis through the CDP-choline pathway (Sha et al., 1998). Thus, PI binding by Sec14 is not essential for Sec14-dependent secretion.

Cho2 and Opi3 are the two enzymes in the pathway for PC synthesis through the methylation pathway. When cells are grown on medium deficient in choline, the methylation pathway is the major contributor to PC synthesis (McMaster and Bell, 1994b). These cells secrete choline into the medium, which can be taken up by the choline transporter Hmn1 and used by the CDP-choline pathway. The simultaneous elimination of Hmn1 and either Cho2 or Opi3 is able to bypass the essential nature of Sec14 (Xie et al., 2001). This probably represents an indirect way of inhibiting the CDP-choline pathway (studies have shown that *de novo* synthesis of PC occurs even in the absence of exogenous choline) (McMaster and Bell, 1994b).

1.4.4 PHOSPHOLIPASE D ACTIVITY

SPO14 is required for the growth of all known Sec14-bypass mutant strains therefore; the normally non-essential *SPO14* is required for Sec14-independent life (Xie et al., 1998).

SPO14 encodes the only yeast PC-PLD activity, which catalyzes the conversion of PC to choline and PA (Waksman et al., 1996). The rate of appearance of spontaneous *sec14^{ts}*-bypass mutants is decreased nearly three orders of magnitude in *sec14^{ts} spo14* strains (Xie et al., 1998). This observation suggests that the bypass of Sec14 requires PLD activity. The introduction of *spo14* into the known "Sec14 bypassers" results in death at 37°C and a block in invertase secretion. Re-introduction of specific Spo14 point mutants demonstrated that catalytic activity is required for Sec14-independent life. Spo14 has been localized primarily to endosomes and the plasma membrane in yeast (Li et al., 2000). It remains unclear if Spo14 functions at the Golgi. To date no electron micrographs have been published of a *sec14^{ts}* strain containing an inactivated *SPO14* and a bypass mutation. While it is presumed that invertase secretion is blocked at the Golgi, it remains unproven. An alternative explanation could be that the invertase accumulates in vesicles that cannot fuse with the plasma membrane.

As Spo14 converts PC to PA and choline, its role in bypass may be either the consumption of PC or the accumulation of PA or a metabolite (i.e. DAG). To investigate this further an ethanol challenge experiment was used (Xie et al., 1998). In the Spo14-catalyzed conversion of PC to PA, water acts as a nucleophile. The addition of ethanol (or another primary alcohol) to cells leads to ethanol competing with water in this reaction, leading to the formation of phosphatidylethanol, a largely inert molecule. Both growth and secretion by the Sec14-bypass strains was inhibited in the presence of ethanol (Xie et al., 1998). As PC is still being consumed by Spo14 at a normal rate, the results suggests that PA production is required in the Sec14-bypass strains.

PA can be dephosphorylated to produce DAG, which raises the possibility that Spo14 activity restores DAG levels that are decreased by the CDP-choline pathway. To distinguish between the effects of DAG and PA, yeast cells were transformed with a bacterial DAG kinase (DGK) gene (Kearns et al., 1997). Expression of bacterial DGK in *sec14^{ts}* cells decreased the non-permissive temperature for these cells from 37°C to 35°C. This is consistent with DAG, and not PA, being the molecule of importance in Sec14-deficient cells. Consistent with this, the addition of water-soluble short-chain DAG caused a substantial increase in invertase secretion by cells carrying the *sec14^{ts}* allele. However, the addition of short-chain DAG had no beneficial effects on the growth or secretion of other *sec* mutants

(Kearns et al., 1997). Short chain DAGs do not alter the rate of phospholipid synthesis and do not appear to be metabolized by yeast, and so could be viewed as a pharmacological mimetic of DAG rather than an actual physiologically relevant DAG molecule (Henneberry et al., 2001; Kearns et al., 1997). While the evidence suggests DAG plays a key role in the regulation of vesicular transport from the Golgi, the levels of endogenous DAG do not change under conditions that alter Sec14-independent vesicular transport. It is possible specific pools of DAG are altered that are masked by the large DAG content found in *S. cerevisiae*.

1.4.5. PHOSPHOLIPASE B ACTIVITY

One line of evidence suggests that PC itself may be toxic to Sec14-deficient cells. Overproduction of *PLB1*, a plasma-membrane phospholipase B, is able to partially rescue growth and restore secretion in *sec14*^{ts} strains (Xie et al., 2001). This suggests that the turnover of PC can partially compensate for the lack of functional Sec14, even when DAG is not over-produced. However, the mechanism by which increased expression of the plasma-membrane localized Plb1 suppresses growth defects associated with the loss of Sec14 is not understood. Perhaps the activity of Plb1 aids in the fusion of vesicles with the membrane.

More recently, a gene referred to as *NTE1* was identified that encodes PLB activity that is localized to the ER. The deacylation of PC by Nte1 is enhanced at high temperatures or in the presence of choline, and the preferred substrate for Nte1 is PC produced by the CDP-choline pathway (Dowd et al., 2001; Murray and McMaster, 2005). Nte1 function *in vivo* appears to be linked to Sec14 function as cells lacking a functional Sec14 do not accumulate GPC, while the reintroduction of Sec14 or Sec14$^{(K66,239A)}$ restores the production of GPC (Murray and McMaster, 2005). This suggests that in addition to negatively regulating Pct1 activity, Sec14 also positively regulates Nte1 activity. Furthermore, increased expression of Nte1 raises the restrictive temperature of *sec14*^{ts} cells, allowing growth at 35°C (Murray and McMaster, 2005). Nte1 activity contributes to the regulation of PC levels and leads to the production of free fatty acids that affect the biophysical properties of the membrane.

1.4.6. PHOSPHATIDYLINOSITOL METABOLSIM

SAC1 (Suppressor of ACtin) was initially identified by mutations that could suppress the growth defects associated with mutations in the gene coding for actin. Sac1 is the only known suppressor of actin (*sac*) mutation that is able to bypass Sec14 (Cleves et al., 1989). Sac1 is an integral membrane protein that localizes to the ER and Golgi and has been characterized as a PIP phosphatase. Loss of Sac1 function results in pleiotropic alterations in lipid metabolism including elevated flux through the CDP-choline pathway, a two- to three-fold increase in DAG production, an increase in PI-4P levels, and inositol auxotrophy (Rivas et al., 1999). In addition, *sac1* mediated Sec14 bypass also requires *SPO14*. To determine if increased DAG lead to an increase in flux through the CDP-choline pathway, bacterial DGK was expressed in *sec14*^{ts} *sac1* cells (Rivas et al., 1999). Alteration of the DAG pools by this method resulted in a decrease in flux through the CDP-choline pathway.

The *sac1* cells accumulate PI-4P at levels 8-10-fold higher than do wild-type cells (Rivas et al., 1999). The accumulation of PI-4P is due to the lack of PI-4P phosphatase activity. The accumulation of PI-4P does not appear to be sufficient for *sac1*-mediated Sec14 bypass, as deletion of *SPO14* or expression of DGK did not alter PI-4P levels but did inhibit bypass. Recent evidence suggests that Sac1 dephosphorylates PI-4P generated primarily by Stt4 (Foti et al., 2001). As Sac1 is a resident of the ER, and Stt4 activity is required at the ER for PS transport, this suggests the site of PI-4P accumulation is the ER (Choi et al., 2006; Voelker, 2005). Alternatively, increased levels of PI-4P may activate Spo14 activity in endosomes or at the plasma membrane. It remains unclear the localization of the Spo14 activity required to support Sec14-independent growth. As a result the precise mechanism of Spo14 in supporting vesicular transport is also unknown.

1.4.7. KES1

Kes1 (Kre11 suppressor) is a protein homologous to the human oxysterol-binding protein (OSBP), and one of seven OSBP homologues (OSH) encoded in the yeast genome (Beh et al., 2001; Jiang et al., 1994). However, only inactivation of *KES1,* and not other *OSH* genes tested resulted in bypass of Sec14 (Fang et al., 1996). Six point mutants of Kes1 that were identified in the original bypass screen are shown in Figure 5 along with a tertiary structure of Kes1. In addition, increased expression of any of the other six yeast Osh proteins

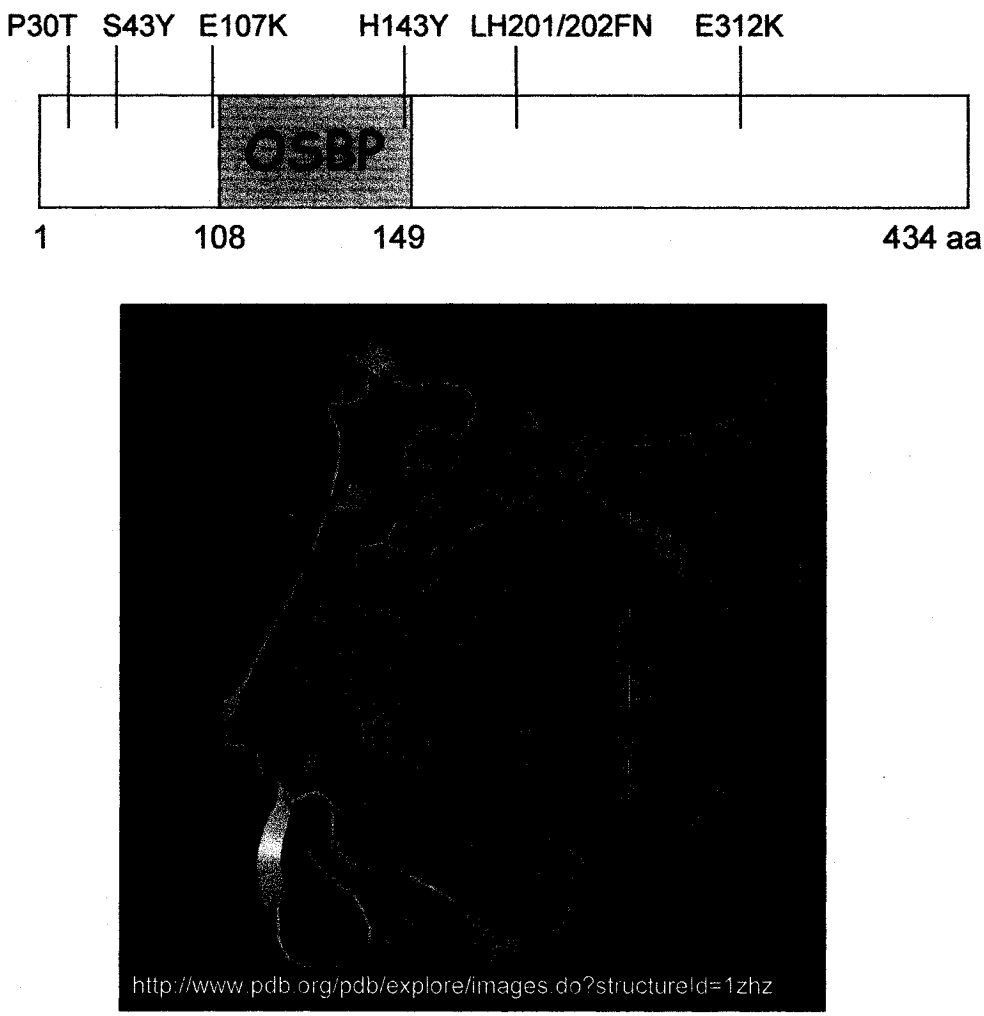


Figure 4. Schematic representation and crystal structure of Kes1. A) Kes1 contains the OSBP signature motif, residues 108-149, that is found in all members of the OSBP family. Six different *kes1* inactivating mutations were found during the "Sec14 bypass" screen and are indicated. B) The three-dimensional structure of Kes1 has been determined with ergosterol residing within the core of the protein.

does not inhibit *kes1*-mediated Sec14 bypass (Beh et al., 2001). Human OSBP has been implicated in the regulation of cholesterol and sphingolipid metabolism (Lagace et al., 1999; Storey et al., 1998), so this suggested that inactivation of *KES1* may alter sterol metabolism that leads to bypass of Sec14. To investigate this further, bulk sterol composition was altered in two ways, using inactivating mutations in *ERG* genes and pharmacologically with zaragozic acid or lovastatin (Fang et al., 1996). None of the alterations in sterol metabolism had any effect on secretion or growth of *sec14*^{ts} strains at 37°C. This suggests that *kes1*-mediated bypass of Sec14 is not related to membrane sterol synthesis. Kes1 is unique among the Sec14 bypassers in that increased levels of Kes1 eliminates bypass mediated by the other mutants. One possible explanation is that Kes1 acts downstream of the CDP-choline pathway (Li et al., 2002). Overexpression of *KES1* phenocopies inactivation of *SPO14*, suggesting Spo14 may be downstream of Kes1. Genetically, Kes1 has been viewed as an inhibitor of Spo14; however, direct biochemical evidence does not exist. This suggests actions of Kes1 may lie upstream of Spo14 function in the secretion pathway (McMaster, unpublished data).

Kes1 binds PI-4,5P_2 (K_d ~ 2.5 μM) and mono-phosphorylated PIPs, the lipid that accumulates in *sac1* cells (Fairn and McMaster, 2005a; Knodler and Mayinger, 2005; Li et al., 2002). Proteomic analysis using yeast cell extracts suggest that Kes1 is the most abundant PI-4P binding protein in yeast (Knodler and Mayinger, 2005). Consistent with this observation, Pik1 generated PI-4P is required for Kes1 to localize to the Golgi (Li et al., 2002). However, in *sac1* cells, Kes1 appears to relocalize to the ER; therefore *sac1* may bypass Sec14 through the redistribution of Kes1 to the ER from the Golgi.

1.4.8. ARF/ARLGAPs

Sec14 is essential and cells lacking Sec14 have pleiothropic alterations in glycerolipid metabolism. To date the only downstream effectors of Sec14-mediated membrane lipid composition in yeast are the ArfGAPs Gcs1 and Age2, which appear to function downstream of Sec14 and Spo14 (Yanagisawa et al., 2002). Gcs1 has both ArfGAP and Arl (Arf-like) GAP activities *in vivo,* and while it is unknown if Age2 has any ArlGAP activity it is known to have ArfGAP activity (Liu et al., 2005; Poon et al., 2001). Genetic and biochemical data suggest that ArfGAP activity is essential for cells, with Gcs1/Age2 activity inhibited by PC and stimulated by DAG and PA (Poon et al., 1999; Poon et al., 2001). As ArfGAPs inactivate

Arf, it is hard to reconcile how activating ArfGAPs, as DAG does, drives vesicular transport from the Golgi. Perhaps DAG prevents excess retrograde transport from the *trans*-Golgi. The other possibility is that attenuating Arl proteins is an important function of DAG to allow secretion. Cells lacking Arf1 have abnormal Golgi structures and do not properly glycosylate invertase but there is no block in invertase secretion. However, there is a block in the maturation of CPY; as CPY and invertase take the same path to the endosomes and then diverge it suggests that endosome to vacuole transport is altered in *arf1* cells, but not transport from the *trans*-Golgi (Gaynor et al., 1998a). Future investigations involving Arl function should help illuminate this situation.

1.4.9. MODEL OF THE SEC14 PATHWAY

The current model of the Sec14 pathway proposes that Sec14 functions not as a transfer protein *per se*, but to maintain Golgi lipid homeostasis (Figure 5). DAG is likely portioned into DAG-enriched and DAG-poor microdomains. While cellular DAG levels remain unchanged, the postulated DAG-enriched membranes are highly competent for secretion from a biophysical standpoint, and DAG also leads to recruitment and activation of molecules like the ArfGAP Gcs1. Currently, Kes1 is interpreted as being an inhibitor of the Arf cycle.

The role of PC metabolism and PC-bound Sec14 in Golgi secretory function is better understood in relation to the involvement of PI and Sec14 function. PC synthesis appears to inhibit secretion from the Golgi through the consumption of DAG. Sec14-PC inhibits the CDP-choline pathway at the rate-limiting step of Pct1. Elevated PC content may also be inhibitory by diluting pro-secretory lipids. Increased expression of *NTE1* suppresses $sec14^{ts}$ but cannot bypass *SEC14*. The PE methylation pathway is not a consumer of DAG, and inactivation of the methylation pathway does not bypass Sec14. Studies of the $sec14^{(K66,239A)}$ mutant demonstrated that PC-only Sec14 can regulate the CDP-choline pathway. The requirement of Spo14 activity is also consistent with the premise that PC is toxic and DAG is stimulatory. Observations that DAG stimulates Gcs1 ArfGAP activity offers a set of potential DAG effectors.

The involvement of PI metabolism and the role of PI-bound Sec14 is less clear. Recently, it was demonstrated that *sec14 cki1* cells have decreased levels of all

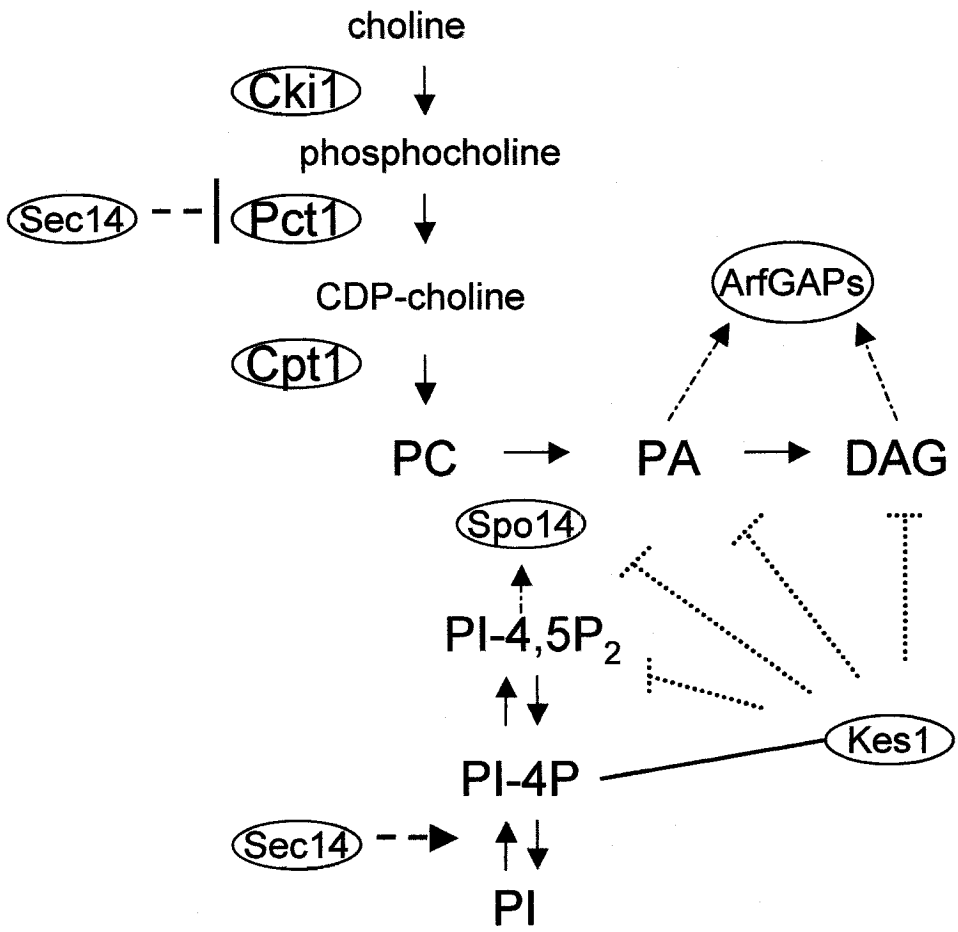


Figure 5. Phospholipid metabolism and the Sec14 pathway. Metabolic steps are indicated with solid arrows. *S. cerevisiae* proteins are indicated by ovals. Experimentally determined inhibitory and stimulatory roles of Sec14 are shown with dashed lines and arrows, respectively. Kes1 is known to bind PI-4P (and PI-4,5P), and proposed inhibitory actions of Kes1 are shown with dotted lines. PI-4,5P_2 is an activator of Spo14 (dotted arrow). DAG and PA have been shown to stimulate ArfGAP activity *in vitro* (dotted arrow).

phosphoinositides. The PI transfer activity of mammalian PITPs is required for the suppression of defects in *sec14*^{ts} mutant cells at non-permissive temperatures. Like mammalian PITPs, Sec14 has also been found to stimulate the human PI 3-kinase activity *in vitro* acting as a co-enzyme. However, mutant Sec14 protein deficient in PI transfer activity is functional in yeast, rescuing both cell viability and secretion. As PI-4,5P_2 is a stimulator of Spo14 activity, PI-bound Sec14 may also indirectly regulate DAG homeostasis.

1.5. SEC14-LIKE PROTEINS AND DOMAINS

1.5.1. *Saccharomyces cerevisiae* SEC14 HOMOLOGUES

The yeast genome encodes five Sec14 homologues, named Sfh1-5 (Sec Fourteen Homolog) (Li et al., 2000). Sfh1 protein has the most similarity with Sec14, being 64% identical at the primary sequence level (Li et al., 2000). However, it displays no PI or PC transfer activity *in vitro,* and is unable to suppress defects of a *sec14*^{ts} strain, likely because it is not expressed in vegetative cells. Sfh2-5 are all ~25% identical and ~45% similar to Sec14 and all display PI transfer activity *in vitro,* but not PC transfer activity. Fluorescence microscopy of GFP-tagged versions of Sfh proteins showed that they are mainly found in the cytosol, with Sfh2 partly enriched in endosomes, and Sfh4 and Sfh5 enriched near the plasma membrane/cortical ER (Schnabl et al., 2003). In the same study, Sec14 was found to be largely cytosolic, with some enrichment at the Golgi.

Increased expression of *SFH2, SFH4,* and *SFH5* is able to suppress growth and secretion defects of *sec14*^{ts} cells at the non-permissive temperature (Li et al., 2000). Increased levels of Sfh2-5 all could restore invertase secretion to ~50% at the non-permissive temperature for *sec14*^{ts}, though Sfh3 could not alleviate the growth defect. Deletion of any individual *SFH* gene has no detrimental effects on growth, nor does the deletion of the entire *SFH1-5* gene set. Deletion of all the *SFH* genes does, however decrease the restrictive temperature for the *sec14*^{ts} allele and prevent the known modes of bypass. Cells deficient for Sfh proteins also have a large decrease in Spo14 activity (Li et al., 2000). Over-expression of Sfh2 could partially rescue *sec14*^{ts} *spo14* cells at the non-permissive temperature and increased levels of Sfh2 or Sfh4 lead to a large increase in Spo14 activity as measured by choline excretion. As previously mentioned, PI-4,5P_2 stimulates Spo14. Stt4 activity appears to be stimulated by Sfh2, while Mss4 activity appears to be stimulated by Sfh5 (Routt et al.,

2005). Increased expression of Sfh5 can also alleviate defects associated with late-acting *sec* mutants $sec9^{ts}$ and $sec15^{ts}$. The growing evidence suggests that Sfh proteins are regulators of PIP synthesis and Spo14 activity. On a mechanistic level, the ability of Sfh2 to rescue *sec14* cells independent of Spo14 could be through the relocalization of Kes1 away from the Golgi by increasing PIP synthesis at the plasma-membrane or ER.

1.5.2. MAMMALIAN SEC14-LIKE PROTEINS AND DOMAINS

The mammalian homologues of Sec14 are referred to as members of the CRAL-TRIO family and include the cellular retinaldehyde protein (CRALBP), supernatant factor (SPF) and α-tocopherol transfer protein (α-TTP) (Bomar et al., 2003; Min et al., 2003; Stocker et al., 2002). The tertiary structures of SPF and α-TTP are very similar to that of Sec14. Like Sec14, CRALBP, SPF and α-TTP bind to PI with about 20-fold greater affinity than PC (Panagabko et al., 2003) . These proteins appear to have evolved additional functions and have high affinities for vitamin A and E and their metabolites. Humans carrying mutations in the α-TTP gene display a dramatic decrease in the circulating levels of vitamin E and possess a number of neuropathological defects associated with vitamin E deficiencies, commonly referred to as Ataxia with vitamin E deficiency (AVED). In addition, the CRAL-TRIO domain is found as part of much larger proteins implicated in cell morphology and polarization pathways. Dbs and Dbl, for example, are Rho GTPases homologues to Cdc24 and their Sec14-homology domains have been shown experimentally to alter the localization (Kostenko et al., 2005; Ueda et al., 2004). Based upon the tertiary structures of Sec14, SPF and α-TTP, the Sec14-homology domain in these proteins likely bind lipid molecules, although the identity of these ligands have yet to be identified.

1.5.3. MAMMALIAN PITPS

The mammalian PITPs are structurally dissimilar to yeast Sec14 and bind to phospholipids with an orientation opposite that of Sec14. Sec14 binds phospholipids with the acyl chains buried within the core of the protein and the head group facing the solvent. PITP α and β bind phospholipids within the lipid head group at the core of the protein and the acyl chains closer to the solvent (Vordtriede et al., 2005). However, like Sec14, PITPs are small (~35 kDa) and have a higher affinity for PI than PC. Sec14 and PITP proteins appear to be an

example of convergent evolution as both PITP α and β are able to suppress Sec14 yeast (Skinner et al., 1993). In addition, both PITPα and Sec14 can increase the formation of PI-3P *in vitro* for a mammalian PI 3-kinase (Jones et al., 1998). Recently, Nir2, a PITP-domain protein, was shown to localize to the Golgi and to be required for secretion. Cells with reduced levels of Nir2 have dispersed Golgi stacks, altered localization of a DAG probe, and defects in export from the *trans*-Golgi (Litvak et al., 2005). A known inhibitor of the CDP-choline pathway (edelfosine) was able to restore secretion in cells where Nir2 protein levels were greatly reduced. Previous work demonstrated that the PITP domain of the *Drosophila* Nir2, homologue rdgB, is essential for its function (Milligan et al., 1997). Whether or not Nir2 can inhibit the CDP-choline pathway is unknown. The data suggest that while metazoans have retained the Sec14 structure, additional PITPs have evolved to perform the roles of Sec14 with regard to phospholipid homeostasis.

1.6. OXYSTEROL BINDING PROTEIN (OSBP) HOMOLOGUES

1.6.1. OSBP

Oxysterols are 27-carbon oxygenated derivatives of cholesterol produced through both enzymatic and non-enzymatic mechanisms. Oxysterols are present in tissues at very low concentrations but are potent signaling molecules within the cell. A large number of their effects are by acting as ligands for nuclear receptors of the liver X receptor family. However, not all effects of oxysterols can be attributed to gene regulation (Bjorkhem and Diczfalusy, 2002; Russell, 2000; Schroepfer, 2000). A search for protein mediators of oxysterol action led to the identification of OSBP.

OBSP was identified as a high-affinity cytosolic receptor for oxysterols, but does not appear to directly alter transcription (Kandutsch et al., 1984; Dawson et al., 1989; Ridgway et al., 1992). OSBP is a 110-kDa protein that translocates to the Golgi apparatus upon treatment of cells with 25-hydroxycholesterol (Ridgway et al., 1992). OSBP overexpression in Chinese hamster ovary cells enhanced the biosynthesis of cholesterol and sphingomyelin (Lagace et al., 1997; Lagace et al., 1999). OSBP contains a couple of protein domains, an N-terminal Pleckstrin-homology (PH) domain and a C-terminal OSBP-related domain (ORD), shared by all OSBP homologues. The PH domain is known to bind PIPs and is required for localization to the Golgi (Levine and Munro, 1998; Levine and Munro, 2002). Recently, a protein-protein

interaction motif was identified that mediates OSBP binding to VAMP-associated protein (VAP) and was mapped to a region just outside of the ORD domain (Amarilio et al., 2005; Kawano et al., 2006; Loewen and Levine, 2005; Loewen et al., 2003; Wyles et al., 2002). VAP binds to proteins containing a FFAT motif (2 phenylalanines in an acid tract); proteins containing this motif include OSBP homologues, and other lipid binding/regulatory proteins such as Nir1-3, CERT and yeast Opi1 (Amarilio et al., 2005; Kawano et al., 2006; Loewen and Levine, 2005; Loewen et al., 2003; Wyles et al., 2002). OSBP appears to influence SM synthesis by stimulating CERT-mediated ceramide transport from the ER to the Golgi (Perry and Ridgway, 2006). The ability of OSBP to enhance SM synthesis requires the targeting to the ER (through its FFAT motif and interaction with VAP) and the Golgi (through its PH domain and interaction with Arf1). Genetic knockdown experiments using RNA interference (RNAi) demonstrated that OSBP is required for CERT optimal CERT-mediated transfer (Perry and Ridgway, 2006). The over-expression and depletion of OSBP reveals that it is not directly responsible for the effects of 25-hydroxycholesterol on cholesterol synthesis (Perry and Ridgway, 2006). Instead, it appears that OSBP acts as a sensor of sterol metabolism and is able to co-ordinate sterol and SM metabolism.

In addition to its role in regulating SM synthesis, OSBP has been characterized as a sterol-responsive regulatory scaffold for two protein phosphatases that attenuate the activity of the extracellular signal-related kinases 1 and 2 (ERK1/2) (Wang et al., 2005b). To date it is unknown if CERT activity is regulated by phosphorylation, as this could be the mechanism by which OSBP regulates CERT activity. Conversely, if the primary function of OSBP is in regulating SM synthesis, perhaps OSBP has evolved additional roles in cell signaling. In this way, OSBP could act as a sterol sensor that co-ordinates membrane synthesis with other cellular activities. An alternative explanation is that sterol binding acts as a conformational switch, which permits additional functions to be revealed. In this way, sterol binding may not be sterol sensing *per se,* but would act as an obligate protein co-factor, similar to prenylation of the Ras family of proteins.

1.6.2. OSBP HOMOLOGUES IN *Saccharomyces cerevisiae*

S. cerevisiae has seven OSBP homologues (Osh) (Beh et al., 2001). Three of these proteins contain a C-terminal OSBP related domain (ORD) and N-terminal extensions

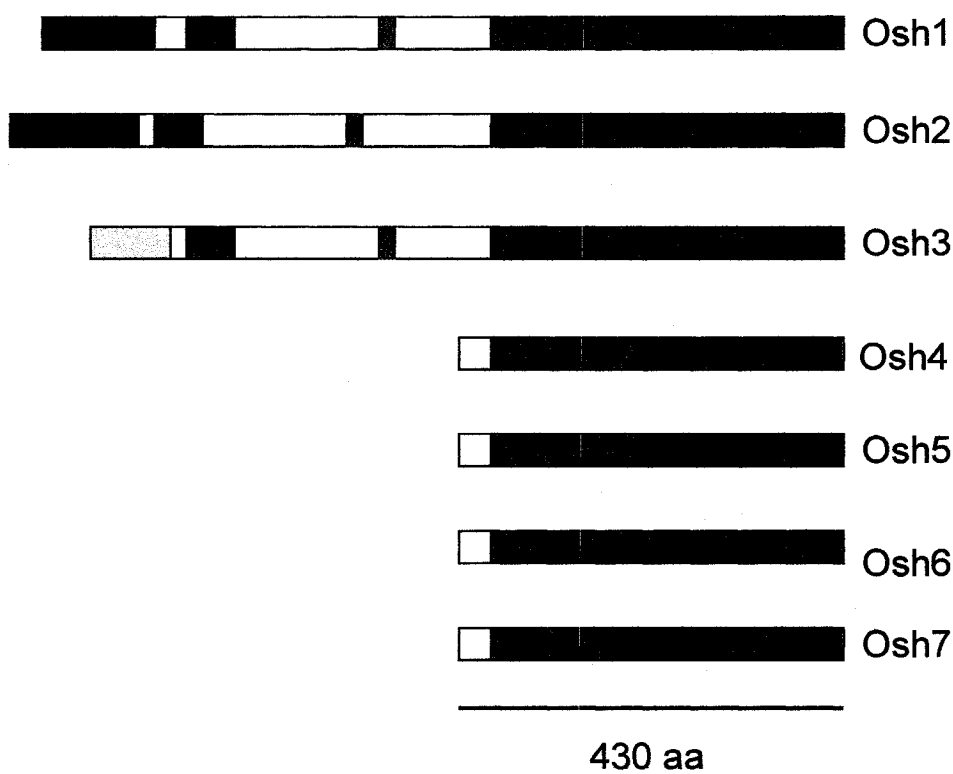


Figure 6. *S. cerevisiae* encodes seven oxysterol binding protein homologues. Schematic representation of the domain structures of the seven Osh proteins are shown. The blue region represents the oxysterol-homology domain with the green strip representing the OSBP signature motif. The Osh proteins can be subdivided into two general groups, long (Osh1-3) and short (Osh4/Kes1-Osh7). Osh1-3 contain additional protein motifs including Ankyrin repeats (black), PH domains (purple), Gold domain (gold) and FFAT motif (red).

containing PH domains and anykyrin repeats (Figure 6). The high molecular-weight yeast Osh proteins also contain a FFAT domain that has been shown to interact with Scs2 (the yeast homologue of VAP) (Loewen et al., 2003). The other four yeast Osh proteins consist of only the ORD. One of these genes, *OSH4*; is also referred to as *KES1* and was described earlier. *KES1* is unique as it encodes the only Osh protein that appears to regulate vesicle biogenesis from the Golgi.

Recent studies involving Osh6 and Osh7 suggest they may influence endosomal sorting or conversely are subject to regulation by Vps4, an endosomal AAA-ATPase (Wang et al., 2005a). The interaction between Osh6/7 and Vps4 appears to be regulated by sterol occupancy of the Osh proteins. When Osh6/7 are bound to ergosterol their interaction with Vps4 is abolished.

Disruption of all seven yeast *OSH* genes results in lethality, suggesting that the Osh proteins may share an overlapping essential function (synergistic roles) (Beh et al., 2001; Beh and Rine, 2004). Experiments employing a controllable expression *OSH2* gene revealed that a cell lacking all seven yeast Osh proteins has a 3.5-fold increase in ergosterol as well as defects in endocytosis (Beh and Rine, 2004). In about half of these cells ergosterol accumulated in an intracellular compartment. Recently, it was demonstrated that *OSH* genes interact genetically with those encoding the small GTPases Cdc42 and Rho1 (Kozminski et al., 2006a). Cdc42 regulates polarization and exocytosis. Increased expression of Osh proteins rescued cells with a thermally labile $cdc42^{ts}$ allele at the non-pemissive temperature. Furthermore, cells deficient for Osh function exhibited a depolarization in actin cytoskeleton, mislocalization of several GTPase (Rho1, Cdc42 and Sec4), and a defect in vesicle fusion at the plasma membrane (Kozminski et al., 2006a).

1.6.3. STRUCTURE

Recently, the lab of James Hurley produced a 3-D crystal structure of Kes1 with and without sterols bound internally, within the core of the protein (Im et al., 2005). The core of the structure is 19 anti-parallel β-sheets that forms a near complete anti-parallel β-barrel. This structure is able to accommodate both sterol and oxysterol ligands. The structure also contains a flexible lid. In the sterol-bound structure the lid protects the sterols from the

solvent. Crystals could not be obtained for the holo protein unless the N-terminal 29 amino acids corresponding to the lid were removed, suggesting the lid is flexible. The ability of Kes1 to bind sterols within the core also suggests that the Osh proteins could be sterol transporters. While it had been previously reported that Kes1 contains a PH-like domain (Li et al., 2002), examination of the tertiary structure clearly indicates there is no PH domain.

1.6.4. POSSIBLE FUNCTION AS STEROL TRANSPORTERS

The transport of newly formed sterols from the ER to the plasma membrane is not inhibited by blocks in vesicular transport (for example, in *sec18^{ts} or sec14^{ts}* yeast cells at the non-permissive temperature) (Baumann et al., 2005). This suggests that the classical vesicular-transport pathway to the plasma membrane is not the only way to transport sterols. Protein-mediated non-vesicular transport could accomplish the movement. Sterol carrier protein 2 is found in Eukaria, Bacteria and Archaea; however, it is not present in *S. cerevisiae* or *S. pombe* (Edqvist and Blomqvist, 2006; Ferreyra et al., 2006). Steroidogenic acute regulatory protein (StAR) related lipid transfer (START) domain proteins are also thought to transport sterols in such a manner. *S. cerevisiae* contain at least one START domain protein, Coq10; however, its *in vivo* function appears to influence polyketide metabolism (Barros et al., 2005). The crystal structures of the START domain and Kes1 demonstrate a similar tertiary structure to accommodate sterol binding (Tsujishita and Hurley, 2000). This suggests that in yeast the Osh proteins could be sterol transporters.

In vitro experiments using Kes1 and several mutant Kes1 proteins showed that Kes1 could bind to cholesterol and oxysterols, with a disociation constant of 300 nM and 50 nM, respectively (Im et al., 2005). Kes1 was also able to extract cholesterol from liposomes and transfer it to acceptor membranes *in vitro* (Raychaudhuri et al., 2006). *In vivo* experiments measuring the uptake of radiolabeled sterols from the medium and conversion to sterol ester in the ER were used to assess *in vivo* transfer activity of Osh proteins. While cells deficient in Osh proteins showed a large reduction in sterol transfer (~50%), it was not completely blocked. Osh3 and 5 appeared to be the largest contributors to the activity. Single deletion mutants of the other *OSH* genes showed little to no loss in transfer activity. In the same report, Kes1 was shown to extract and transfer PI-4,5-P_2, though to a lesser degree than

cholesterol. This raises the possibility that other Osh proteins may transfer lipids other than sterols.

1.6.5. OSBP RELATED PROTEINS IN HUMANS

The OSBPs (oxysterol binding proteins) and ORPs (OSBP-related proteins) constitute an enigmatic protein family that is united by a signature domain that binds oxysterols, sterols or possibly phospholipids (Olkkonen and Levine, 2004). In humans there are 12 genes that, through pre-mRNA splicing, code for at least 16 predicted ORPs (Jaworski et al., 2001; Lehto et al., 2001; Lehto and Olkkonen, 2003). OSBP and ORPs are usually grouped into 6 subfamilies based upon homology (reviewed by Olkkonen and Levine, 2003; Figure 7). With the exception of Orp2, all are long Orps, with variable N-terminal regions. In addition to the FFAT motif and PH domains, two Orps (5 and 8) are predicted to contain a C-terminal transmembrane domain. Previous work demonstrated that ORP1S can phenocopy Kes1 in yeast, however its although homologue ORP2 could not (Xu et al., 2001).

Two splice variants of ORP1 have been identified: one consisting of the C-terminal ligand binding domain only, ORP1S (437 aa), and a long N-terminally extended form, ORP1L (950 aa), containing three ankyrin repeats and a PH domain (Johansson et al., 2003). Expression profiles demonstrate that ORP1S predominates in heart and skeletal muscle, while ORP1L is more abundant in the brain and lung. In mammalian cell culture, ORP1L localizes to the late endosomal compartments and appears to influence endosomal trafficking through Rab7 by regulating its GTP-GDP cycling (Johansson et al., 2005). Transport of fluorescent dextran to the late endosomes and vacuolation of the late endosomes by the *H. pylori* protein Vac A are both Rab7- dependent processes and are inhibited in cells over-expressing ORP1L (Johansson et al., 2005).

The cell biology of the ORP family as a whole is in its infancy, and assessing the available information, it appears that any single model for their actions will likely be insufficient.

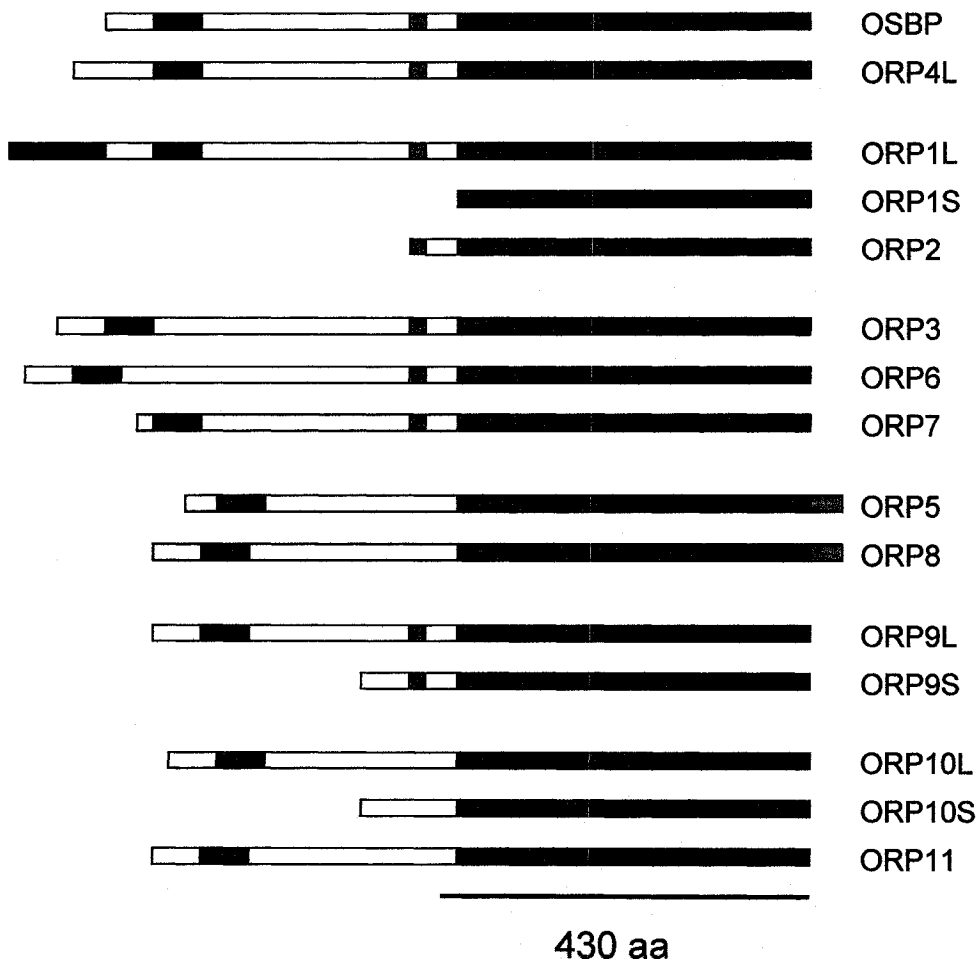


Figure 7. The Human Oxysterol Binding Protein Related Protein Family. Schematic representation of the domain structures of the 12 members of the ORP family. The ORPs can be further grouped into 6 subfamilies based upon gene structure and sequence homology. The blue region represents the OSBP homology domain with the green strip representing the OSBP signature motif. Additional protein motifs are located within the N-terminal region of the proteins, including Ankyrin repeats (black), PH domains (purple), and FFAT motif (red). Orp5 and 8 contain predicted transmembrane domains at the C-terminus (orange). Splice variants of interest for this work are ORP1S, ORP9S and ORP10S.

CHAPTER 2. MAJOR OBJECTIVES

Kes1 is a member of an enigmatic protein family in *S. cerevisiae* referred to as the oxysterol binding protein homologues (Osh). Osh proteins are identified by a common structural domain that binds sterols/oxysterols and have an overlapping essential function currently thought to be related to sterol metabolism. Kes1 is a unique member of the family, as it is the only member that negatively regulates secretion from the *trans*-Golgi network. The mechanism of this inhibition is unknown. The objective of this study was to identify the mechanism of Kes1 function. Through the identification of novel genetic interactions involving *SEC14* (encoding a regulator of phospholipid metabolism) *CKI1* (encoding the enzyme for the first step in the CDP-choline pathway) and *KES1,* the function of Kes1 was investigated. In addition, four human Kes1 homologues were characterized to investigate the degree of evolutionary conservation of function from yeast to human Kes1-like proteins.

CHAPTER 3. MATERIALS AND METHODS

3.1.1. MATERIALS

Reagents

All molecular biology reagents used in this study were purchased from New England Biolabs (NEB), Invitrogen, or Qiagen. Custom oligonucleotides were purchased from Invitrogen or Integrated DNA technology (IDT). All materials used in the preparation of bacteria or yeast media were purchased from Difco or Q-biogene. [Methyl-^{14}C]choline chloride was purchased from American Radiolabelled Chemicals. [γ-^{33}P]ATP and Express protein labeling mix ([^{35}S]methionine and cysteine) were purchased from Perkin Elmer. Phospholipids were purchased from Avanti Polar Lipids except for phosphorylated phosphatidylinositols, which were purchased from Echelon. Affinity resins where purchased from Clontech (Talon), Sigma (rabbit IgG agarose), Stratagene (calmodulin agarose) and GE Healthcare (glutathione sepharose). Antifungal compounds were purchased from Sigma (G418, referred to as KAN, L-canavanine, referred to as CAN, and hygromycin B), Werner BioAgents (nourseothricin, referred to as NAT) and PCR Inc. (5-fluoroorotic acid, referred to as FOA). Protease inhibitors used in this study included complete and EDTA-free tablets (Roche), as well as leupeptin, pepstatin A and phenylmethylsulfonyl fluoride (PMSF) (Sigma).

Yeast media

Rich medium was YEPD (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose); on occasion dextrose was replaced by raffinose or galactose. Minimal medium was SD (2% dextrose, 0.67% bacto-yeast nitrogen base without amino acids), supplemented to satisfy the seven most commonly occurring auxotrophies (20 mg/L adenine sulfate, 20 mg/L L-histidine HCl, 100mg/L L-leucine, 30 mg/L L-lysine, 20 mg/L L-methionine, 20 mg/L L-tryptophan, and 20 mg/L uracil). For maintenance of a particular plasmid, the appropriate supplement was omitted.

Diploids were sporulated in liquid sporulation medium (1% potassium acetate, 0.1% bacto-yeast extract, 0.05% dextrose).

Creation of yeast strains and plasmids

Described below is the creation of several yeast strains in which selected genes have been replaced by selectable nutritional or drug-resistance markers. Described below is the first replacement of a given gene with a specific marker. In some cases the gene replacement was repeated in additional strains using the conditions described below.

CMY209 (*ckil*::NAT^R)

To generate a chromosomal replacement of *CKI1*, the NAT^R gene from pAG25 (Euroscarf) was amplified by PCR using primers 5'-cki1::NAT (5'-TGTAAGAAGTTACTCGCTCGGTTACCAAGCAAGGTCCAGAACATGGAGGCCC AGAATACCC) and 3'-cki1::NAT (5'-TTACAAATAACTAGTATC-GAGGAACTTGAAAGAGCTGAAATTTCAGTATAGCGACCAGCATTCAC). The linear product was transformed into yeast and nourseothricin-resistant tranformants were screened by PCR of genomic DNA using primers hybridizing 500 bp upstream and downstream of the *CKI1* ORF, followed by an analytical restriction enzyme digest using KpnI.

CMY210 (*kes1*::NAT^R)

To generate a chromosomal replacement of *KES1*, the NAT^R gene from pAG25 was amplified by PCR using primers 5'-kes1::NAT (5'-ATGTCTCA-ATACGCAAGCTCATCCTCATGGACTTCTTTCTTGAAGTCAATCACATGGAGGC CCAGAATACCC) and 3'-kes1::NAT (5' TTACAAAACAAT-TTCCTTTTCTTCGTCCCACAACTCTCTTTGGAATCTCCACAGTATAGCGACCAG CATTCAC). The linear product was transformed into yeast and nourseothricin- resistant tranformants were screened by PCR of genomic DNA using primers hybridizing 500 bp upstream and downstream of the *KES1* ORF, followed by an analytical restriction enzyme digest using KpnI.

CMY212 (*ckil*::NAT^R *sec14::LEU2*) and CMY213 (*kes1*::NAT^R *sec14::LEU2*)

To generate a chromosomal replacement of *SEC14,* the *LEU2* gene was amplified from pRS415 vector by PCR using primers 5'-sec14::LEU2 (5'-

ATGGTTACAGTATGTTGTTGCTTTTATTTACTTTTTCTTTTTTTGACATTCGAG GAGAACTTCTAGTATATC) and 3'-sec14::LEU2 (5'-TCATTTCATC-GAAAAGGCTTCCGGAGCTTCACCTTCCGGTCCAATTTCAAGAAGGTATTGACT TAAAC). The linear product was transformed into yeast and Leu+ transformants were screened by PCR of genomic DNA using primers hybridizing 500 bp upstream and downstream from the *SEC14* ORF to detect *sec14::LEU2*.

CMY510 (*sec14-1^{ts}*:NAT^{R} *cki1::LEU2*)

To generate a chromosomal replacement of *CKI1* in the CMY503 (*sec14-1^{ts}*:NAT^{R}) strain, the *LEU2* gene was amplified from pRS415 vector by PCR using primers 5'-cki1::LEU2 (5'-GTAAGAAGTTACTCGGTCGGTTACCAAGCAAGG-TCCAGAGAGGAGAACTTCTAGTATATCCAC) and 3'-cki1::LEU2 (5'-TTACAAATAACTAGTATCGAGGAACTTGAAAGAGCTGAAATTTTTCAAGAAG GTATTGACTTAAACTCC) The linear product was transformed into yeast and Leu+ transformants were screened by PCR of genomic DNA using primers hybridizing 500 bp upstream and downstream from the *CKI1* ORF to detect *cki1::LEU2*.

GFY101 (*sec14-1^{ts}*:NAT^{R} *kes1::LEU2*)

To generate a chromosomal replacement of *KES1* in the CMY503 (*sec14-1^{ts}*:NAT^{R}) strain, the *LEU2* gene was amplified from pRS415 by PCR vector using primers 5'-kes1::LEU2 (5'-CTCCATCGATTATGTGGTTCTACAAAATTGGAGG-AGAACTTCTAGTATATCCAC) and 3'-kes1::LEU2 (5'- CCGTTCGCGTTT-TACTAGCTGCAGCATTTTTGGGTTCAAGAAGGTATTGACTTAAACTCC). The linear product was transformed into yeast and Leu+ transformants were screened by PCR of genomic DNA using primers hybridizing 500 bp upstream and downstream from the *KES1* ORF to detect *kes1::LEU2*.

CMY512 (*sec14-1^{ts}*:NAT^{R} *cki1::LEU2 kes1::*HYG^{R})

To generate a chromosomal replacement *KES1* in the CMY510 strain, the hygromycin-resistance cassette was amplified from pAG32 (Euroscarf) by PCR vector using primers 5'-kes1::HYG (5' ATGGTTACAGTATGTTGTTG-

Table 1. Yeast strains used in this study

Yeast Strain	Genotype	Source
BY4741	**a** *his3 leu2 met15 ura3*	EUROSCARF; Brachmann et al. (1998)
Y2454	α *mfa1D::MFRA-pr-HIS3 can1 his3 leu2 ura3 lys2*	Boone Lab; Tong et al. (1999)
CMY209	Y2454; *cki1::*NAT^R	this study
CMY210	Y2454; *kes1::*NAT^R	this study
CMY212	CMY209; *sec14::LEU2*	this study
CMY213	CMY210; *sec14::LEU2*	this study
CMY214	Y2454; *pct1::*NAT^R	this study
CMY215	Y2454; *cpt1::*NAT^R	this study
CMY503	Y2454; *sec14-1^{ts}:*NAT^R	McMaster Lab; Curwin (unpublished)
CMY510	CMY503; *cki1::LEU2*	this study
CMY512	CMY510; *kes1::*HYG^R	this study
GFY101	CMY503; *kes1::*HYG^R	this study
GFY300	CMY510; *trs33::*KAN^R	this study
GFY303	CMY510; *trs65::*KAN^R	this study
GFY306	CMY510; *trs85::*KAN^R	this study
GFY309	CMY510; *ypt31::*KAN^R	this study
GFY312	CMY510; *ypt32::*KAN^R	this study
GFY320	CMY512; *trs33::*KAN^R	this study
GFY323	CMY512; *trs65::*KAN^R	this study

GFY326	CMY512; *trs85*::KAN^R	this study
GFY329	CMY512; *ypt31*::KAN^R	this study
GFY332	CMY512; *ypt32*::KAN^R	this study
GFY340	GFY101; *trs33*::KAN^R	this study
GFY343	GFY101; *trs65*::KAN^R	this study
GFY346	GFY101; *trs85*::KAN^R	this study
GFY349	GFY101; *ypt31*::KAN^R	this study
GFY352	GFY101; *ypt32*::KAN^R	this study
SEY6210	*MATα leu2 ura3 his3 trp1 lys2 suc2*	Emr Lab; Robinson et al. (1998)
AAY102	SEY6210; *stt4::HIS3* [pRS415-stt4-4 *(stt4-4^{ts} LEU2 CEN6)*]	Emr Lab; Audhya et al. (2000)
AAY104	SEY6210; *pik1::HIS3* [pRS314-pik1-83 *(pik1-83^{ts} TRP1 CEN6)*]	Emr Lab; Audhya et al. (2000)
AAY1139	SEY6210; pik1::HIS3 + [pRS415-pik1-139 (*pik1-139^{ts} LEU2 CEN6*)	Emr Lab; Sciorra et al., (2005)
VSY446	SEY6210; *trs130-(33aa truncation)-HA^{ts}:HIS3MX6*	Emr Lab; Sciorra et al., (2005)
VSY468	SEY6210; *ypt32::TRP1 ypt31-101^{ts}:URA3*	Emr Lab; Sciorra et al., (2005)
GFY201	AAY104; *kes1*::KAN^R	this study
GFY207	AAY102; *kes1*::KAN^R	this study
GFY209	VSY446; *kes1*::KAN^R	this study
GFY211	VSY468; *kes1*::KAN^R	this study

BY4741, sac1	BY4741; *sac1*::KAN^R	EUROSCARF; Winzeler et al. (2001)
GFY212	BY4741; *sac1*::KAN^R *kes1*::NAT^R	this study
R1158	*MAT***a** *ura3::CMV-tTA his3 leu2 met15*	OPEN BIOSYSTEMS; Mnaimneh et al. (2004)
GFY213	R1158; *prPIK1::kanMX-tet07-TATA*	OPEN BIOSYSTEMS; Mnaimneh et al. (2004)
GFY214	GFY213; *kes1*::HYG^R	this study
GFY215	R1158; *prFRQ1::kanMX-tet07-TATA*	OPEN BIOSYSTEMS; Mnaimneh et al. (2004)
GFY216	GFY215; *kes1*::HYG^R	this study
GFY217	BY4741; *kes1*::KAN^R	EUROSCARF; Winzeler et al. (2001)
GFY218	GFY217; *frq1*::NAT^R	this study
CBY926	SEY6210; *osh1Δ::KAN^R osh2Δ::KAN^R osh3Δ::LYS2 osh4Δ::HIS3 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3* [*osh4-1^{ts} TRP1 CEN6*]	Beh Lab; Beh & Rine (2004)
GFY400	CBY926; [pESC-URA *(URA3 CEN6)*]	this study
GFY401	CBY926; [pESC-KES1 (*URA3 CEN6 KES1)*]	this study
GFY402	CBY926; [pESC-ORP1S (*URA3 CEN6* human ORP1S)]	this study
GFY403	CBY926; [pESC-ORP2 (*URA3 CEN6* human ORP2)]	this study
GFY404	CBY926; [pESC-ORP9S (*URA3 CEN6* human ORP9S)]	this study
GFY405	CBY926; [pESC-ORP10S (*URA3 CEN6* human ORP10S)]	this study
CMY102	*MATα sec14-1^{ts} ura3 his3 trp1 leu2*	McMaster Lab; Xu et al. (2001)

GFY406	CMY102; *spo14::*KAN^R	this study
GFY407	GFY406; *cki1::LEU2*	this study
GFY408	GFY406; *kes1::*HYG^R	this study
GFY409	GFY407; *kes1::*HYB^R	this study
CMY136	CMY102; *kes1::HIS3*	this study
AF-1A2	*MAT***a** *trp1 his3 ura3 leu2 can1 ade2 ade3::hisG*	Dobson Lab; Flaman (unpublished)
SF294-1C	X2180; *sec7-1^{ts}*	Schekman Lab; Novick et al. (1980)
SF282-1D	X2180; *sec18-1^{ts}*	Schekman Lab; Novick et al. (1980)

CTTTTATTTACTTTTTCTTTTTTTGACATTCGCCTCGTCCCCGCCGGGTCAC) and 3'-kes1::HYG (5'-TTACAAATAACTAGTATCGAGGAACTTGAAAGAGCT-GAAATTCGTACAAAGATGGCATCGACC) The linear product was transformed into yeast and Leu+ transformants were screened by PCR of genomic DNA using primers 500 bp upstream and downstream from the *KES1* ORF to detect *kes1::LEU2.*

GFY series of triple mutants (*sec14-1^{ts}*:NAT^{R} *ckil::LEU2*)

Gene replacement with the KAN^{R} cassette was performed by amplifying the replacement allele *xxx1*::KAN^{R}-from the genome knockout strain collection obtained from Euroscarf. Each KAN^{R} disrupted allele was amplified by PCR using the primer pairs below, the linear product was transformed into yeast, and G418-resistant transformants were screened by PCR of genomic DNA. Primers used were *ypt31* (5'-GGTTTCTTGTTCTTGCTCGAT) (5'-GGCTAAAAAT-GGTTTGACGA), *ypt32* (5'-TGAAGAATCTGGAAGCAGTGA) (5'-TCAT-TGTCGTACCCCATCCTT), *trs85* (5'-TGTTTCTCAACGTGCTCTGGT) (5'-AACTTTCAACTCCAAGTGCG), *trs65* (5'-TTACGGTAGCGGAAATAGGGA) (5'-TTTCGTCATCATCTTTGGCG), *trs33* (5'-TCGATGCGGCCTTTTATATG) (5'-TTACAGGTATGGAGGGCAAA).

CMY400 (*sec14-1^{ts} spo14::*KAN^{R})

The *SPO14* gene in strain CMY102 (*sec14-1^{ts}*) was targeted for deletion/replacement by amplifying *spo14::*KAN^{R} from the genomic DNA of the *spo14::*KAN^{R} strain in the Euroscarf collection by PCR. Primers used hybridized 500 base pairs upstream of the start site for the ORF and 450 base pairs downstream from the stop codon. The primers used were 5'-SPO14-(500) (5'- TGGTCCTCTCCGTCACAAT) and 3'-SPO14-(450) (5'-CTGGATTTCCTT-ACTCATTGG). The linear PCR product was transformed into cells of strain CMY102 and G418-resistant colonies were screened by PCR to verify the presence of *spo14::*KAN^{R}.

GFY218 (*kes1::*KAN^{R} *frq1::*NAT^{R})

To delete/replace *FRQ1* in a GFY217 strain, the NAT^{R} cassette was amplified by PCR from pAG25 vector using primers 5'-frq1::NAT (5' ATGGGAGCCAAGACG-

TCAAAGCTTTCCAAAGATGACCTGACAGACATGGAGGCCCAGAATACCC) and 3'-frq1::NAT (5'-TCATATTAAGCCATCGTAAAGGTTTAAGGCACCAATAATAG-ACAGCAGTATAGCGACCAGCAT) The linear product was transformed into yeast cells and NAT-resistant transformants were screened by PCR of genomic DNA using primers that hybridize 150 bp upstream and downstream from the *FRQ1* ORF to detect *frq1::*NAT^R.

Yeast and bacterial expression plasmids

Unless otherwise stated, yeast genes were amplified using HiFi Platnium Taq polymerase (Invitrogen) in a polymerase chain reaction using genomic DNA from a wild-type strain as the template. In general, in addition to the open reading frame of the gene in question, 500 base pairs up and downstream were also amplified. Primers used for PCR were often designed to include restriction enzyme sites to aid in sub-cloning. DNA fragments produced by PCR were initially cloned into pCR2.1-TOPO
using the TOPO-TA cloning kit (Invitrogen) and then subcloned into the appropriate yeast vector using restriction sites located within the yeast vector and the pCR2.1-TOPO vector.

Human and yeast genes expressed in bacteria were also amplified, TA-cloned and sub-cloned similar to the production of yeast vectors. However, only the open reading frames were cloned into the bacterial expression vectors. For PCR of ORP1S and ORP2 (Xu et al., 2001), plasmids already produced by past members of the McMaster lab were used as templates. For PCR of ORP9S and ORP10S, cDNA was obtained from Kazusa DNA Research Institute (Kisarazu, Japan) and used as the template for PCR reactions and cloning.

Plasmids generated were sequenced by Cortec DNA Service Laboratories (Kingston, ON), SeqWright (Houston, TX) or the DalGEN sequencing facility (Halifax, NS).

3.1.2. METHODS

DNA techniques

Plasmid DNA was isolated from DH10B or DH5α *E. coli* cells using QIAprep Spin Miniprep Kit (QIAGEN). DNA fragments excised from agarose gels were purified using

the Glass Milk and washed with NaI supplied as a kit, GeneCleanII (Bio101). Plasmid or genomic DNA was isolated from yeast using the Y-DER (yeast DNA extraction reagent) Kit (Pierce).

Yeast transformation

Plasmid DNA was transformed into yeast using the lithium acetate/polyethylene glycol method (Gietz et al., 1995). Yeast were grown to early logarithmic phase in 10 mL of YEPD medium and collected by centrifugation at 3000 rpm for 5 min in a table-top centrifuge. Cells were washed with 1 mL of 100 mM lithium acetate, 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA (LiAc/TE), and cell pellets were resuspended in 50 μL of LiAc/TE and transferred to a microfuge tube containing 50 μg heat-denatured herring sperm DNA and 1-1.5 μg of plasmid DNA. After 3-5 minutes of incubation at room temperature, 300 μL of 40% polyethylene glycol 3400 in LiAc/TE was added and the tubes were incubated at 25°C for 60 minutes. The majority of strains used in this study contained temperature-sensitive mutations and therefore the strains were not subjected to heat shock. However, in cases in which non-temperature-sensitive cells were used, they were subjected to an additional incubation at 42°C for 15 minutes. The transformation mixture was subjected to centrifugation at 12000 x *g* for 1 minute, resuspended in 200 μL of TE, and the entire mixture was spread equally over two selective-medium agar plates and incubated at either 25°C or 30°C.

For targeted disruption of yeast genes, linear DNA was transformed in the same fashion as plasmid DNA. However, when the selectable marker used encoded a drug-resistance cassette (i.e. KAN^R), cells were allowed to grow for at least 4 hours in YEPD medium after the transformation. Cells were then pelleted by centrifugation, resuspended in TE and spread equally on medium containing the appropriate concentrations of drug.

Bacterial transformations

Chemically competent DH5α *E. coli* cells (25 μL) were incubated with 0.1-0.2 μg of plasmid DNA for 5 minutes at 4°C. Tubes were transferred to 42°C for 30 seconds, followed by the addition of 200 μL SOC (0.5% Yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20mM glucose) medium. Cells were incubated with

Table 2. Plasmids used in this study.

Plasmid	Description	Source
pRS415, pRS425, pRS416, pRS426	yeast shuttle vectors	Hieter Lab; Sikorski & Hieter. (1989)
pDB31	pRS416 *TPI1* prom:*SUC2*	Schekman Lab; Brada & Schekman. (1988)
pTL511	pRS416 *PHO5* prom., GFP, PHOsh2 (169 aa = 256-424 aa) ; VNSKL linker, PHOsh2 repeat, GFP	Levine Lab; Roy & Levine. (2000)
pFAPP	pRS416 *PRC1* prom. GFP, PH FAPP	Emr Lab; Stefan (unpublished)
pGFP-Snc1	promoter of *TPI;* expresses GFP-*SNC1*	Pelham Lab; Lewis et al. (2000)
pESC-ura, pESC-leu, pESC-trp	galactose inducible yeast expression vectors	Startegene
pCKI-415	*CKI1* under control of own promoter	McMaster Lab; Howe (unpublished)
psec14-PC	expresses Sec14 (K66A, K239A)	Bankaitis Lab; Philips et al. (1999)
pCTY-201	*SFH2* under control of own promoter	Bankaitis Lab; Li et al. (2000)
pB325	*SPO14* expression vector	Engebrecht Lab; Rudge et al. (2002)
pSLS1	yeast expression vector with *ADE3*	Dobson Lab; Flaman (unpublished)
pSLS1-NCR1	yeast expression vector with *ADE3, NCR1*	Dobson Lab; Flaman (unpublished)
pET23b	bacterial expression vector	Novagen; Studier et al. (1990)
pGEX-3x	bacterial expression vector	GE Healthcare; Kaelin et al. (1992)
pGEX-4T1-SMAD4	bacterial expression vector	Nachtigal Lab; Dunfield et al. (2002)

pGAL-PIK1-TAP	galactose inducible expression of Pik1 fused with TAP tag (Prot. A & 6xHIS)	Open Biosystems; Gelperin et al. (2005)
pGAL-KES1-TAP	galactose inducible expression of Kes1 fused with TAP tag (Prot. A & 6xHIS)	Open Biosystems; Gelperin et al. (2005)
pBSKII-kes1	disruption cassette, *kes1::HIS3*	McMaster Lab; Xu et al. (2001)
pRS416-SEC14	*SEC14* under control of own promoter	McMaster Lab; Murray & McMaster. (2005)

Table 3. Plasmids constructed for this study.

Plasmid	ORF/ Gene	Primers Used/ Construction	Restriction Sites Used	Backbone
pSLS-KES1	*KES1*	5'-CGCGGATCCCTCCATCGATTATGTGGTTCTAC 3'-	BamHI, SalI	pSLS1
pGDF1	ORP1S	5'-GCTGGATCCATGTCCGAAAAAGACTGTGGTG 3'-CCTGTCGACATAAATGTCAGGCAAATTGAAG	BamHI, SalI	pET23a
pGDF2	ORP1S (1-273aa)	5'-GCTGGATCCATGTCCGAAAAAGACTGTGGTG 3'-CCTGTCGACCGTGGCAGGGTCAACACTG	BamHI, SalI	pET23a
pGDF3	ORP1S (1-176aa)	5'-GCTGGATCCATGTCCGAAAAAGACTGTGGTG 3-GCTGTCGACTTTGGGTTCTGCTTCTACAC	BamHI, SalI	pET23a
pGDF4	ORP1S (67-437aa)	5'-GCTGGATCCACTGAATACATGGAGCATACTTAC 3'-CCTGTCGACATAAATGTCAGGCAAATTGAAG	BamHI, SalI	pET23a
pGDF5	ORP1S (67-370aa)	5'-GCTGGATCCACTGAATACATGGAGCATACTTAC 3'-CCTGTCGACATTTTCCATGGCTCTGATGTC	BamHI, SalI	pET23a
pGDF6	ORP1S (67-273aa)	5'-GCTGGATCCACTGAATACATGGAGCATACTTAC 3'-CCTGTCGACCGTGGCAGGGTCAACACTG	BamHI, SalI	pET23a
pGDF7	ORP1S (134-370aa)	5'-GCTGGATCCCAGGTCAGCCATCACCCACCAATC	BamHI, SalI	pET23a
pGDF8	ORP1S (134-273aa)	5'-GCTGGATCCCAGGTCAGCCATCACCCACCAATC	BamHI, SalI	pET23a
pGDF9	ORP1S (160-437aa)	5'-GCTGGATCCTATCCCAAACTGAAATTC 3'-CCTGTCGACATAAATGTCAGGCAAATTGAAG	BamHI, SalI	pET23a
pGDF10	ORP1S (160-303aa)	5'-GCTGGATCCTATCCCAAACTGAAATTC 3'-CCTGTCGACTTCATCCCACTCCTCAGAGGTG	BamHI, SalI	pET23a
pGDF11	ORP1S (160-273aa)	5'-GCTGGATCCTATCCCAAACTGAAATTC 3'-CCTGTCGACCGTGGCAGGGTCAACACTG	BamHI, SalI	pET23a

pGDF12	ORP1S (192-437aa)	5'- GCTGGATCCACAAATCCCACCTGCTGTG 3'-CCTGTCGACATAAATGTCAGGCAAATTGAAG	BamHI, SalI	pET23a
pGDF13	ORP1S (192-370aa)	5'- GCTGGATCCACAAATCCCACCTGCTGTG 3'-CCTGTCGACATTTTCCATGGCTCTGATGTC	BamHI, SalI	pET23a
pGDF14	ORP1S (192-303aa)	5'- GCTGGATCCACAAATCCCACCTGCTGTG 3'-CCTGTCGACTTCATCCCACTCCTCAGAGGTG	BamHI, SalI	pET23a
pGDF15	ORP1S (192-273aa)	5'- GCTGGATCCACAAATCCCACCTGCTGTG 3'-CCTGTCGACCGTGGCAGGGTCAACACTG	BamHI, SalI	pET23a
pGDF16	ORP1S (324-437aa)	5'- GCTGGATCCCCCACGGCCTCC 3'-CCTGTCGACATAAATGTCAGGCAAATTGAAG	BamHI, SalI	pET23a
pGDF35	ORP9S	5'-GCTGGATCCATGGTAGAATCAATTAAACAC-3' 5'-GCTGTCGACATGCTTGGCAGCACCAAGACG-3'	BamHI, SalI	pET23b
pGDF36	ORP10S	5'-GCTGGATCCATGGAAATGAATTCTAAGAGT-3' 5'-CCTGTCGACGTGTGCTTTCCAGAGGGGATTG-	BamHI, SalI	pET23b
pGDF37	ORP9S	pGDF35 was digested and the ORP9S fragment was subcloned	BamHI, NotI	pGEX-4t1
pGDF38	ORP10S	pGDF36 was digested and the ORP10S fragment was subcloned	BamHI, NotI	pGEX-4t1
pGDF39	ORP9S	5'-GCTCTCGAGATGGTAGAATCAATTAAACAC-3' 5'-CCAAAGCTTCTAATGCTTGGCAGCACCAAG -3'	XhoI, HindIII	pESC-ura
pGDF40	ORP10S	5'- GGACTCGAGATGGAAATGAATTCTAAGAGT-3' 5'-CCTAAGCTTTCAGTGTGCTTTCCAGAGGGG-3'	XhoI, HindIII	pESC-ura

shaking at 30°C for 30-60 minutes and were then spread on LB plates containing the appropriate antibiotic, normally ampicillin (100 μg/mL).

Electroporation of DH10B cells (Invitrogen) were electroporated as directed by the manufacturer, except 30 μL of cells and 1 μL of ligation reaction were used. The mixture was electroporated in a 0.1-cm gap electroporation cuvette using a Bio-Rad Genepulser II set at 1.56 kV.

Yeast genetic techniques

Synthetic lethal screen of *kes1* using UV mutagenesis

Cells of the yeast strain AF-1A2 (*kes1::HIS3 ade2 ade3*) were transformed with pSLS-KES1, with transformants selected on minimal medium lacking uracil. Cells were grown to mid-logarithmic phase, at which time the cell density was estimated by measuring OD_{600}. Cells were diluted to 20 cells/μL and 50 μL of mixture was plated on YEPD agar medium. After the plates dried (~10 minutes), cells were subjected to 10 mJ/cm^2 of ultraviolet light (UV Stratalinker 1800). This dose of UV light killed about 50% of the yeast cells through the mutagenesis of genomic DNA. After being subjected to UV light, plates were placed in the dark overnight (to inhibit cellular DNA-repair machinery).

The screen selects for colonies based upon the colour of the colony. The parental strain is *ade2 ade3* and the colonies have a typical off-white colour. The pSLS-KES1 plasmid carries a wild-type *ADE3* gene and colonies of cells with this vector will appear red. The *ADE2* and *ADE3* genes encode two genes in the biosynthetic pathway of adenine. Cells lacking a functional *ADE2* gene accumulate phosphoribosylaminoimidazole (AIR), which gives the yeast a red colour. Ade2 is a phosphoribosylamino-imidazole carboxylase that converts AIR into phosphoribosylaminoimidazolecarboxylate (CAIR), while Ade3 is further upstream in the biosynthetic pathway and required for the formation of AIR.

Non-sectoring red colonies were re-streaked onto YEPGalactose medium to inhibit plasmid segregation, and if no white colonies were present this re-streaking was repeated. Colonies that remained red at this point were streaked onto plates containing 5-FOA; at this point all colonies grew and none remained red. Of the 300,000 yeast that

were originally mutagenized, none contained a mutation that was synthetic lethal with *kes1*.

Synthetic genetic array (SGA) analysis

Synthetic genetic array (SGA) analysis is a term used to describe one type of genetic screen which can be performed using high-throughput robotics developed in the lab of Charles Boone, University of Toronto (Tong et al., 2001). SGA analysis involves mating cells of a "query" strain containing a mutation of interest (where the gene has been replaced with a dominant selectable marker, for example the nourseothricin-resistance cassette) to cells from a deletion collection of approximately 4700 individual gene-replacement strains in which a single gene has been replaced with a *kanMX4* kanamycin-resistance cassette. The procedure generates double-mutant haploid progeny which if unable to grow, suggests that while each mutation on its own is not lethal, the combination of mutations results in a lethal situation. Through screening the entire deletion collection this way, a large number of functional and genetic interactions can be identified. An overview of this procedure is illustrated in Figure 9. Due to my interest in the bypass of an essential gene through inactivation of *KES1*, I expanded SGA analysis to include the use of double mutants as the query strain, resulting in the production of triple mutants for synthetic lethality determination.

The SGA procedure commences with the growth of the query strain as a lawn on YEPD medium containing NAT to select for the NAT^R-replaced query deletion of interest. Cells from the resulting lawn are crossed to cells from each of the 4700 deletion-collection mutant strains through a robotic pinning procedure. Diploids are selected on medium containing both NAT and KAN. Diploids are sporulated and haploid *MAT***a** progeny are selected through the use of a "magic-marker" gene and the *can1Δ* mutant allele. The *MFA1pr-HIS3* magic marker fusion gene allows the expression of the *HIS3* gene from the Mating Factor A (*MFA1)* promoter. Both the query and deletion-collection strains have *his3Δ1* genotype, but only the *MATα* query strain contains the magic-marker gene. Transcription initiation from the *MFA1* promoter only occurs in *MAT***a** haploid cells, and not in diploids or *MATα* haploids, so the magic marker allows the selection of *MAT**a*** haploids through the selection for histidine prototrophy. The cells obtained in the

final step must have undergone mating to obtain the magic marker and sporulation to express the *HIS3* gene.

To further insure no diploids grow in the final step of the SGA analysis, L-canavanine is added to the medium. The *CAN1* gene encodes an arginine permease which facilitates the uptake of arginine from the medium, but it is also the only route of entry for canavanine, an arginine analog (Whelan et al., 1979). In the presence of canavanine, *CAN1* strains incorporate canavanine into proteins in the place of arginine, leading to aberrant protein function and lethality. The *can1Δ* allele blocks the uptake of canavanine, allowing the cells to live. In the SGA analysis, diploid strains are *CAN1/can1Δ* heterozygotes and the *can1Δ* allele is recessive, meaning the diploids are sensitive to canavanine, while *can1Δ* haploids are not.

To select for *MAT**a*** haploids by the presence of each of the markers previously described, sporulated diploids were transferred to Yeast Nitrogen Base synthetic minimal medium lacking histidine and arginine but containing canavanine (haploid selection medium). Resulting haploids were next transferred to haploid selection medium containing KAN (200 μg/mL). These KAN-resistant colonies were transferred to haploid selection medium containing KAN and NAT (100 μg/mL) to select for *MAT**a*** double-mutant haploid progeny. In cases were the query strain was a double mutant, leucine was omitted from the final round of selection.

To learn more about the biology surrounding *SEC14* and *KES1* a series of SGA screens were performed. Previous work demonstrated that the nonessential *SPO14* gene, that encodes the only yeast PC phospholipase D enzyme, is required for the viability of *sec14 cki1, sec14 kes1* and *sec14 sac1* bypass strains (Xie et al., 1998). To identify genes that, like *SPO14* are required for the viability of 'Sec14 bypass' the following query strains were generated and used for SGA analysis: CMY209 (*cki1::*NAT^R), CMY210 (*kes1::*NAT^R), CMY212 (*cki1::*NAT^R *sec14::LEU2*), and CMY213 (*kes1::* NAT^R *sec14::LEU2*). Screens were performed 3 or 4 times, and any gene that demonstrated a synthetic lethal or synthetic sick interaction 2 or more times was investigated further through random-spore analysis.

Figure 8. Synthetic genetic array - double mutants. A *MATα* strain carrying a mutation of interest (*kes1::*natR) replaced with a dominant selectable marker for nourseothricin resistance and a *MFA1pr-HIS3* reporter is crossed to an ordered array of *MATa* viable yeast deletion mutants. In the yeast single-deletion collection each gene is replaced by a dominant kanamycin-resistance marker. After mating, diploids are selected for in the presence of kanamycin and nourseothricin. Diploids are transferred to sporulation medium to induce the formation of meiotic spore progeny. Spores are transferred to synthetic medium lacking histidine to ensure that only haploids resulting from the mating and sporulation procedure will grow. In select progeny the *MFA1pr-HIS3* will be present; in haploids of the *MATa* type this construct provides a functional *HIS3* gene and allows the cells to produce their own histidine. This promoter will not express in *MATα* cells or in diploids. Double mutants containing both gene deletions are selected for on minimal medium lacking histidine and including kanamycin and nourseothricin. Growth of the double mutants was monitored every 24 h at 30°C.

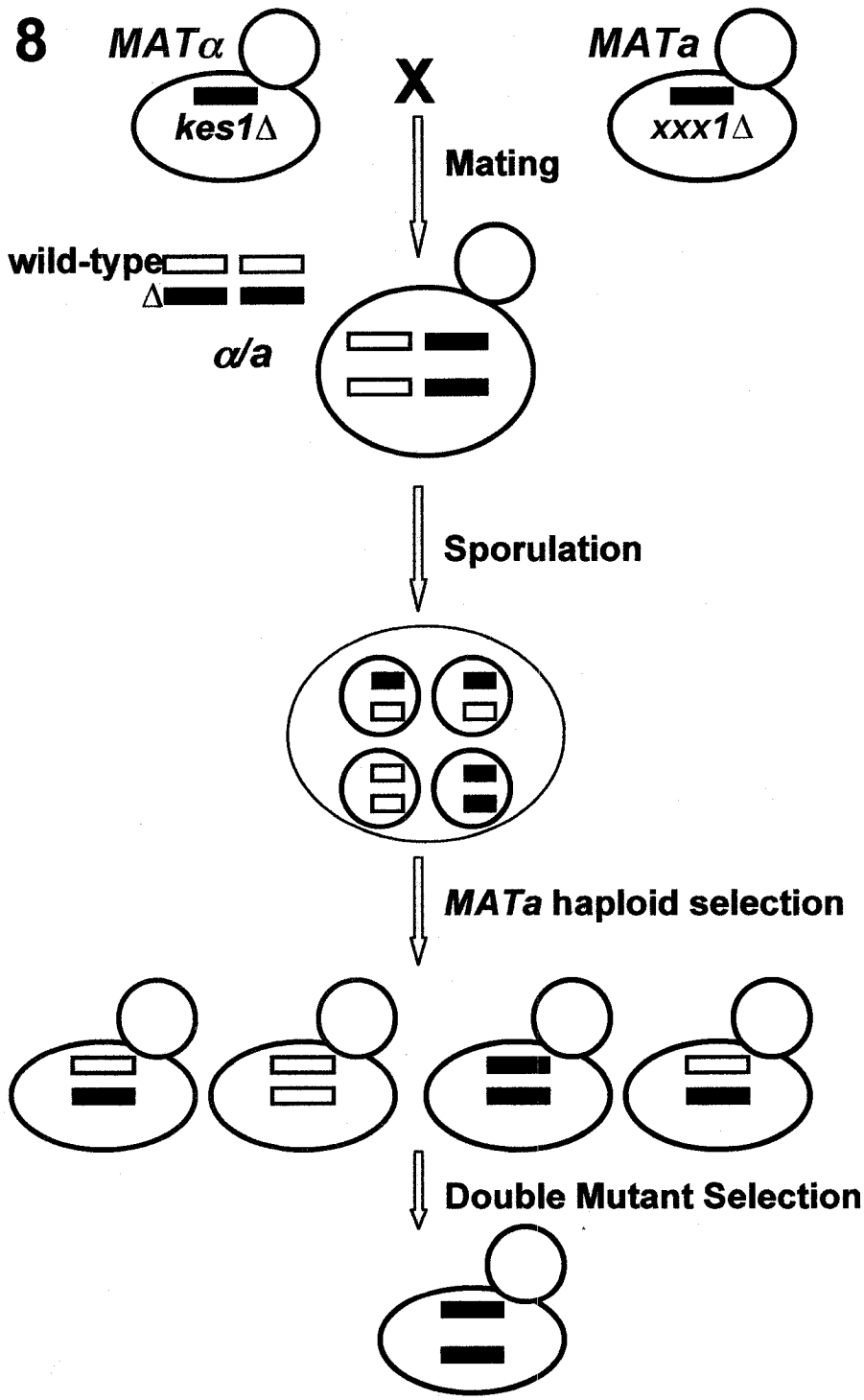
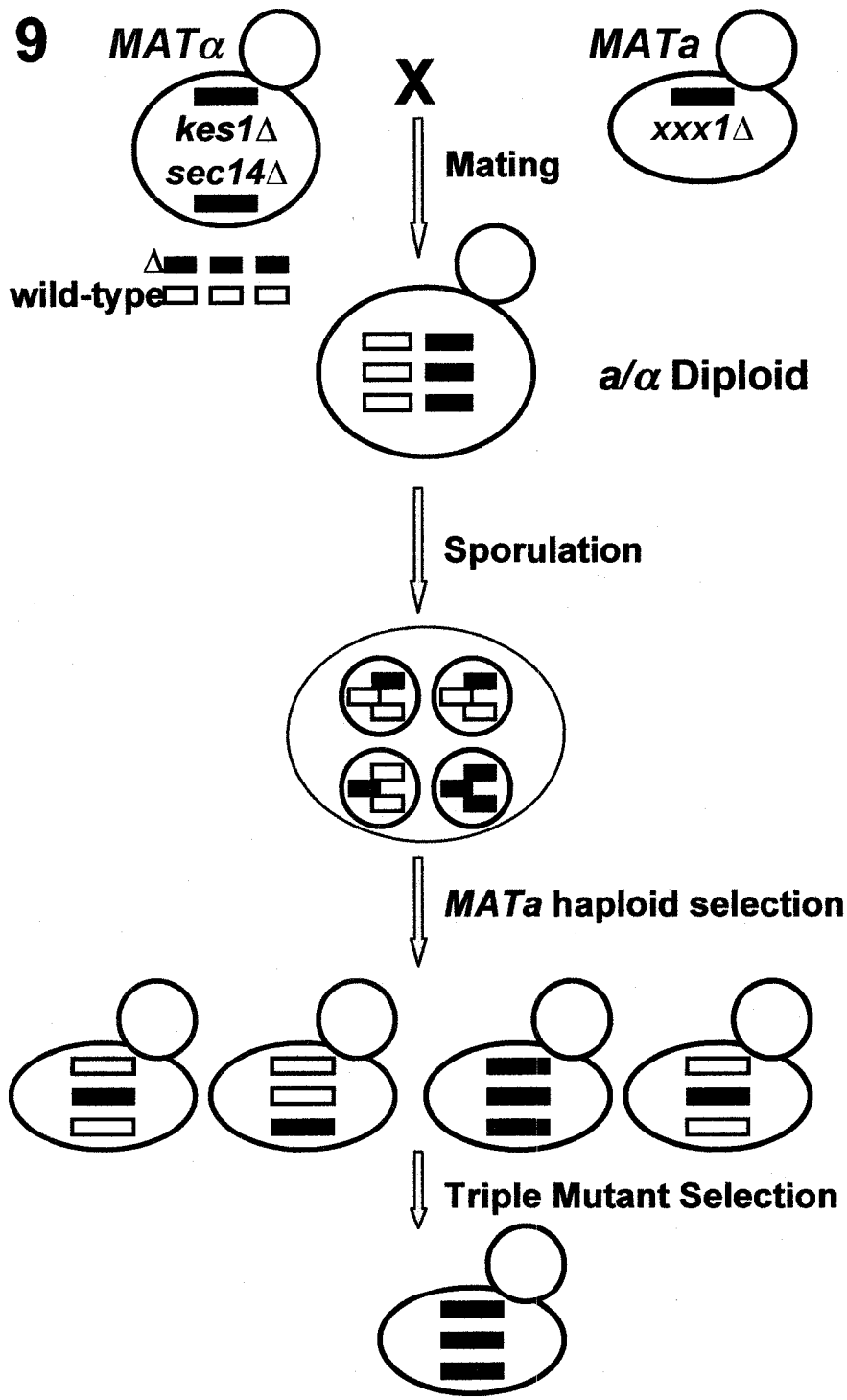

8
MATα
kes1Δ
X
MATa
xxx1Δ
Mating
wild-type
Δ
α/a
Sporulation
MATa haploid selection
Double Mutant Selection

Figure 9. Synthetic genetic array - triple mutants. A *MATα* strain carrying a mutation of interest (*kes1*::natR) replaced with a dominant selectable marker for nourseothricin resistance and a *MFA1pr-HIS3* reporter is crossed to an ordered array of *MATa* viable yeast deletion mutants. In addition the query strain also contains a second gene replacement, *sec14::LEU2*. In the yeast single-deletion collection each gene is replaced by a dominant kanamycin- resistance marker. After mating, diploids are selected for in the presence of kanamycin and nourseothricin. Diploids are transferred to sporulation medium to induce the formation of meiotic spore progeny. Spores are transferred to synthetic medium lacking histidine to ensure that only haploids resulting from the mating and sporulation procedure will grow. In select progeny the *MFA1pr-HIS3* will be present; in haploids of the *MATa* type this construct provides a functional *HIS3* gene and allows the cells to produce their own histidine. This promoter will not express in *MATα* cells or in diploids. Triple mutants containing all three gene replacements are selected for on minimal medium lacking histidine and leucine and including kanamycin and nourseothricin. Growth of the triple mutants was monitored every 24 h at 30°C.


9
MATα
kes1Δ
sec14Δ
X
MATa
xxx1Δ
Mating
Δ
wild-type
a/α Diploid
Sporulation
MATa haploid selection
Triple Mutant Selection

Spore isolation based upon hydrophobicity

Spores were isolated from diploids essentially as described by Guthrie and Fink (1991). Yeast cultures were sporulated in liquid medium for 3-5 days. 3 x 10^7 cells were collected by centrifugation and resuspended in 1 mL of 100 μg/mL of zymolyase (ICN) and incubated at 30°C for 20 minutes. 500 μL of culture was transferred to a 1.5 mL-polypropylene microfuge tube, and washed with 1 mL sterile water. The resulting pellet was resuspended in 100 μL of sterile water and mixed with a vortex at maximum for 2 minutes. The supernatant was discarded and the microfuge tube was rinsed gently 3 times with 1 mL of sterile water. 1 mL of 0.01% NP40 was added to the tube, and was sonicated in a water-bath sonicator for 2 minutes (instead of a probe sonicator). The resulting mixture was plated on the appropriate selective medium in 1:10 serial dilutions.

Random sporulation of yeast

A small clump of cells the size of an inoculating needle was resuspended in 200 μL of 1 M sorbitol containing 40 μg of zymolyase. Glass beads were added and the tubes were incubated at 30°C for 1 hour with gentle rocking. To the tube, 1 mL of sterile water was added and the mixture was placed on a vortex for 1 minute. The sample was then diluted 1:50 and plated on the appropriate agar medium. When random-spore analysis was used to verify a genetic interaction, plates were imaged with a VersaDoc and colonies were counted using the colony-counting option the Quantity One software (BioRad). To ensure the accuracy of the colony-counting algorithm random plates were counted by hand for comparison.

Serial dilution to assess yeast growth

To visualize cell growth under varying conditions, serial dilution spots were used. Yeast cultures were grown to logarithmic phase at 25°C or 30°C, depending upon the strains used, in selective minimal medium or in YEPD as indicated. Cell cultures were diluted to an absorbance at 600 nm (OD_{600}) of 0.4/mL in the appropriate medium, then serially diluted 1 in 10 and 3 μL of each dilution was spotted onto solid agar medium. Plates were incubated at the appropriate temperature for 48-96 hours depending upon the

experiment. Images of the plates were acquired by scanning with a CanoScan D1250 U2 (Canon) or using a VersaDoc (Bio-Rad).

Preparation of yeast for electron microscopy

Yeast cultures were grown to logarithmic phase at 25°C in minimal selective medium, shifted to 25°C or 37°C for a 1-hour incubation, and 10 OD_{600} units were collected. Cells were washed once with PBS and fixed in a solution of 1.5 % $KMnO_4$ for 20 minutes at room temperature. Cells were washed an additional three times with phosphate buffered saline. Embedding, image capture and processing was performed by the Electron Microscopy Facility of the Faculty of Medicine, Dalhousie University.

Live-cell imaging

Cells were transformed with a GFP or DsRED construct of interest. Cells were grown to early logarithmic phase and shifted to 37°C for further incubation if needed. For immobilization and maintainance of all cells in a single plane of focus, cells were mounted on agarose slabs as follows two % agarose in PBS was heated to 95°C to dissolve, then approximately 300 µL was placed on a microscope slide. Another slide was immediately placed on top of the agarose to flatten the thin agarose pad evenly. After a couple of minutes the top slide was carefully removed. Excess agarose was trimmed from the glass slide to a 400-mm^2 using a scalpel. Two-three µL of cells resuspended in mounting medium were placed on the agarose pad, and then a coverslip was placed on top and sealed with clear nail polish. Slides were viewed immediately.

Yeast subcellular fractionation

Typically, yeast cells were grown in 100 mL of selective or YEPD medium to logarithmic phase and collected by centrifugation at 1000 x *g* at 4°C for 10 minutes. Pellets were resuspended in 25 mL of GTE buffer (20% glycerol, 50 mM Tris-HCl [pH7.4], 1 mM EDTA) or GHE buffer (20% glycerol, 50 mM HEPES [7.4], 1 mM EDTA) and collected by centrifugation at 1000 x *g* at 4°C. Pellets were resuspended in 700 µL of GTE or GHE buffer supplemented with complete protease inhibitor (Roche) and 1 mM PMSF. The cell mixture was transferred to tubes containing approximately 300-400 µL of 0.5-mm acid-washed glass

beads (Sigma). The tubes were placed on the bead beater (BioSpec) and were mechanically shaken for 2 minutes at 4°C. Cell homogenates were centrifuged at 700 x *g* for 1 minute to collect unbroken cells in a pellet. The supernatant was removed, and the beads and pellet were washed with an additional 500 µL of buffer. On occasion, the resulting cell extract was transferred to an ultracentrifuge tube and subjected to centrifugation at 450,000 x *g* (TLA120 rotor) for 15 minutes at 4°C to produce supernatant essentially free of organelles and membranes. The remaining pellet contains the microsomal fraction, Golgi and other light membrane components and was resuspended by Dounce homogenization in 500 µL GTE buffer. All fractions were used immediately or stored at -80°C.

Lowry protein determination

Protein concentrations were determined by the method of Lowry (Lowry et al., 1951). Both samples and bovine serum albumin (BSA) standards were added to test tubes to which 100 µL of 2% sodium deoxycholate was added, followed by the addition of distilled water to a total volume of 1 mL. Next, equal amounts of 1 % copper sulfate and 2 % potassium sodium tartarate were mixed and added to 2 % sodium carbonate (in 0.1 M sodium hydroxide) in a ratio of 1:50, and 4 mL of this solution was added to each tube. Tubes were incubated at room temperature for 10 minutes. Folin's phenol reagent was diluted 2:1 with distilled water and 500 µL was added to each tube and mixed immediately. The tubes were incubated at 60°C for 10 minutes and allowed to cool to room temperature, and absorbance was monitored at 730 nm.

SDS PAGE and western blot analysis

Proteins were separated by SDS-PAGE along with prestained or unstained markers (Benchmark, Invitrogen). Gels used for this study ranged from 5% to 20% acrylamide depending upon the experiment. Gradient gels were supplied by BioRad. Gels were stained by Coomassie or silver nitrate.

Proteins were transferred to PVDF membrane (GE Healthcare) ii 25 mM Tris-HCl, 200 mM glycine, 1% SDS, 20% methanol either overnight at 25 volts or for 2 hours at 60 volts. The membrane was washed with distilled water and blocked by drying

overnight at room temperature, or in PBS (or occasionally TBS) containing 0.1 % Tween 20 and 5% skim milk powder. Membranes were incubated for 1 hour in PBS/Tween20/1% milk powder containing the appropriate antibody. Membranes were rinsed twice with PBS/Tween20, and then 3 times for five minutes with PBS/Tween20. When secondary antibodies were used, membranes were incubated for 1 hour in PBS/Tween20/1% skim milk powder containing 1:10,000 dilution of the horse radish peroxidase-coupled secondary antibody, followed by 4-5 washes with PBS/Tween20. Proteins were detected using an enhanced chemiluminescence (ECL) (GE Healthcare) or PicoECL (BioRad) kit and membranes were exposed to X-Omat Blue film (Kodak).

Metabolic labeling of yeast lipids

PC synthesis through the CDP-choline pathway was monitored by labeling cells with [^{14}C]choline chloride. Yeast cultures (3 mL) were grown to mid-logarithmic phase in minimal selective medium and then grown in the presence of 3 μCi [^{14}C] choline for 1 hour. After 1 hour, lipids were extracted using the Folch method (Folch et al., 1957). The cells were collected by centrifugation at 3000 x *g* for 5 minutes in a tabletop centrifuge. Pellets were washed with 1 mL of water. Cell pellets were resuspended in 1 mL of $CHCl_3/CH_3OH$ (1:1, v/v), and transferred to a 1.5-mL bead-beater screw cap tube ~1/6 filled with glass beads. Cells were disrupted by bead beating for 2 minutes at 4°C. The cell extract was transferred to a test tube, and the beads were washed twice with 750 μL of $CHCl_3/CH_3OH$ (2:1, v/v) and combined with the cell extract. 100 μL of the combined fractions (~2.5 mL) was removed and counted in a scintillation counter to determine total radiolabel uptake. To the remaining extract 500 μL of $CHCl_3$ and 1.5 mL of water were added and tubes were mixed by placing them on a vortex for 30 seconds. Tubes were subjected to centrifugation at 2500 rpm in a microfuge for 5 minutes to facilitate phase separation. The upper phase (aqueous) was removed for TLC analysis if desired, or discarded along with the protein layer that accumulates at the interface. The lower phase (organic) was split, with 100 μL being counted in the scintillation counter and the rest used for lipid phosphorus determination.

The aqueous phase isolated from the lipid extraction was dried overnight in a rotary vacuum, resuspended in 20 μL of water and spotted on Silica Gel G thin-layer chromatography plates (Fisher Scientific). The aqueous choline metabolites were separated using the solvent system of methanol, 0.6% NaCl, and 4.2 M ammonium hydroxide (10:10:1, v/v). Plates were scanned for radioactivity with a Bioscan Image Scanner, and regions containing radioactivity were scraped and the silica was transferred to vials for scintillation counting.

Sphingolipid synthesis was measured by labeling cells with [^{3}H]serine. Cultures were grown in minimal medium at 30°C to logarithmic phase, from which 3 OD_{600} units of cells were harvested and washed 3x with minimal medium (subjected to centrifugation at 3000 rpm for 5 minutes to obtain a pellet). Cells were resuspended in 0.5 ml of minimal medium followed by the addition of 20 μCi of [^{3}H]serine. After 30 minutes of incubation with shaking at 30°C, cell growth was stopped by the addition of 2 ml of ice-cold 10 mM sodium azide, 10 mM sodium fluoride. Cells were harvested by centrifugation and the pellets were washed once with water. Cells were resuspended in 500 μL $CHCl_3/CH_3OH/H_20$ (10:10:3), ¼ volume of glass beads were added and cells were lysed by bead beating for 2 minutes at 4°C. Cell debris was separated from the resulting mixture by centrifugation at 13000 x *g* for 5 minutes at 4°C, and the supernatant was transferred to a new eppendorf tube. The pellet was re-extracted by the addition of $CHCl_3/CH_3OH/H_20$ (10:10:3) which was combined with the first extract and dried under nitrogen. Next, the dried lipids were dissolved in 100 μl of water-saturated butanol, then the butanol was extracted with 50 μl of water. The butanol phase was transferred to a new tube and saved. The aqueous phase was extracted twice more with 100 μl of water-saturated butanol, and all the butanol phases were combined and dried under nitrogen. Lipids were dissolved in 110 μl of $CHCl_3/CH_3OH/H_20$ (10:10:3) with 10 μl removed for scintillation counting. The 100 μL was split into two, with one part saved for TLC and the other dried under nitrogen and resuspend in 0.1 M potassium hydroxide and incubate at 37°C for 1 hour to deacylate the phospholipids. The lipid were extracted twice with butanol, the butanol phases were pooled, dried under nitrogen and resuspend in 50 μl of $CHCl_3/CH_3OH/H_20$ (10:10:3). Five μl was removed for scintillation counting. Equal

disintegrations/minute/lane were spotted on a Silica 60 TLC plate and separated using solvent system 1, CHCl3/CH3OH/0.25%KCl (55:45:10) or system 2 CHCl3/CH3OH/4.2N ammonium hydroxide (9:7:2). Plates were sprayed with Enhance (NEN Life Sciences) and exposed to film at -80°C.

Lipid phosphorous determination

The method developed by Ames and Dubin was used to determine phospholipid mass (Ames and Dubin, 1960). From the organic-phase sample, 500 μL was dried down under nitrogen. Standards were prepared by adding 0-40 nmol of potassium dihydrogen phosphate to test tubes. Water (300 μL) and percholic acid (150 μL) were added and samples were incubated overnight at 160°C in a heating block. Following the incubation, 700 μL of water was added and samples were incubated at room temperature for 20 minutes, followed by the addition of 150 μL of 10 % ascorbic acid (w/v) and 500 μL of 0.9% ammonium molybdate. Samples were mixed by placing them on a vortex, and incubated at 45°C for 30 minutes. The absorbance was measured at 820 nm using a spectrophotometer.

Quanitative invertase assay

To measure invertase secretion, yeast cultures were grown to logarithmic phase at 25°C in 5 mL of YEPD or selective medium. This culture was collected by centrifugation at 2500 x *g* for 5 minutes and the pellets were washed twice with medium containing 0.1% glucose prewarmed to 25°C or 37°C. Cultures were grown for an additional two hours at the given temperature. Invertase secretion was measured using the standard method (Goldstein and Lampen, 1975) with modifications (Bankaitis et al., 1989). Briefly, the 5-mL culture was harvested by centrifugation in a clinical centrifuge at 700 x *g* for 5 minutes, washed twice with 1 mL of 10 mM cold sodium azide, and resuspended in 500 μL of sodium azide. Suspensions were split equally and transferred into new tubes. To one tube, an additional 250 μL of 10 mM sodium azide was added (external tube) and to the other tube 250μL of 10 mM sodium azide, 0.2% Triton X-100 was added (total tube), and placed at -80°C for 10 minutes. The absorbance of the external tubes was determined at 600 nm. Aliquots of total and external tubes were set up in triplicate with

equal number of cells and 0.1 M sodium acetate (pH 5.1) was added to give a total volume of 200 μL. Tubes were equilibrated to 30°C and 50 μL of 0.5 M sucrose was added. Samples were incubated for 30 minutes at 30°C and the reaction was terminated by the addition of 300 μL of 0.2 M sodium phosphate (pH 7.0) containing 10 mM *N*-ethylmaleimide (NEM) and boiling for 3 minutes. The invertase assay was a coupled assay, invertase converts sucrose into glucose and fructose, and the glucose produced can be determined using a standard glucose oxidase reaction. Tubes were incubated at 30°C and 2 mL of fresh glucostat reagent (0.1M potassium phosphate [pH7.0] 4.34 U glucose oxidase, 2.5 μg/uL, 0.1 mM NEM, 150 μg/mL *O*-dianisidine, and 1 mg/mL horseradish peroxidase) was added. Samples were incubated for 30 minutes (or less) at 30 °C and the reaction was terminated by the addition of 2 mL of 6 N HCl. Absorbance was measured at 540 nm and the ratio of invertase secreted was determined by dividing external activity by total activity.

In-gel invertase assay

To determine the glycosylation status of invertase, and in-gel assay was employed as described by Gabriel and Wang (Gabriel and Wang, 1969). Yeast cultures were grown to mid-logarithmic phase in 10 mL of YEPD medium. Cells were washed twice with 0.1% glucose YEPD prewarmed to 25°C or 37°C, and incubated an additional two hours. After two hours, cells were converted to spheroplasts by resuspending them in 100 μL of 1.4 M sorbitol, 50 mM Tris-HCl (pH 7.5) 10 mM sodium azide, 2 μL/mL β-mercaptoethanol, 25 μg/mL zymolyase and incubating at 37°C for 45 minutes. Cells were collected by micro-centrifugation at 700 x *g* for 5 minutes. The supenatant (~100 μL) was transferred to a new tube (external) and placed on ice. The pellet was resuspended in 100 μL of 0.1% SDS, 0.5% Triton X-100, 10 mM sodium azide and vortexed for 10-15 seconds. The debris was collected by microcentrifugation at 14,000 x *g* for 5 minutes and the supernatant was transferred to another tube (internal). To both samples 10 μL of glycerol was added along with a small amount of bromophenol blue. Half of each sample (~55 μL) was loaded on a 5% SDS-PAGE gel, and the samples were resolved by electrophoresis at 80 volts/cm. The gel was then washed with distilled water and

incubated in a 0.1 M sodium acetate (pH 5.2) for 15 minutes. Next, the gel was incubated in a 0.1 M sucrose for 2 hours at 37°C, followed by washing with water. The gel was then incubated in 0.1 M iodoacetamide for 15 minutes at room temperature, followed by washing with water for 5 minutes. Finally, the gel was soaked in 0.1% 2,3,5-triphenyl tetrazolium chloride, 0.5 M NaOH until bands appeared. The reaction was stopped by washing the gel several times in 7.5 % acetic acid.

FM4-64 staining of the vacuole

To visualize the morphology of the vacuole, FM4-64 staining was performed as described by Vida and Emr (Vida and Emr, 1995). Yeast cultures were grown to mid-logarithmic phase and the equivalent of 3 OD_{600} of cells were harvested. Cells were resuspended in 300 μL of prewarmed minimal medium containing 32 μM FM4-64 and incubated at 37°C for 10 minutes. Cells were collected by microcentrifugation for 30 seconds, supernatant was discarded and cells were resuspended in 300 μL of fresh pre-warmed medium and incubated for an additional 30 to 60 minutes. To terminate the trafficking of the FM4-64, 1.2 mL of 10 mM sodium azide and 10 mM sodium fluoride was added to the cell suspension. Samples were viewed with a fluorescent microscope using the appropriate filters to visualize FM4-64.

CPY secretion

The CPY secretion assay was developed based upon the secretion assay for Kar2 developed by Belden and Barlowe (Belden and Barlowe, 2001; Fairn and McMaster, 2005a). Cells were grown to mid-logarithmic phase in medium appropriate for the experiment. Cells were harvested, washed and resuspended in fresh prewarmed medium to a cell density of 0.5 OD_{600}. After growth for 1 to 3 hours, 1 mL of each culture was collected by centrifugation at 14000 x *g* for 1 minute and 800 μL of supernatant was transferred to a new eppendorf tube. Proteins in the medium were precipitated by the addition of 200 μL of 50% trichloroacetic acid (TCA) and incubation at 4°C for 30 minutes. In experiments in which minimal medium was used, 1 mg of BSA was added to serve as carrier protein. The precipitated proteins were collected by centrifugation at 14,000 x *g* for 15 minutes at 4°C. Pellets were washed twice with 100% acetone, with

proteins being collected by centrifugation at 14,000 x *g* for 5 minutes at room temperature between washes. Pellets were air dried for 5 minutes at room temperature and resuspended in 40 μL of SDS-PAGE sample buffer supplemented with 50 mM Tris-HCl (pH 8.8). Equal volumes were resolved by SDS-8%PAGE and processed for immunoblotting. An anti-CPY monoclonal antibody was purchased from Molecular Probes and used at the recommended concentration of 0.5 μg/mL.

Hsp150 secretion

Wild-type and mutant cells were grown to mid-logarithmic phase and concentrated to 3 OD^{600}/mL in minimal medium lacking cysteine and methionine and labeled with 150 μCi of ^{35}S Expre$^{35}S^{35}S$ Mix protein label (Perkin Elmer) for 10 minutes. Cells were chased by incubation in 10 mM cysteine and 10 mM methionine for 30 minutes. To monitor Hsp150 secretion, 1 mL of each culture was collected by centrifugation at 14,000 x *g* for 1 minute and the supernatant was transferred to a new eppendorf tube. Proteins in the medium were precipitated by the addition of 230 μL of 50% TCA and 1 μg of BSA and incubation at 4°C for 30 minutes. The precipitated proteins were collected by centrifugation at 14000 x *g* for 15 minutes at 4°C. Pellets were washed twice with 100% acetone, with proteins being collected by centrifugation at 14,000 x *g* for 5 minutes at room temperature between washes. Pellets were air dried for 5 minutes at room temperature and resuspended in 30 μL of SDS-PAGE sample buffer supplemented with 50 mM Tris-HCl (pH 8.8). The total volume was loaded on an SDS-8%PAGE and the gel was dried under vacuum onto filter paper. The gel was exposed to a phosphorimager screen for 1-2 days and the image was captured and processed using Quantity One software (Bio-Rad). Yeast strains generally secrete less than 10 proteins into the medium with Hsp150 being the only one greater than 100 kDa.

Latrunculin B halo assay

Latrunculin B (BIOMOL) is a toxin produced by *Latrunculia magnifica* (a red sea sponge) that binds to monomeric actin and results in the inhibition of actin polymerization (Spector et al., 1983; Coue et al., 1987). Due to its cost, when using it to investigate actin dynamics in yeast a halo assays was used. The cells of interest were

grown to logarithmic phase in YEPD medium, harvested and resuspended in 2 ml of medium. Next, in a microwave heat a sterile solution of 2% low-melting-point agarose was heated and placed in a 55°C water bath. Once the agarose cooled 2 ml of it was combined with the 2 ml of cells and poured onto a YEPD solid medium plate. While the agar solidified, 11 μL of LatB solution was spotted onto 10-mm round disk. Disks were placed on freshly solidified agar, which was then incubated for 2 to 4 days (or to when a lawn appeared). Growth differences can be detected by eye and halo size were measured (Ayscough et al., 1997; Reneke et al., 1988).

Lipid protein overlay assay

To assess phospholipid-binding by various proteins a standard lipid protein overlay assay as described by Dowler et al.was used (Dowler et al., 2000; Dowler et al., 2002). Typically, 1 nmol of phospholipid was immobilized on Hybond-C nitrocellulose membranes (GE Healthcare). Blots were dried for 1 hour and blocked in 3% fatty-acid-free BSA, Tris-HCl-buffered saline (pH 7.4) for 1 hour. Purified proteins (10 nM) were incubated at 4°C for 12 hours (or room temperature for 1 hour) in 3% BSA/TBS. Blots were washed ten times in TBS/0.1% Tween 20 over the course of an hour and incubated with primary antibody (1:5000) in 3% BSA/TBS/0/1% Tween 20 for one hour. Blots were washed 6 times over the course of 30 minutes, followed by incubation with secondary antibody under the same conditions as primary antibody. Finally, blots were washed 6 times and lipid binding was determined by ECL as described in the Western blotting section.

***In vitro* Pld2 assay**

The PE/PS-specific PLD activity was assayed by a method developed by the Liscovitch lab was used (Waksman et al., 1997). Cells were grown to mid-logarithmic phase and 25 OD_{600} units of cells were harvested, washed with water and resuspended in 250 μL lysis buffer (PBS, EDTA-free complete protease inhibitor supplemented with 1 mM PMSF). Cells were mechanically lysed by the addition of an equal volume of glass beads, followed by 2 minutes of bead beating at 4°C. Cell extracts were clarified by centrifugation at 14000 x *g* for 5 minutes at 4°C. Protein concentrations were determined

by microBCA assay (Pierce). Six carbon nitrobenzoxadiazole (C6-NBD) phophatidylethanolamine (PE) was used as a substrate. NBD-PE (supplied dissolved in chloroform) and was dried under nitrogen prior to use. NBD-PE was dissolved in 1.5 mM Triton X-100 to a final concentration of 2.4 mM. The Pld2 assay contained 0.3 mg/mL of protein (whole cell extract), 35 mM HEPES (pH 7.2), 150 mM NaCl, 400 μM C6-NBD-PE, 1 mM EDTA, 5 mM EGTA, 7 mM $CaCl_2$ in a total volume of 120 μL. The reaction was terminated by the addition of 300 μL of chloroform:methanol:water (100:100:0.6). To resolve NBD-PE from NBD-PA, samples were concentrated and separated by thin-layer chromatography using the solvent system chloroform/methanol/water (65:25:4). The identity of NBD-PE and NBD-PA in the samples was determined using standards (Avanti).

***In vitro* Pik1 assay**

Cell extracts (34 μg of protein) or *in vitro* transcribed and translated Pik1 was used to assay Pik1 activity. The method used was based on the method developed by Flanagan and Thorner (Flanagan and Thorner, 1992; Hendricks et al., 1999). PI 4-kinase activity was measured by the incorporation of radioactivity from [γ-^{33}P]-ATP to PI-4P. Separation of PI-4P from ATP was accomplished by organic extraction and thin layer chromatography. The typical reaction volume was 100 μL and contained 0.25 mM PI. PI is supplied dissolved in chloroform and prior to making micelles the PI was dried under nitrogen and resuspended in 10 mM β-mercaptoethanol. The final reaction mixture contained 0.25 mM PI, 0.25% Trition X-100, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM [γ-^{33}P]ATP ($3x10^7$ cpm/μmol) and enzyme. The micelles were prepared by bath sonication prior to the addition of protein sample or ATP. The micelles were stored at 4°C until used and were discard after two weeks. All assay components except ATP was added to 2.0-mL tubes and equilibrated to 34°C just prior to the addition of ATP. Initiation of the assay was performed by the addition of 10 μL of 10 mM [γ-^{33}P]ATP and was allowed to continue for 20 minutes at 34°C. The reaction was terminated by the addition of 350 μL of 1M $MgCl_2$ and 150 μL of $CHCl_3$/CH_3OH/0.1 N HCl (3:47:48) followed by mixing using a vortex at maximum speed, and samples were placed on ice. To separate PI-4P from the excess of ATP substrate, organic extraction was used. To the

600-μL sample, 1 mL of $CHCl_3/CH_3OH$ (2:1, v/v) was added, material was mixed and phases were separated by centrifugation. The upper (aqueous) phase was transferred to a new tube and re-extracted in the same way. The two organic phases were combined and back-extracted by the addition of 500 μL of $CHCl_3/CH_3OH$/0.1 N HCl (3:47:48). The final volume typically ranged from 1.1 to 1.2 mL, 1 mL was transferred to a 1.5-mL eppendorf tube and dried in a centrifugal vacuum concentrator (Speed Vac). Samples were resuspended in 10 μL $CHCl_3/CH_3OH$ (2:1, adjusted to 0.1 N HCl) and spotted on a silica-gel TLC plate (LK5D Whatman). The solvent system used for TLC chromatography was $CHCl_3/CH_3OH$/0.1 N HCl/NH_4OH (45:45:10:5, v/v). Plates were scanned for radioactivity with a Bioscan Image Scanner, and regions containing radioactivity were scraped and the silica was transferred to vials for scintillation counting.

***In vivo* analysis of phosphoinositides**

Phosphoinositide analysis was performed by Chris Stefan in the lab of Dr. Scott Emr (Howard Hughes Medical Institute/University of California, San Diego) as previously described (Gary et al., 2002). Wild-type and mutant cells were grown to early logarithmic phase at 26°C in minimal medium with the appropriate supplements. Five OD_{600} equivalent of cells were harvested, washed with inositol-free minimal medium and resuspended in inositol-free minimal medium. Cells were incubated with shaking at the appropriate temperature (26°C or 38°C) for 10 minutes, followed by the addition of 60 μCi of myo-[2-^{3}H]inositol (GE Healthcare). After 10 minutes, excess myo-inositol was added to a final concentration of 300 μg/mL and cells were immediately collected by centrifugation for 2 minutes at 8000 rpm. Cells were resuspended in inositol free minimal medium containing 250 μg/mL unlabelled myo-inositol and incubated with shaking for an additional 30 minutes at 26° or 38°C. Cells were harvested and resuspended in 4.5% (v/v) ice-cold perchloric acid, incubated on ice for 15 minutes, collected by centrifugation at 16,000 x *g* for 10 minutes at 4°C and resuspended in 50 μL of water (Whiteford et al., 1996). Phospholipids were deacylated by using methylamine as described by Hawkins et al (Hawkins et al., 1986). Briefly, to the 50-μL sample, 0.5 mL of methylamine reagent (methylamine, methanol, 1-butanol, 4:4:1, v/v) was added and the mixture was incubated

in a 53°C heat block for 50 minutes. Unreacted methylamine was then removed by drying in a Speed Vac, and the dried pellet was resuspended in 300 μL sterile water. After a second sequence of drying and resuspension in 300 μL water, an equal volume of 1-butanol/ethylether/formic acid ethyl ester (20:4:1) was added. The samples were mixed by vortex for 5 min and collected by centrifugation at 14,000 x *g* for 2 min. The aqueous phases containing the [^{3}H]glycerophosphoinositides were transferred to new tubes and re-extracted with 1-butanol/ethyl ether/formic acid ethyl ester (20:4:1). Finally, the aqueous phases were collected and dried in a Speed Vac. Samples were resuspended in sterile water and 18 x 10^6 cpm quantities of [^{3}H]glycerophosphoinositides were analyzed using an anion-exchange Partisphere SAX column (Whatman Inc.) coupled to a gold high-performance liquid chromatography (HPLC) system (Beckman Coulter Inc.) and an on-line radiomatic detector (Packard Instrument Company). Fractions were collected every 40 seconds.

Co-purification of Kes1-TAP and protein binding partners

Tandem affinity purification (TAP) procedure as described by Rigaut et al. was used (Rigaut et al., 1999). Yeast strains used in this study containing TAP-tagged genes were purchased from Open Biosystems. Four litres of cells were grown to OD_{600} of 2 in YEPD at room temperature with mixing on a stir plate. Cells were collected by centrifugation at 1000 x *g* for 15 minutes at 4°C and washed once with water. Cells were stored at -80°C if not used immediately. Cell pellets were resuspended in an equal volume of Buffer A containing 20 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5 mM phenyl-methylsulfonyl fluoride (PMSF) and 1/50 (v/v) EDTA-free complete protease inhibitor, and lysed by passing the suspension three times through a French press at 10,000 psi. The concentration of KCl was adjusted to 0.2 M with the addition of 1/9 volume of 2 M KCl solution. The extract was cleared by centrifugation at 25,000 x *g* for 30 minutes at 4°C and the supernatant was transferred to a 50-mL Falcon tube. A rabbit IgG-agarose bead suspension (Sigma) 400 μL was washed three times with Buffer B (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40) and added to the supernatant. The composition of the supernatant was also adjusted to 10 mM Tris-HCl, 150 mM NaCl, 0.1% NP-40. Rabbit IgG beads and the cell extract were

incubated for 2 hours or overnight at 4°C. The suspension was transferred in step-wise fashion to 10-mL econoprep disposible columns (Bio-Rad) and drained by gravity flow. Beads were washed with 30 mL of Buffer B followed by 10 mL of TEV cleavage buffer (Gibco). Cleavage of the TAP tag was done in the same column with 1 mL of Tobacco Etch Virus (TEV) cleavage buffer and 100 units of acTEV recombinant protease. The beads were incubated for 2 hours at 16°C and the eluate was recovered by gravity flow.

Next, 200 µL of calmodulin-agarose suspension was washed with calmodulin binding buffer (10 mM Tris-HCl [pH 8.0], 10 mM β-mercaptoethanol, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1 % NP-40) and added to the 1 mL of eluate plus 3 mL of calmodulin binding buffer and 3 µL of 1 M $CaCl_2$. This mixture is incubated for 1 hour at 4°C followed by washing with 30 mL of calmodulin binding buffer. Finally, the proteins where eluted from the resin using 1 mL calmodulin elution buffer (10 mM Tris-HCl, [pH 8.0], 10 mM β-mercaptoethanol, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 0.1% NP-40, 2 mM EGTA). Samples were concentrated using TCA precipitation or by using a ReadyPrep Cleanup Kit (Bio-Rad).

Purification of GST-tagged proteins

Glutathione S-transferase (GST)-tagged proteins were purified from BL21 (DE3) pLYS *E. coli*. Cells were grown overnight in 10 mL of LB medium plus 100 µg/mL ampicillin and 34 µg/mL chloraphenicol at 37°C. The following morning 4 mL of overnight culture was added to 400 mL of LB medium plus ampicillin and chloramphenicol and cells were grown to mid-logarithmic phase at 37°C. Next, 400 µL of 0.5 M isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture, transferred to 30°C and grown an additional 4-5 hours. Cells were harvested by centrifugation at 2500 x *g* at 4°C for 10 minutes, washed once with PBS and stored at -80°C if not used immediately. Cells pellets were resuspended in 15-20 mL of bacterial protein extraction reagent (B-PER, Pierce), 1/50 (v/v) complete protease inhibitor and passed repeatedly through 16.5, 18.5, and 21.5 gauge needles. Lysates were cleared by centrifugation at 27,000 x *g* at 4°C for 20 minutes and the supernatants were transferred to fresh tubes. Glutathione fast-flow Sepharose (GE Healthcare) was resuspended and

500 μL of slurry was transferred to a 15-mL Falcon tube. The resin was sedimentated in a tabletop centrifuge at 1500 rpm for 2 minutes, storage buffer was discarded, and resin was washed with 10 mL of ice-cold PBS and resuspended in 400 μL of PBS. Resin and cleared cell extract were incubated together with mixing for 1 hour at room temperature or overnight at 4°C. Resin was sedimentated in a tabletop centrifuge at 1500 rpm for 2 minutes washed twice for 10 minutes with 10 mL of PBS, and transferred to a disposable column; wash solution was then removed by gravity flow. Resin was washed with an additional 10 mL of PBS, and the GST-fusion protein was eluted with 25 mM reduced glutathione in PBS (pH 7.4) with 500-μL fractions collected and stored at -80°C

Purification of His6x-tagged proteins

Hexahistidine (His6x) tagged proteins were purified from BL21 (DE3) pLYS *E.coli.* Cells were grown overnight in 10 mL of LB medium plus 100 μg/mL ampicillin and 34 μg/mL chloraphenicol at 37°C. The following morning 4 mL of overnight culture was added to 400 mL of LB medium plus ampicillin and chloramphenicol and cells were grown to mid-logarithmic phase at 37°C. Next, 400 μL of 0.5 M isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture which was transferred to 30°C and grown an additional 4-5 hours. Cells were harvested by centrifugation at 2500 x *g* at 4°C for 10 minutes, cells were washed once with PBS, and stored at -80°C if not used immediately. Cells pellets were resuspended in 15-20 mL of bacterial protein extraction reagent (B-PER, Pierce), 1/50 (v/v) EDTA-free complete protease inhibitor and passed repeatedly through 16.5, 18.5, and 21.5 gauge needles. Lysates were cleared by centrifugation at 27000 x *g* rpm at 4°C for 20 minutes and the supernatants were transferred to fresh tubes. Talon resin (Chelated cobalt, Clontech) was resuspended and 500 μL of slurry was transferred to a 15-mL Falcon tube. The resin was sedimentated in a table-top centrifuge at 1500 rpm for 2 minutes, storage buffer was discarded, and resin was washed with 10 mL of ice-cold Talon wash buffer (50 mM sodium phosphate, pH 7.0, 300 mM sodium chloride) and resuspended in 400 μL of Talon wash buffer. Resin and cleared cell extract were incubated together with mixing for 1 hour at room temperature or overnight at 4°C. Resin was sedimentated in a tabletop centrifuge at 1500 rpm for 2 minutes, washed three times for 10 minutes with 10 mL of Talon wash buffer,

and transferred to a disposable column; wash solution is removed by gravity flow. Resin was then washed with an additional 10 mL of Talon wash buffer containing 5 mM imidazole, and the protein was eluted with Talon wash buffer containing 150 mM imidazole, with 500-μL fractions collected and stored at -80°C

Vps10 half-life determination

Cultures were grown to early logarithmic phase in 10 mL of YEPD medium at which point cycloheximide (Sigma) was added to a final concentration of 50 μg/mL (Cereghino et al., 1995). Samples were taken at 1 and 3 hours, cells were lysed by mechanical bead beating and protein levels were determined by microBCA. Equal protein amounts were resolved by SDS-PAGE and immunoblotting. The anti-Vps10 antibody was used following manufacturers recommendation (Molecular Probes).

Phosphorylation status of GFP-Snc1

Wild-type and mutant cells expressing GFP-Snc1 were grown to mid-logarithmic phase in minimal medium lacking uracil to ensure plasmid maintenance. Typically, 4-8 OD_{600} unit equivalents of cells were collected by centrifugation at 3000 rpm in a tabletop centrifuge, resuspended in lysis buffer and homogenized by mechanical bead beating. The composition of the lysis buffer was 50 mM Tris-HCl (pH7.5), 10% glycerol, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM NaF 1/50 (v/v) complete protease inhibitor, 10 μg/mL leupeptin, 20 μg/mL pepstatin, 0.2 mM sodium orthovanadate, 15 mM sodium pyrophosphate, 5 mM β-glycerophosphate. Whole cells and beads were separated from the cell extract by centrifugation at 700 x *g* for 5 minutes at 4°C. Extracts were transferred to eppendorf tubes and the appropriate volumes of 4x SDS-PAGE running buffer were added. Samples were resolved by SDS-PAGE using stacked 10% and 15% acrylamide gels. Proteins were transfered to PVDF and detected using an anti-GFP antibody (Amersham).

***in vitro* transcription and translation**

Plasmids containing a T7 bacterial promoter and an ORF of interest were used as template for *in vitro* transcription coupled to translation following the manufacturers

protocol (Promega). Briefly, 40 μL of rabbit reticulolysate was combined with 0.5 μg of plasmid DNA plus 2 μL of 1 mM methionine solution and made up with water up to 50 μL. Tubes were incubated at 30°C for 60-90 minutes and used immediately.

CHAPTER 4. RESULTS – SEC14 AND KES1

4.1.1. CHARACTERIZATION OF *kes1*-MEDIATED SEC14-BYPASS

To try to learn more about the biology surrounding Kes1 and Sec14 a series of synthetic lethal screens were employed. Synthetic lethality should identify genes with similar functions.

The yeast strain AFY-1A2 (*ade2 ade3*) is an adenine auxotroph with two enzymatic steps in the adenine biosynthetic pathway inactivated and thus grows normally in the presence of adenine but is unable to grow in its absence. When transformed with a plasmid carrying a functional *ADE3* gene the cells still are unable to grow in the absence of adenine, but the cells accumulates a red pigment that is distinguishable by visual inspection. This provides a visually tractable system that is the basis for identifying potential synthetic lethal interaction partners. Cells of the yeast strain GFY1 (*ade2 ade3 kes1::HIS3*) containing pSLS-KES1 (carrying *KES1* and *URA3*) were grown to mid-logarithmic phase and spread on YEPD solid medium at a density of 500 cells per plate. The cells were then exposed to UV light to mutagenize genomic DNA and allowed to grow for 3 days in the dark. The cells grew on rich medium containing uracil, so there was no selective pressure to maintain the pSLS-KES1 plasmid. Progeny within a given yeast colony that do not receive the plasmid during mitosis will not have a functional *ADE3* gene and will not accumulate any pigment, and likewise for any cells in the lineage of this cell. During the course of this genetic screen, of the 300,000 colonies screened, none of the colonies examined remained red over extended periods of growth. As the plasmid is the sole source of *ADE3* and *KES1* in this strain, none of the mutated colonies required a functional *KES1* for viability. The results are consistent with no single loss-of-function mutation forms a synthetic lethal pair with *kes1*, though additional rounds of screening could identify gain-of-function mutations or partial loss-of-function mutations in essential genes.

4.1.2. SYNTHETIC GENETIC ARRAY (SGA) ANALYSIS, SINGLE MUTANTS

The creation of the non-essential yeast single-mutant deletion collection (Winzeler et al., 1999)allowed the development of robotics for high-throughput synthetic lethal screens, referred to as SGA analysis (Tong et al., 2001). The inability to identify synthetic lethal interaction partners for *kes1* by using UV mutagenesis and colour screening suggested that there were no synthetic lethal interacting partners for *kes1*. SGA analysis is a complimentary method to investigate synthetic interactions with *kes1*. SGA analysis has the benefit of knowing the identity of the genes being tested directly. Furthermore, instead of colour screening, SGA analysis compares the relative growth rates of double mutants to those of the single mutants, with the advantage being that more subtle genetic interactions are detectable. Inactivation of *KES1* is known to bypass the loss of *SEC14* (Fang et al., 1996), as does inactivation of the genes of the CDP-choline pathway for PC synthesis (*CKI1, PCT1,* and *CPT1)* (Cleves et al., 1991). To learn more about the biology associated with *KES1,* SGA analysis of strains with inactivated *KES1, CKI1, PCT1* and *CPT1* was performed using the four single-mutant strains CMY209, 210, 214, 215, with the results listed in Table 4. Consistent with the previous synthetic lethal screen, no genes were found to form a synthetic lethal pair with *KES1* by SGA analysis.

To confirm impairment of the CDP-choline pathway, cells of strains CMY209 (*cki1*::NAT^R), CMY214 (*pct1*::NAT^R), and CMY215 (*cpt1*::NAT^R) were grown to logarithmic phase at 30°C and cultures labeled for 1 hour with [^{14}C] choline after which lipids were extracted and counted (Figure 10). All three mutants have an impaired incorporation of choline into PC, with *cki1* reduced to 15%, *pct1* reduced to 2% and *cpt1* reduced to 40%; these values are similar to previously reported values (Skinner et al., 1995). No gene formed a synthetic lethal pair with *cki1;* however 24 genes formed a synthetic lethal/sick pair with *pct1,* with 12 of them also identified in the *cpt1* screen (Table 4). Pct1 is the rate-limiting enzyme of the pathway and the sole enzyme catalyzing this step in the pathway. Consistent with this fact, *pct1* has the largest number of synthetic lethal interactions. The genes common to both *pct1* and *cpt1* in general are of

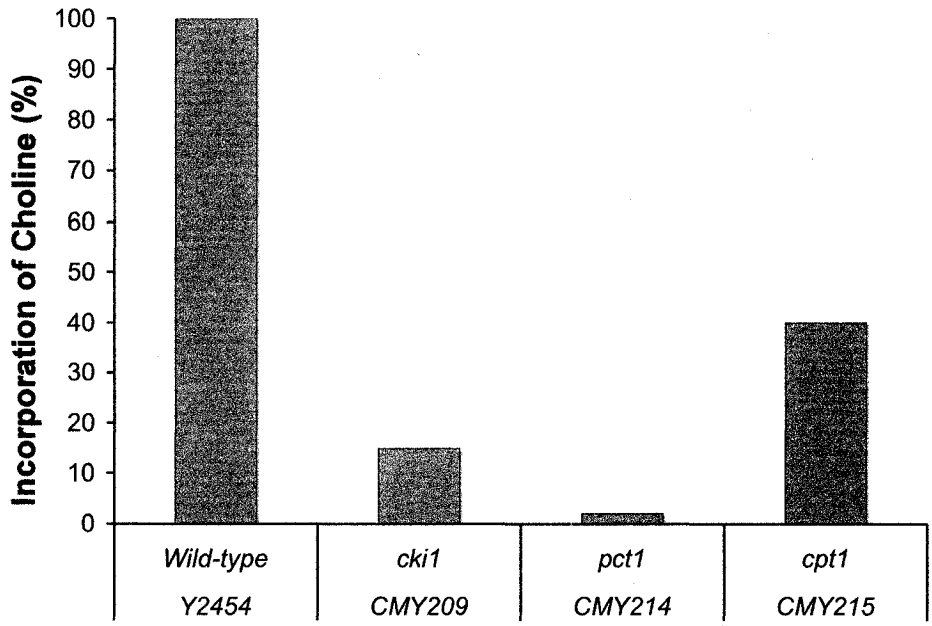


Figure 10. Phosphatidylcholine synthesis by the CDP-choline pathway. Wild-type cells and CDP-choline pathway mutants were grown to log phase at 30°C in minimal medium supplemented with 10 μM choline and then incubated in the presence of [^{14}C]choline for 1 h. Lipids were extracted and quantified by scintillation counting. Values represent percent incorporation compared to wild-type.

Table 4. Synthetic lethal results for *pct1* and *cpt1*

ORF	Common Name(s)	Description	SL partner(s)
YLR025w	*SNF7, VPS32*	subunit of ESCRT III	*pct1, cpt1*
YMR077c	*VPS20*	subunit of ESCRT III	*pct1, cpt1*
YKL080w	*VMA5*	vacuolar ATPase V1 domain subunit C	*pct1, cpt1*
YLR148w	*PEP3, VPS18*	HOPS complex, vacuolar biogenesis	*pct1, cpt1*
YMR231w	*PEP5, VPS11*	HOPS complex, vacuolar biogenesis	*pct1, cpt1*
YDR323c	*PEP7, VPS19*		*pct1, cpt1*
YPL045w	*VPS16*	HOPS complex, vacuolar biogenesis	*pct1, cpt1*
YLR417w	*VPS36*	Component of the ESCRT-II complex	*pct1, cpt1*
YLR240w	*VSP34, PEP15*	Phosphatidylinositol 3-kinase	*pct1, cpt1*
YER144c	*UBP5*	Putative ubiquitin-specific protease	*pct1, cpt1*
YPR159w	*KRE6*	required for beta-1,6 glucan biosynthesis	*pct1, cpt1*
YOR036w	*PEP12, VPS6*	t-SNARE for vesicular intermediates traveling between the Golgi apparatus and the vacuole	*pct1, cpt1*
YGR020c	*VMA7*	vacuolar ATPase V1 domain subunit F	*pct1*
YML001w	*YPT7, VAM4*	Rab protein, endosomal fusion	*pct1*
YKL041w	*VPS24*	subunit of ESCRT III	*pct1*
YGL095c	*VPS45*	Protein of the Sec1/Munc-18 family, essential for vacuolar protein sorting	*pct1*
YLR396c	*VMA5, VPS33*	HOPS complex, vacuolar biogenesis	*pct1*
YHR012w	*VPS29*	retromer complex essential for endosome-to-Golgi retrograde transport	*pct1*
YBR112c	*SSN6, CYC8*	General transcriptional co-repressor, acts together with Tup1	*pct1*
YCR084c	*TUP1*	General repressor of transcription, forms complex with Cyc8,	*pct1*
YJL176c	*SWI3*	Subunit of the SWI/SNF chromatin-remodeling complex, Required for *INO1* expression	*pct1*

YGR105w	*VMA21*	Required for V-ATPase function, localized to the ER	*pct1*
YBR127c	*VMA2*	vacuolar ATPase V1 domain subunit B	*pct1*
YHR060w	*VMA22*	Required for V-ATPase function, localized to the ER	*pct1*

the Vacuole Protein Sorting (*VPS*) class. Trafficking to the Golgi is a multi-step process and the gene products are spread over several of the steps. The genes unique to the *pct1* screen were additional *VPS* types, *VMA* (vacuolar ATPase) members and genes involved in chromatin remodeling and transcription.

4.1.3. SGA ANALYSIS OF SEC14-BYPASS

To investigate the biology surrounding "Sec14 bypass" two additional strains were created and subjected to SGA analysis. Due to the observation that *ckil* and *kes1* have no synthetic lethal interactions, the strains CMY212 (*ckil::*NAT^R *sec14::LEU2*) and CMY213 (*kes1::*NAT^R *sec14::LEU2*) were created, transformed with an empty pESC-ura or pESC-KES1 (containing a galactose inducible *KES1*) and transferred to SC-galactose-ura medium (Figure 11). Consistent with previous results, increased *KES1* expression prevented *ckil*-mediated bypass of Sec14 and restoration of *KES1* prevents *kes1*-mediated Sec14 bypass (Fang et al., 1996; Li et al., 2002).

SGA analysis was performed using both CMY212 and 213 and the resulting triple mutants were monitored for growth. This procedure identified 33 genes required to support *ckil*-mediated Sec14 bypass and 16 genes required for *kes1*-mediated bypass of Sec14, with 12 genes in common (Tables 5 & 6 and Figure 12).

Previously it was reported that all modes of Sec14 bypass required *SPO14,* the sole yeast PC-phospholipase D (PLD) enzyme (Xie et al., 1998), and *spo14* was one of the 12 genes common to both screens. With the exception of *VPS4,* none of the other genes shared by the two screens have known roles in vesicular transport, nor do any of the genes required for *kes1*-mediated Sec14 bypass. Conversely, of the 21 genes specifically required for the viability of the *sec14 ckil* strain, most have described roles in vesicular trafficking from the Golgi. This is consistent with the major function of Sec14 being regulation of Golgi-derived vesicular transport. Genes common to both screens also have known roles in the maintenance of the yeast cell wall. Secretion and cell polarity are both critical for the proper production and maintenance of the cell wall in a growing yeast cell.

Figure 13 is a synthetic lethality interaction network diagram for *sec14*. The top half of the diagram shows genes involved in cell wall production and cell polarity,

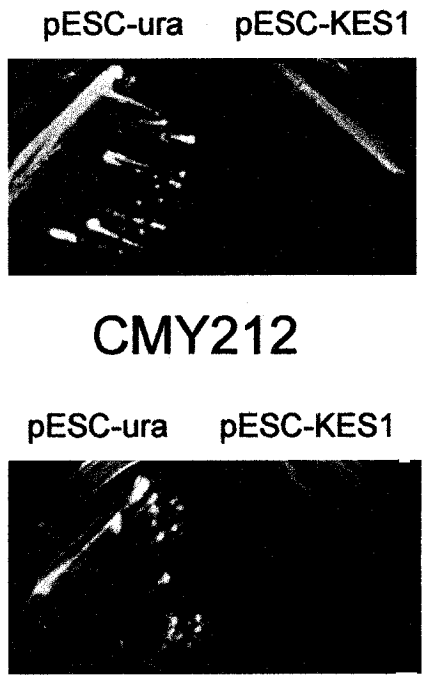


Figure 11. Kes1 dosage and bypass of Sec14. The strains CMY212 (*ckl1::*natR *sec14::LEU2*) and CMY213 (*kes1::*natR *sec14::LEU2*) were transformed with either empty pESC-ura vector or pESC-KES1. Single colonies from an SC-URA plate were streaked on SGal-URA medium to induce expression of *KES1* and incubated 72 h at 30°C.

Table 5. Synthetic lethal interactions identified for *sec14 cki1*

ORFs	Common Name(s)	Description
YKR031c	*SPO14, PLD1*	Phospholipase D, catalyzes the hydrolysis of phosphatidylcholine, producing choline and phosphatidic acid
YBR164c	*ARL1, DLP1*	Soluble GTPase with a role in regulation of membrane traffic; similar to ARF
YPL051w	*ARL3*	GTPase of the Ras superfamily required to recruit Arl1 to the Golgi; similar to ADP-ribosylation factor
YHL031c	*GOS1*	v-SNARE protein involved in Golgi transport
YOR070c	*GYP1*	Cis-golgi GTPase-activating protein (GAP) for Rab family. Role in vesicle docking and fusion
YOL018c	*TLG2*	t-SNARE involved in retrograde transport; endosomes to Golgi
YDR108w	*TRS85, GSG1*	Subunit of TRAPP (transport protein particle); Roles in transport into and out of the Golgi
YGR166w	*TRS65, KRE11*	Subunit of TRAPP (transport protein particle); Roles in transport into and out of the Golgi
YOR115c	*TRS33*	Subunit of TRAPP (transport protein particle); Roles in transport into and out of the Golgi
YER031c	*YPT31, YPT8*	GTPase of the Ypt/Rab family; role in budding of post-Golgi vesicles from the trans-Golgi
YJL145w	*SFH5*	Putative phosphatidylinositol transfer protein (PITP); Sec14 Homolog
YLR380w	*SFH2, CSR1*	Putative phosphatidylinositol transfer protein (PITP); Sec14 Homolog
YDR293c	*SSD1, CLA1, MCS1*	Cell integrity; TOR pathway
YKR001c	*VPS1*	GTPase required for vacuolar protein sorting
YDR320c	*SWA2, AUX1*	Clathrin-binding protein required for uncoating of clathrin-coated vesicles
YNR051c	*BRE5*	Ubiquitin protease cofactor; involved in vesicular transport
YHR129C	*ARP1, ACT5*	Actin-related protein of the dynactin complex
YNL298W	*CLA4, ERC10*	Cdc42 activated signal transducing kinase of the PAK (p21-activated kinase) family
YKR020W	*VPS51, VPS67*	Component of the GARP (Golgi-associated retrograde protein) complex
YOR311c	*HSD1*	High-copy suppressor of *sly1* defects
YGL147c	*RPL9a*	Protein component of the large (60S) ribosomal subunit
YNL136w	*EAF7*	Subunit of the NuA4 histone acetyltransferase complex
YLR371w	*ROM2*	GDP/GTP exchange protein (GEP) for Rho1 and Rho2
YMR307w	*GAS1*	Beta-1,3-glucanosyltransferase, required for cell wall assembly
YNL238w	*KEX2*	Subtilisin-like protease (proprotein convertase)

YDR496c	*PUF6*	Binds *ASH1* mRNA at PUF consensus sequences in the 3' UTR and represses its translation
YGL007w		Hypothetical protein
YIL110w	*MNI1*	Putative S-adenosylmethionine-dependent methyltransferase
YLR454w	*FMP27*	Localizes to the mitochondria
YDL133w		Hypothetical protein
YPR069c	*SPE3*	Spermidine synthase
YPL086c	*ELP3*	Histone acetyltransferase subunit of the Elongator complex
YPR173C	*VPS4, CSC1, END13*	AAA-type ATPase required for efficient late-endosome to vacuole transport

Table 6. Synthetic lethal interactions found for sec14 kes1

ORFs	Common Name(s)	Description
YKR031c	*SPO14, PLD1*	Phospholipase D, catalyzes the hydrolysis of phosphatidylcholine, producing choline and phosphatidic acid
YLR371w	*ROM2*	GDP/GTP exchange protein (GEP) for Rho1 and Rho2
YMR307w	*GAS1*	Beta-1,3-glucanosyltransferase, required for cell wall assembly
YNL238w	*KEX2*	Subtilisin-like protease (proprotein convertase)
YDR496c	*PUF6*	Binds *ASH1* mRNA at PUF consensus sequences in the 3' UTR and represses its translation
YGL007w		Hypothetical protein
YIL110w	*MNI1*	Putative S-adenosylmethionine-dependent methyltransferase
YLR454w	*FMP27*	Localizes to the mitochondria
YDL133w		Hypothetical protein
YPR069c	*SPE3*	Spermidine synthase
YPL086c	*ELP3*	Histone acetyltransferase subunit of the Elongator complex
YPR173c	*VPS4, CSC1, END13*	AAA-type ATPase required for efficient late-endosome to vacuole transport
YHL007c	*STE20*	Signal-transducing kinase of the PAK (p21-activated kinase) family
YDR159w	*SAC3, LEP1*	Forms a complex with Thp1 that is involved mRNA export from the nucleus
YPL026c	*SKS1*	Serine/threonine protein kinase
YOR283w		Hypothetical protein

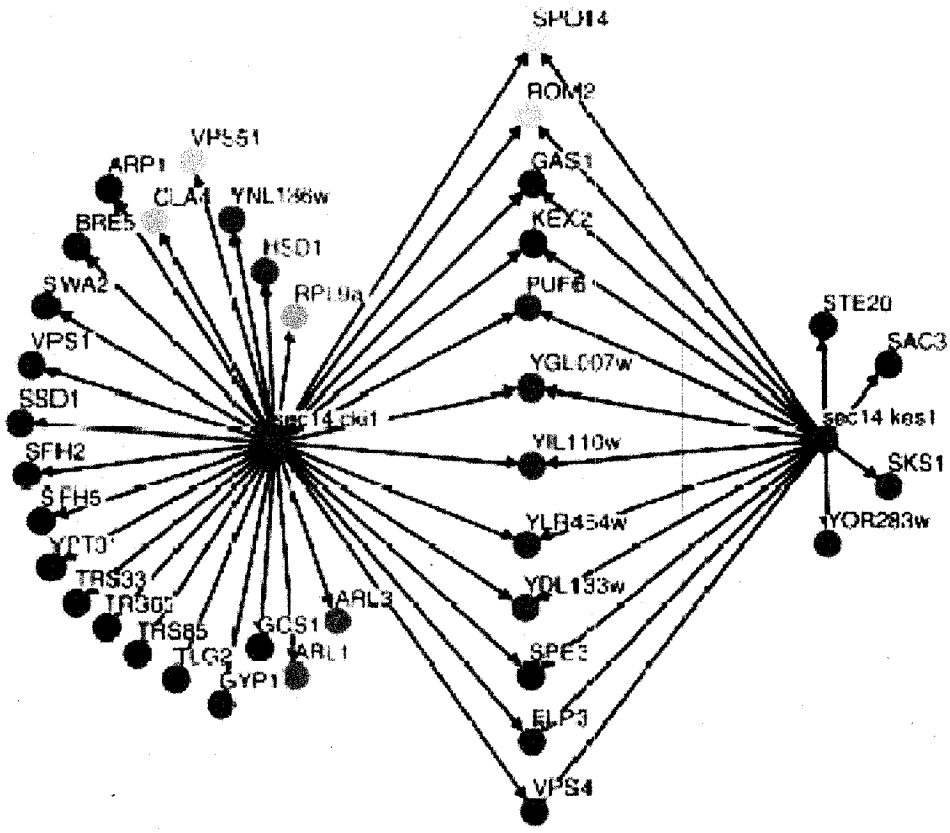


Figure 12. Synthetic lethal interactions formed with *sec14*. The diagram displays the synthetic interactions discovered for the query strains CMY212 (*sec14-1^{ts}*:nat^{R} *ckl1::LEU2*) and CMY213 (*sec14^{ts}*:nat^{R} *kes1::LEU2*). Genes are represented as nodes and interactions as lines with genes coloured based on Gene Ontology function: blue, metabolism; light purple, cell organization and biogenesis; dark purple, transport; pink, protein transport; yellow; budding; green, cell cycle; orange, protein biosynthesis; light blue, sporulation; gold, protein amino acid phosphorylation; grey, unknown.

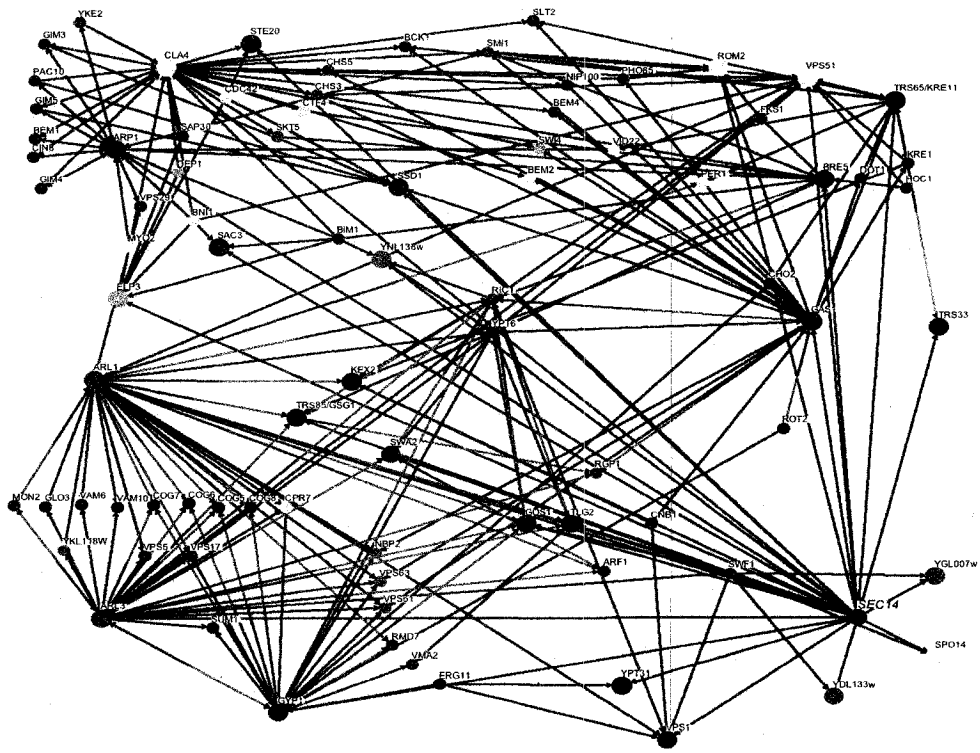


Figure 13. Genetic interaction network for *sec14*. The network was assembled with the Osprey program using the sec14-bypass screen results displayed in Figure 12 and previous results available from the GRID database. Genes are represented as nodes and interactions as edges. Genes are colored based on Gene Ontology function. blue, metabolism; light purple, cell organization and biogenesis; dark purple, transport; pink, protein transport; yellow; budding; green, cell cycle; orange, protein biosynthesis; light blue, sporulation; gold, protein amino acid phosphorylation; grey, unknown. All genes displayed have at least two annotated interactions. A total of 269 genes and 430 genetic interactions were identified.

whereas the bottom half shows genes involved in vesicular transport to and from the Golgi. The interaction network can be loosely subdivided into cause and effect modules. Cells lacking *sec14* function have enfeebled transport into and out of the Golgi, so additional mutations compounding this transport problem will kill the cell. Cells with reduced rates of secretion have difficulty sustaining their cell walls, with any further defects in cell-wall maintenance resulting in cell death. The *sec14* interaction network consists of 230 genes comprising 430 interactions; for simplicity, Figure 13 shows only genes with two or more genetic interactions.

Previous work had identified four genes whose inactivation prevents the growth of "Sec14 bypass" strains (Xie et al., 1998; Yanagisawa et al., 2002; Li et al., 2002). These were: (i) the previously mentioned *SPO14*; (ii) the Arf/Arl GTPase-activating protein (GAP) gene *GCS1* required for growth and secretion by *sec14*^{ts} *cki1* and *sec14*^{ts} *kes1* cells at the non-permissive temperature of 37°C (Yanagisawa et al., 2002); (iii) *ARF1* encoding the more prevalent of the two ADP-ribosylation factor which was required for *sec14*^{ts} *kes1* growth, (Li et al., 2002) and; (iv) the ArfGAP gene *AGE2* required for growth of *sec14*^{ts} *sac1* cells (Yanagisawa et al., 2002). As mentioned above, SGA analysis identified *SPO14* as required for both *sec14 kes1* and *sec14 cki1* yeast but did not identify *GCS1* or *ARF1*. Construction of *sec14::LEU2 cki1::NAT*^{R} *gcs1::KAN*^{R} and *sec14::LEU2 kes1::NAT*^{R} *age2::KAN*^{R} strains revealed that they were viable at 30°C, the temperature at which our SGA analysis was performed, but were inviable at 37°C, the temperature used for analysis of strains carrying the *sec14*^{ts} allele. The results demonstrate that the genetic interactions between *SEC14* and *ARF1, GCS1,* and *AGE2* are subtle and only present themselves when cells are incubated at elevated temperatures.

4.1.4. *sec14 cki1* CELLS REQUIRE YPT31 AND THE TRAPPII COMPLEX

The major known defect in cells with deficient Sec14 function is an inability to effectively transport vesicles out of the *trans*-Golgi due to a mismanagement of Golgi lipid composition (Bankaitis et al., 1989; Cleves et al., 1991; McGee et al., 1994). Results from our SGA analysis confirmed that a major role for Sec14 is the regulation of Golgi-derived vesicular transport, and connected Sec14 regulation of Golgi lipid content with proteins that regulate vesicle fission. To further investigate the new genetic interactions

identified in this work, isogenic strains were constructed using the *sec14-1^{ts}:NAT^{R}* strain as the parent and disrupting genes of interest.

From the SGA analysis, loss of function of the Rab GTPase Ypt31 as well as non-essential components of the Transport Protein Particle II (TRAPPII) Trs33, Trs65/Kre11, and Trs85/Gsg1 were identified as specifically being required for *ckil*-mediated Sec14 bypass. TRAPPII is an essential Golgi-associated complex containing 10 subunits (the essential subunits being Bet3, Bet5, Trs20, Trs23, Trs31, Trs120, and Trs130) (Sacher et al., 2001; Sacher et al., 1998). The TRAPPII complex has been described as possessing guanine-nucleotide exchange activity toward the highly similar Ypt31/32 pair (Jones et al., 2000; Morozova et al., 2006). The Ypt31/32 Rab GTPases function as an essential pair and control exit from the *trans*-Golgi as well the cycling of the v-SNARE Snc1 between the Golgi and plasma membranes (Benli et al., 1996; Chen et al., 2005; Jedd et al., 1997). To confirm these findings, strains were constructed using the *sec14-1^{ts}* allele and were characterized at permissive and non-permissive temperatures.

Wild-type cells, *sec14^{ts}* mutants and single-deletion mutants *trs33, trs65, trs85, ypt31* and *ypt32* were grown to mid-logarithmic phase in YEPD medium and washed, and equal numbers of cells were serially diluted, spotted on YEPD agar medium, and allowed to grow for 3 days at 30°C or 37°C. All cells grow normally at 30°C, while at 37°C the *sec14^{ts}* cells died and the *trs85* cells grew poorly (Figure 14a). To assess the secretion efficiency of the single mutants the secretion of invertase was measured. Briefly, cells were grown to mid-logarithmic phase in YEPD medium, washed, resuspended in 0.1% glucose YEPD and grown at 37°C for 2 hours. At this time, the cultures were harvested and the extracellular and total invertase activities were determined and used to estimate the secretion efficiency. All the mutants secrete 75% of their invertase or more over the course of 2 hours, which is similar to the behaviour of to the wild type cells (Figure 14b). While the *trs85* mutant appeared to have difficulties growing at elevated temperatures, this problem did not appear to affect its ability to secrete.

With a baseline obtained for the growth and secretion of the single mutants at elevated temperatures, the *sec14^{ts} ckil TRAPPII* triple mutants were examined. Cells were grown to mid-logarithmic phase in YEPD, harvested, serially diluted and spotted on

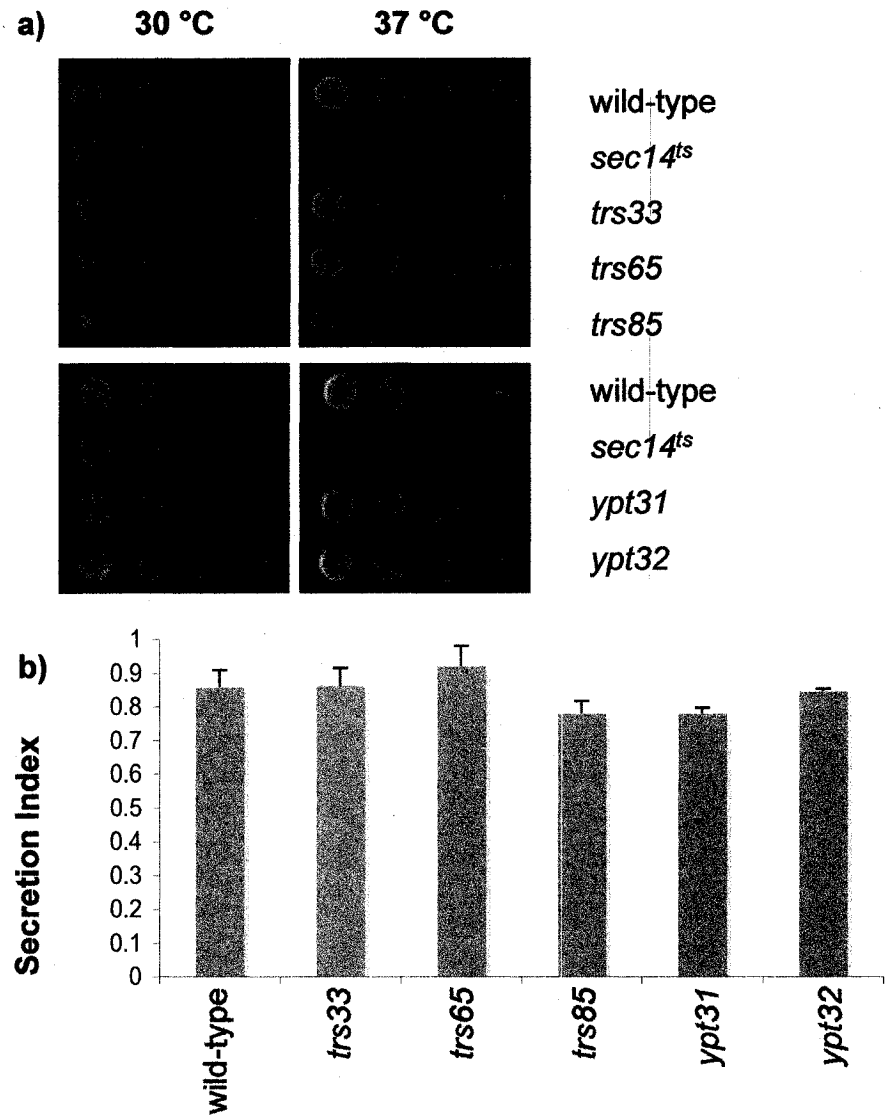


Figure 14. Cells lacking genes required to support Sec14-independent growth grow and secrete normally. The indicated single-deletion strains from the BY4741 background were assayed for growth and invertase secretion at 37°C. a) Cultures were grown to log phase, serially diluted and spotted on YEPD agar medium and allowed to grow for 48-72 h at the indicated temperatures. b) Log-phase cells were harvested and washed in low-glucose YEPD to induce the *SUC2* gene and incubated at 37 °C for 2 h. The secretion index was determined by measuring the activity of the extracellular invertase compared to the total. Data are the average of at least three independent experiments ± S.E.M.

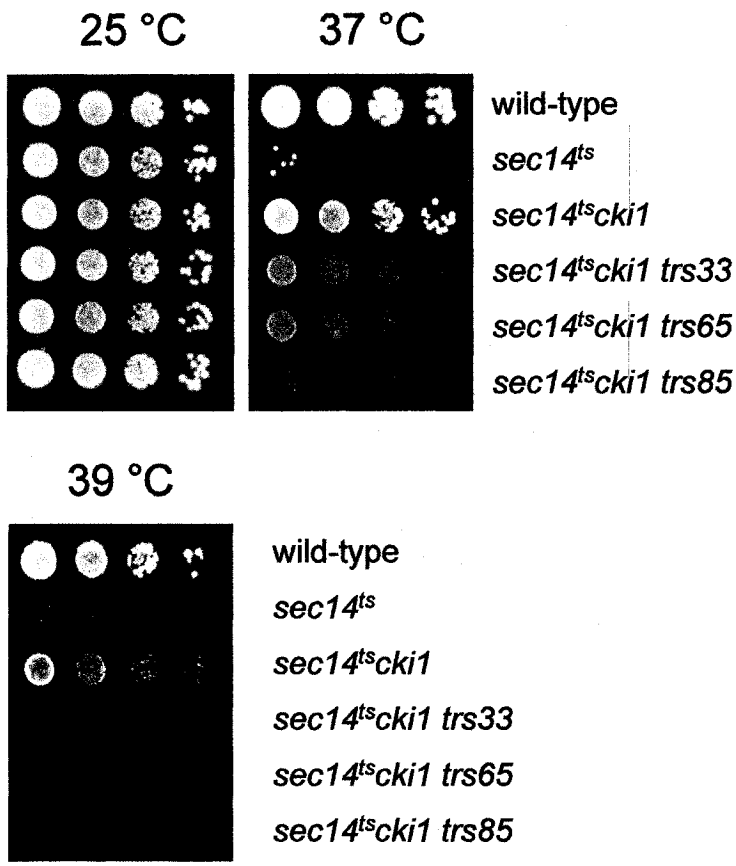


Figure 15. Nonessential TRAPP II components are required for *cki1*-mediated Sec14 bypass. Cells of the isogenic *sec14^{ts}* strains indicated were grown to mid-logarithmic phase and harvested, and 10-fold serial dilutions were spotted on YEPD solid medium and incubated for 72 h at the indicated temperatures.

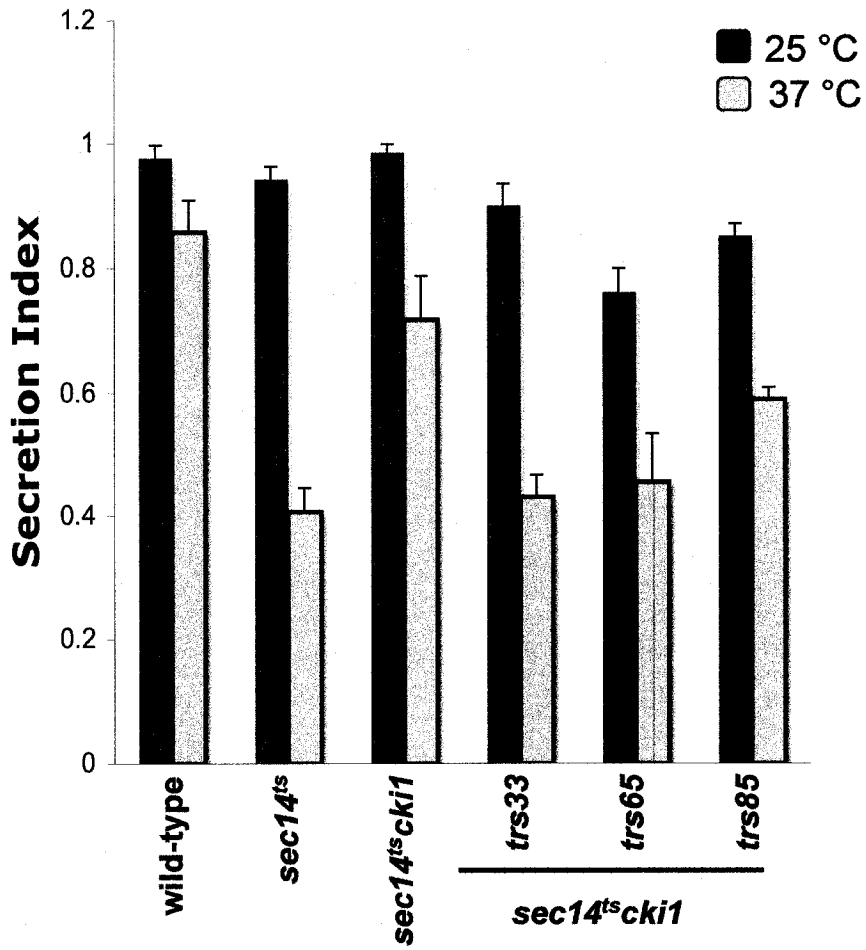


Figure 16. Loss of TRAPP II components compromises secretion by *sec14^{ts} ckil* cells. Cells of the isogenic *sec14^{ts}* strains indicated were grown to mid-logarithmic phase at 25°C, harvested and washed in low-glucose (0.1%) YEPD to induce expression of the *SUC2* gene, encoding the invertase enzyme, and grown for an additional 2 hours at the indicated temperature. The secretion index was determined by measuring the activity of the extracellular invertase compared to the total. Data are the average of at least three independent experiments ± S.E.M.

YEPD solid medium. After 3 days of growth at 30°C, 37°C or 39°C cells containing *sec14^{ts}* died *sec14^{ts} ckil* cells remained viable and *sec14^{ts} ckil trs33, trs65, or trs85* triple mutants grew slower at 37°C and appeared to die at 39°C (Figure 15). Consistent with this observation, when the secretion efficiency of these triple mutants was examined each one had reduced efficiency at the non-permissive temperature for the *sec14^{ts}* allele (approaching the levels of *sec14^{ts}* single-mutant cells) (Figure 16). To further assess the genetic interaction between *SEC14* and the TRAPP II complex, a temperature-sensitive allele for an essential member of TRAPP II, *trs130^{ts}*, was utilized. Diploids were constructed by mating *sec14^{ts}* cells with *trs130^{ts}* cells resulting in a diploid (*SEC14/sec14^{ts} TRS130/trs130^{ts}*). These cells heterozygous for both *SEC14* and *TRS130* displayed growth defects at the non-permissive temperature (Figure 17). In contrast diploid cells haploinsufficient for either *SEC14* or *TRS130* were normal at the elevated temperatures. The combination of two heterozygous genes resulting in death is a very strong genetic interaction, which will be referred to as "synthetic haploinsufficiency" for the remainder of this thesis. The data indicate that the combined functions of Sec14 and TRAPP II are important for secretion from the Golgi.

YPT31 and *YPT32* form an essential gene pair whose simultaneous inactivation results in an inability to secrete from the *trans*-Golgi. The protein products share 81% identity and 90% similarity (Jedd et al., 1997), yet only *ypt31* was revealed by SGA analysis to form a synthetic lethal interaction with *sec14 ckil*. To investigate further, haploid cells of *sec14^{ts} ckil ypt31* and *sec14^{ts} ckil ypt32* genotype were constructed. The *sec14^{ts} ckil ypt31* cells demonstrated severe growth impairment at 37°C, while the *sec14^{ts} ckil ypt32* cells displayed no growth or secretion defects when compared to the parental *sec14^{ts} ckil* cells (Figures 18 & 19), confirming the SGA analysis. Consistent with the impairment of growth at the non-permissive temperature, the secretion of invertase was defective in *sec14^{ts} ckil ypt31* cells but not in *sec14^{ts} ckil ypt32* cells. Therefore dispite their high degree of similarity, Ypt31 and Ypt32 proteins do not appear to be interchangeable.

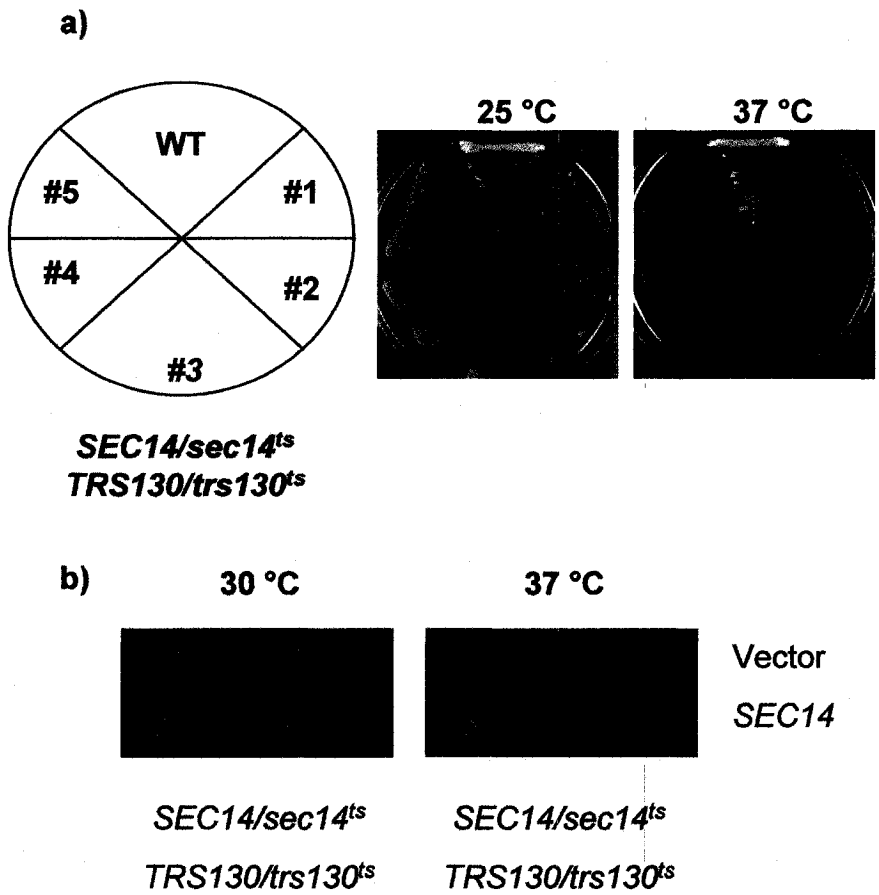


Figure 17. *SEC14* and *TRS130*, encoding an essential TRAPPII-specific component, constitute a synthetic haploinsufficient pair. Five (#1-#5) independently isolated diploids (*SEC14/sec14^{ts}, TRS130/trs130^{ts}*) were streaked on YEPD solid medium and allowed to grow for 72-hours at 25°C or 37°C. A *SEC14/sec14^{ts}, TRS130/trs130^{ts}* diploid was transformed with either pRS416 vector or pRS416-SEC14 and grown to logarithmic phase at 25°C. Identical numbers of cells were serially diluted and spotted on complete medium lacking uracil to ensure plasmid maintenance. Images were captured after 72 h of growth at the indicated temperature.

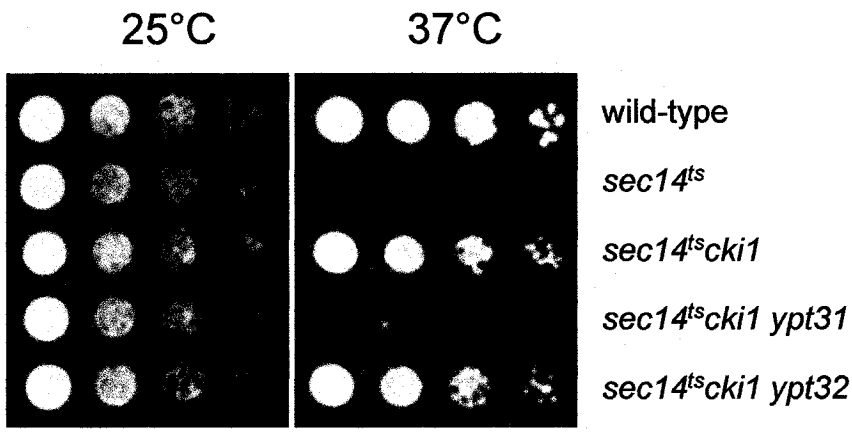


Figure 18. The Rab protein Ypt31 but not Ypt32 is required for *cki1*-mediated Sec14 bypass. The isogenic *sec14^{ts}* cells indicated were grown to mid-logarithmic phase and harvested, and 10-fold serial dilutions were spotted on YEPD solid medium and incubated for 72 h at the indicated temperatures.

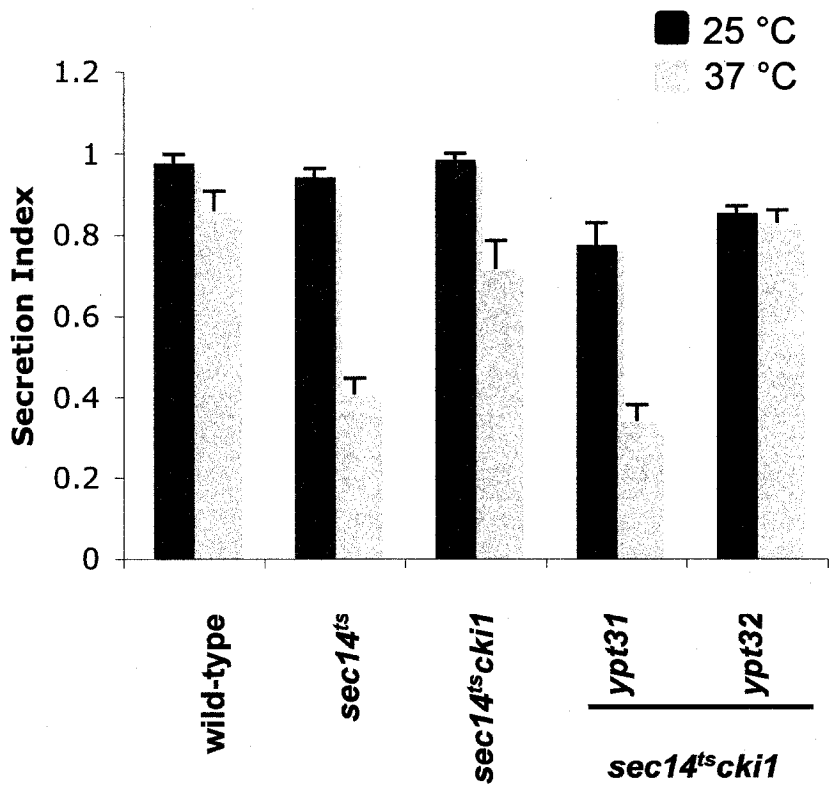


Figure 19. Loss of *YPT31* but not *YPT32* compromises secretion by *sec14^{ts} cki1* cells. The isogenic *sec14^{ts}* strains indicated were grown to mid-logarithmic phase at 25°C, harvested and washed in low-glucose (0.1%) YEPD to induce expression of the *SUC2* gene, and grown for an additional 2 hours at the indicated temperature. The secretion index was determined by measuring the activity of the extracellular invertase compared to the total. Data are the average of at least three independent experiments ± S.E.M.

4.1.5. PC-ONLY SEC14 AND SFH2 SUPPRESSES GROWTH DEFECTS

Previous reports have demonstrated that both Sfh2 and PC-only Sec14 can bypass the essential requirement of Sec14 (Li et al., 2000; Phillips et al., 1999). With Sfh2 only displaying PI transfer ability and Sec14$^{(K66,239A)}$ only able to transfer PC ("PC-only" Sec14), it would be reasonable to believe that they suppress *sec14^{ts}* defects differently. Plasmids carrying *SEC14, sec14$^{(K66,239A)}$ and SFH2* were transformed into CMY309 (*sec14^{ts} ckil ypt31*)cells, which were then grown to mid-logarithmic phase, serially diluted, spotted on minimal solid medium and incubated at 25°C and 37°C for 3 days. Figure 20 shows that the expression of *SEC14, sec14$^{(K66,239A)}$* or *SFH2* all suppress the growth defects at the non-permissive temperature.

4.1.6. INCREASED DOSAGE OF YPT31 DOES NOT SUPPRESS *sec14^{ts}* DEFECTS

Together with the proposed role of the TRAPPII complex as a GEF for Ypt31/32, my data demonstrate Ypt31 and its GEF are required for *ckil*-mediated Sec14 bypass. To examine the Sec14-Ypt31 relationship further, the genes encoding Ypt31 and a GTP-bound constitutively activated mutant of Ypt31 (Ypt31Q72L) (supplied by Nava Segev, University of Illinois) were transformed into *sec14^{ts}* or *trs130^{ts}* cells which were grown to mid-logarithmic phase, serially diluted and spotted onto solid medium, and incubated for 3 days at 25°C, 35°C or 37°C to allow growth. Both increased expression of Ypt31 and expression of the GTP-bound constitutively activated mutant of Ypt31 rescued the *trs130^{ts}* cells (Sciorra et al., 2005), but were unable to rescue *sec14^{ts}* cells (Figure 21). While Trs130 function is essential, and defects appear to be suppressed by elevated Ypt31 activity, the same is not true for cells with compromised Sec14 function. This difference implies that the sole essential function of Trs130 is to activate Ypt31, whereas Sec14 has additional roles, possibly in addition to regulating TRAPPII.

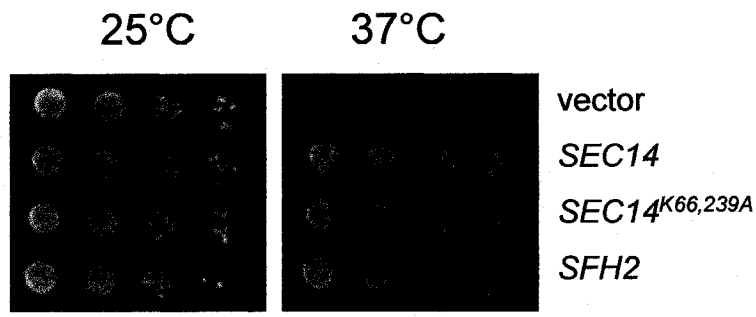


Figure 20. *SFH2* or *SEC14*$^{(K66,239A)}$ can suppress defects associated with *sec14*^{ts} *ckil ypt31* cells. Cells of the GFY309 (*sec14*^{ts}:natR *ckil1::LEU2 ypt31::*kanR) strain were transformed with plasmids expressing the indicated genes or with empty vector. Cells were grown to mid-logarithmic phase in minimal medium lacking uracil and harvested, and 10-fold serial dilutions were spotted on SC solid medium lacking uracil and incubated for 72 h at the indicated temperatures.

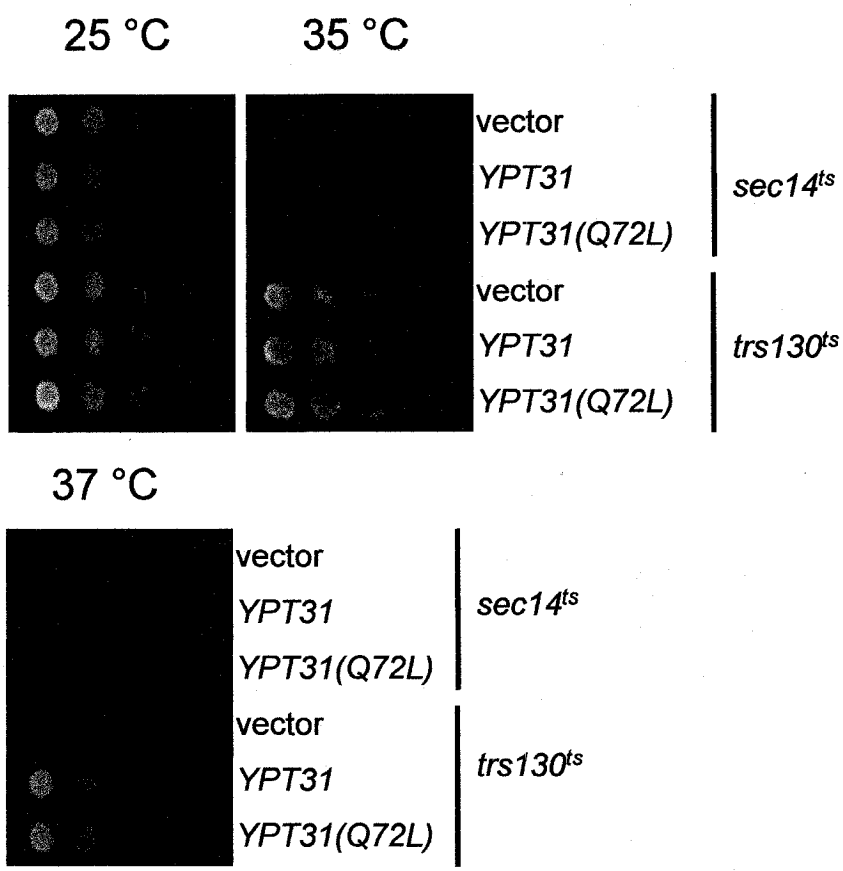


Figure 21. Increased dosage of *YPT31* alleviates defects associated with *trs130*^{ts} but not *sec14*^{ts}. Cells of the *sec14*^{ts} or *trs130*^{ts} strains indicated were transformed with the plasmids indicated. Cells were then grown to log-phase in minimal medium lacking uracil to ensure plasmid maintenance and harvested, and 10-fold serial dilutions were spotted on SC solid medium lacking uracil and incubated for 72 h at the indicated temperatures.

4.1.7. *sec14 kes1* CELLS DO NOT REQUIRE *YPT31* OR THE NON-ESSENTIAL TRAPP II COMPONENTS

From the SGA analysis, Ypt31 and the non-essential components of the TRAPP II complex were identified to be required for *ckil*-mediated Sec14 bypass, but not *kes1*-mediated bypass. To confirm the specificity of Ypt31 and TRAPPII in Sec14 bypass, I constructed each of the triple mutants carrying the *sec14*^{ts} allele. Figure 22 shows the isogenic *sec14*^{ts} *kes1 ypt31, ypt32, trs33, trs65,* and *trs85* triple-mutant cells grew normally at 37°C. This demonstrates that *kes1*-mediated bypass of Sec14 does not require Ypt31 or the non-essential TRAPP II components, consistent with the SGA analysis. Consistent with the cell growth results, *sec14*^{ts} *kes1 ypt31, ypt32, trs33, trs65,* and *trs85* triple-mutants also secreted invertase at rates comparable to that of wild-type cells (~85%) at the non-permissive temperature for the *sec14*^{ts} allele (Figure 23).

4.1.8. THE MODES OF SEC14-BYPASS – DISTINCT AND ADDITIVE

With the results in hand, my hypothesis was that Kes1 and the CDP-choline pathway negatively regulate secretion by different mechanisms. To further test this hypothesis I sought to determine if simultaneous inactivation of the CDP-choline pathway and *KES1* alters the growth of *sec14*^{ts} cells. The strain CMY512, *sec14-1*^{ts}*:NAT*^{R} *ckil::LEU2 kes1::HYG*^{R}, was therefore created to investigate properties of a "double Sec14 bypass". CMY512 cells, along with isogenic strains of interest, were grown to mid-logarithmic phase in YEPD at 25°C, harvested, serially diluted, spotted on YEPD solid medium and grown for 3 days at 25°C, 37°C, and 39°C. Figure 24 shows that the three bypass strains grew normally at all three temperatures.

4.1.9. INVERTASE SECRETION OF SEC14-BYPASS STRAINS

Previous reports demonstrated that the "Sec14 bypass" strains, while able to grow, displayed decreases in the secretion efficiency of invertase (Cleves et al., 1991). Invertase secretion was therefore measured for the isogenic *sec14*^{ts} strains of interest *sec14*^{ts}, the single-bypass strains *sec14*^{ts} *ckil* and *sec14*^{ts} *kes1* and the double-bypass strain *sec14*^{ts} *ckil kes1*. Under the permissive temperature for the *sec14*^{ts} allele, all strains

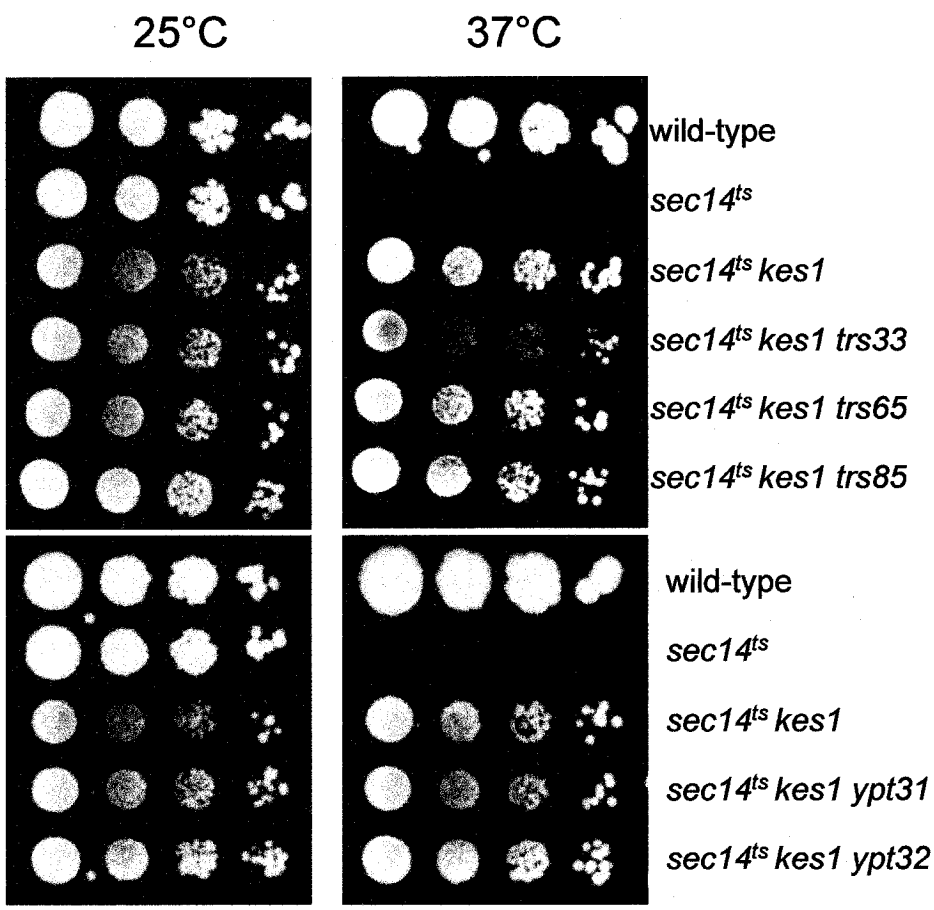


Figure 22. Nonessential TRAPP II components and Ypt31 are not required for *kes1*-mediated Sec14 bypass. The isogenic *sec14^{ts}* strains indicated were grown to mid-logarithmic phase and harvested and 10-fold serial dilutions were spotted on YEPD solid medium and incubated for 72 h at the indicated temperatures.

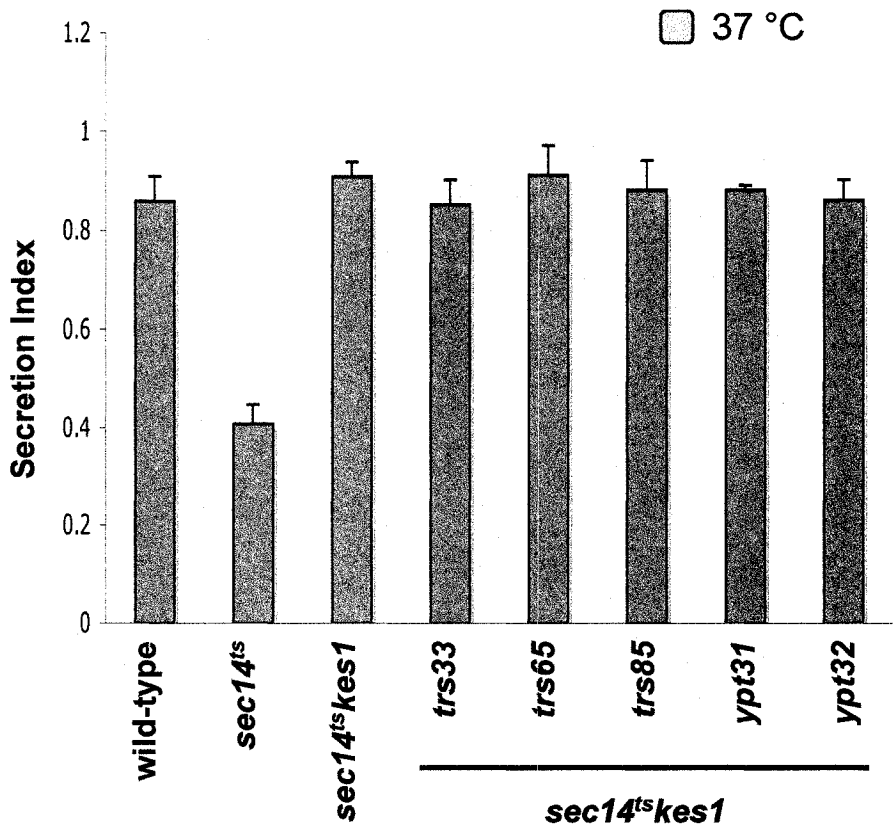


Figure 23. Loss of TRAPP II components or *YPT31* does not compromise secretion by *sec14^{ts} kes1* cells. Cells of the isogenic *sec14^{ts}* strains indicated were grown to mid-logarithmic phase at 25°C, harvested and washed in low glucose (0.1%) YEPD to induce expression of the *SUC2* gene, and grown for an additional 2 hours at the indicated temperature. The secretion index was determined by measuring the activity of the extracellular invertase compared to the total. Data are the average of three separate experiments ± S.E.M.

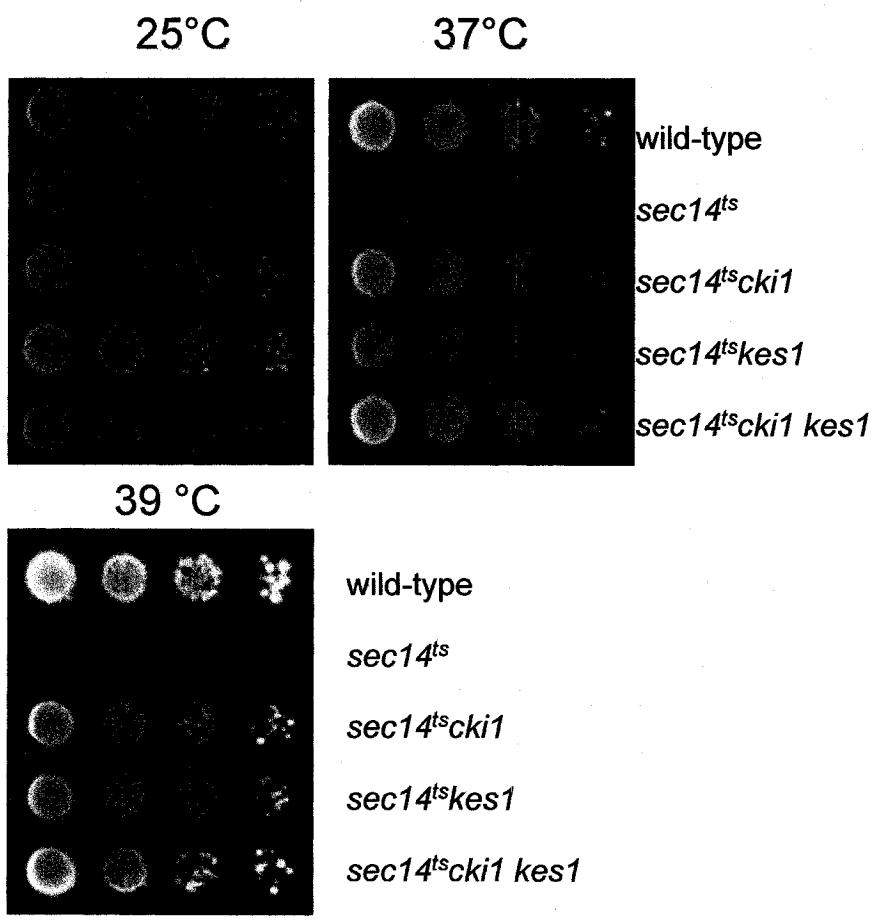


Figure 24. The "Sec14 double-bypass" strain is viable. Cells of the isogenic *sec14^{ts}* strains indicated were grown to mid-logarithmic phase and harvested, and 10-fold serial dilutions were spotted on YEPD solid medium and incubated for 72 h at the indicated temperatures.

displayed similar secretion efficiency. At the non-permissive temperatures for the *sec14^{ts}* allele, the bypass strains displayed different results. The invertase secretion index for the *sec14^{ts}* strain decreased to 40% and 22% at 37°C and 39°C, respectively (Figure 25). Secretion by the *sec14^{ts} ckil* strain decreased moderately from 70% to 55% at 37°C and 39°C, while the secretion indices for *sec14^{ts} kes1* and *sec14^{ts} ckil kes1* cells remained unchanged at around 90%. This observation would suggest that inactivation of *KES1* is a stronger bypasser of Sec14 function.

4.1.10 ELECTRON MICROSCOPY OF SEC14-BYPASS STRAINS

Previous results indicated that regardless of the presence of an inactivated *CKI1* gene, *sec14^{ts}* cells have severe defects in subcellular morphology (Chang et al., 2002). Subcellular morphology of the *sec14^{ts}* and *sec14^{ts} ckil* cells appeared to be normal when the cells were grown at the permissive temperature of 25°C; however, defective subcellular morphology became evident in *sec14^{ts}* and *sec14^{ts} ckil* cells within 1 hour following a shift to the *sec14^{ts}* restrictive temperature of 37°C. Vacuoles in the *sec14^{ts} ckil* cells were small and less well defined. Consistent with this observation, *sec14^{ts}* cells, regardless of mechanism of bypass, have multiple small vacuoles opposed to the normal 1-3 large vacuoles (Figure 26). While the modes of Sec14 bypass are additive, Sec14 may regulate an additional pathway that influences vacuolar fusion that is not subject to the regulation of Kes1 of the CDP-choline pathway.

4.1.11. FM4-64 STAINING OF VACUOLAR MEMBRANES IN SEC14-BYPASS STRAINS

Cells carrying the *sec14^{ts}* allele have a defect in Lucifer yellow uptake at non-permissive temperatures, but not in FM4-64 uptake (Vida and Emr, 1995). FM4-64 staining of vacuoles in the single- and double-bypass strains was less well defined, and there appeared to be a hazy cytosolic staining at the non-permissive temperature for the *sec14^{ts}* allele. Consistent with the electron micrographs, the simultaneous inactivation of *kes1* and *ckil* did not appear to restore vacuole structure to wild-type configuration as greater than 95% of cells contained small fragements vacuoles (Figure 27).

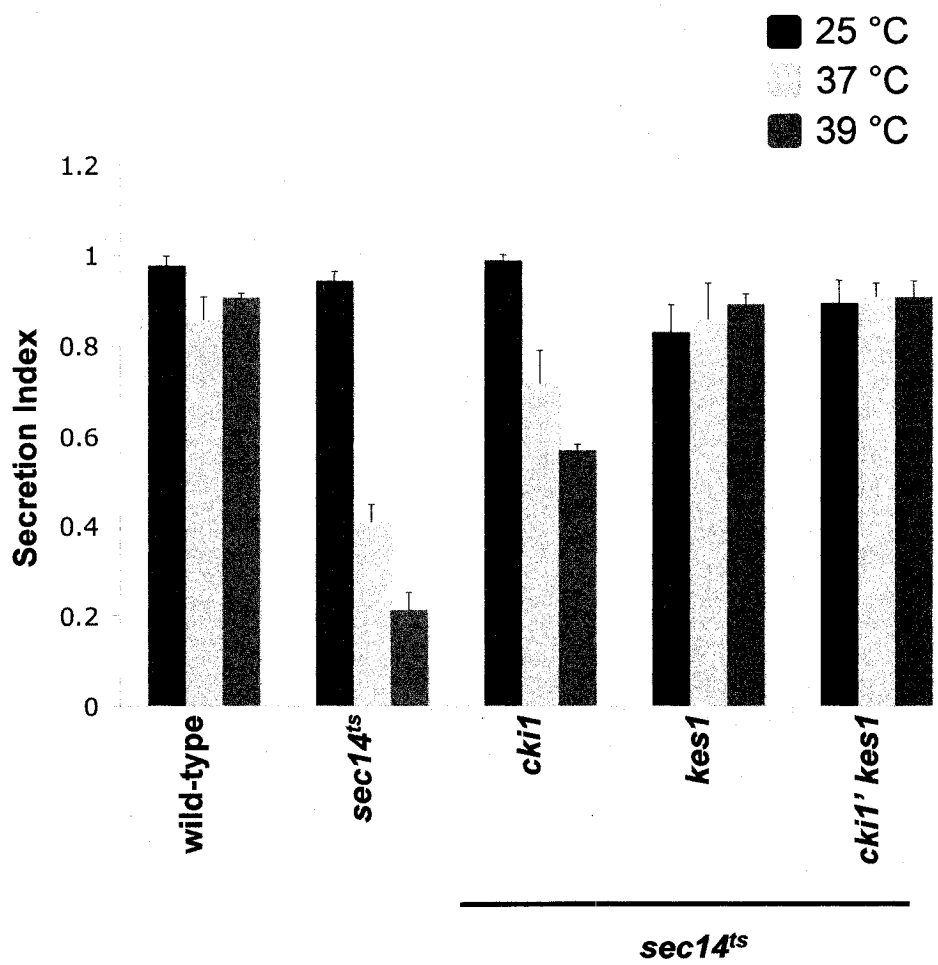
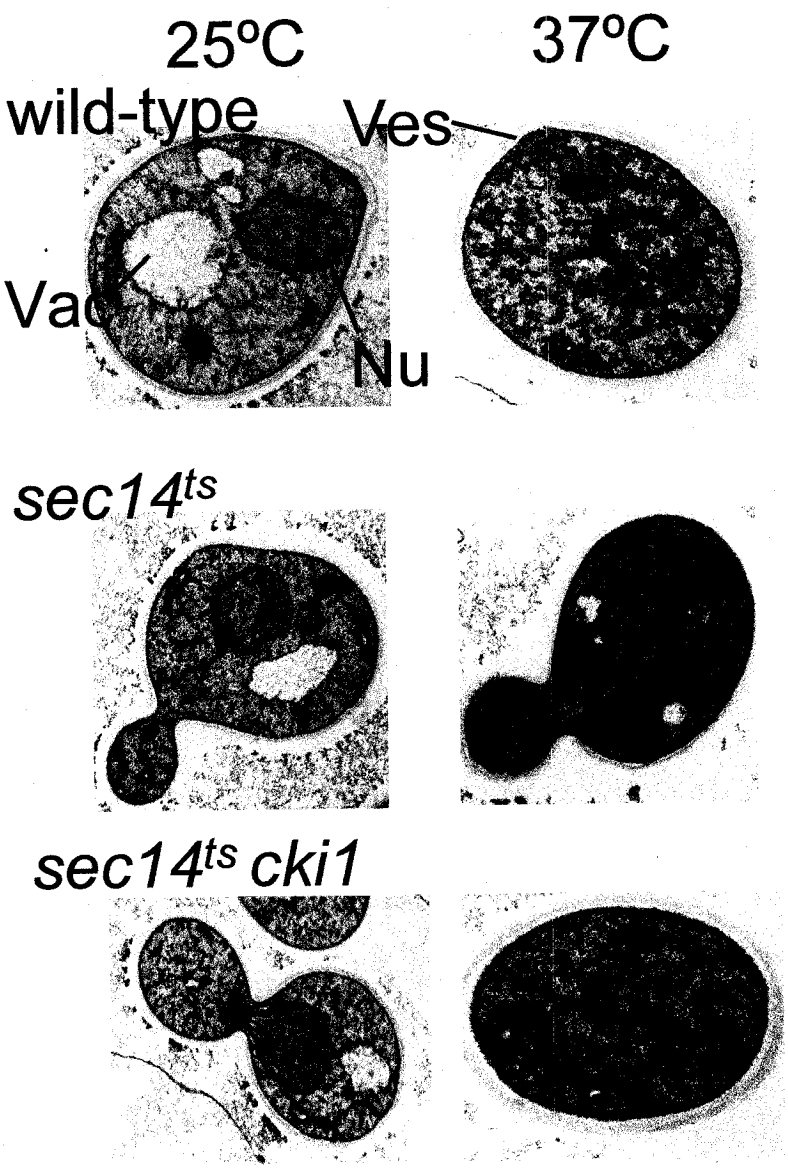
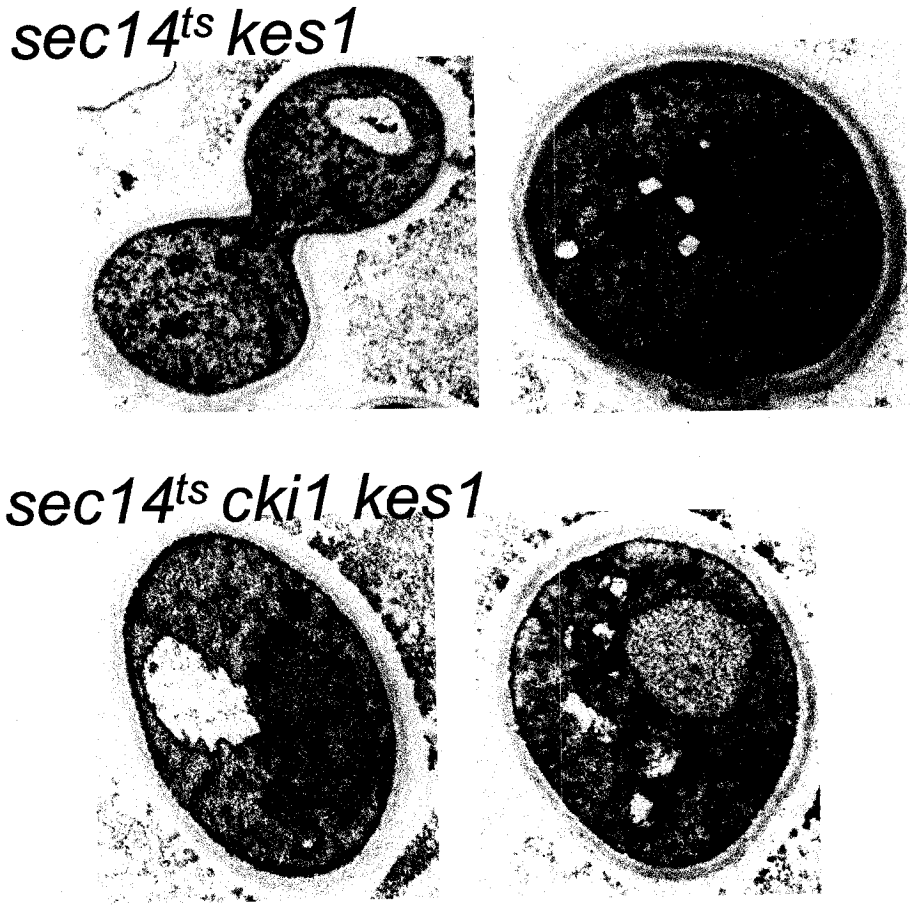


Figure 25. Kes1 is a stronger inhibitor of secretion than is the consumption of DAG. Cells of the isogenic *sec14^{ts}* strains indicated were grown to mid-logarithmic phase at 25°C, harvested and washed in low glucose (0.1%) YEPD to induce expression of the *SUC2* gene, and grown for an additional 2 hours at the indicated temperature. The secretion index was determined by measuring the activity of the extracellular invertase compared to the total. Data are the average of at least three independent experiments ± S.E.M.

Figure 26. Electron microscopy. Cells of a) wild-type b) *sec14*^{ts} c) *sec14*^{ts} *cki1* d) *sec14*^{ts} *kes1* and e) *sec14*^{ts} *cki1 kes1*. Cells were grown in YEPD medium to mid-logarithmic phase and processed for EM. At 25 °C there were no obvious differences between the wild-type and the isogenic *sec14*^{ts} cells. At 37 °C, wild-type cells were unchanged. *sec14*^{ts} cells accumulated vesicles and aberrant membrane structures, and the vacuole is rarely visible, consistent with previous findings. Sec14-bypass strains did not accumulate vesicles or aberrant membranes, but large vacuoles were rarely observed. Nucleus (Nu), Vacuole (Vac) exocytic vesicles (Ves).


25°C
37°C
wild-type
Ves
Vac
Nu
sec14ts
sec14ts cki1


sec14ts kes1
sec14ts cki1 kes1

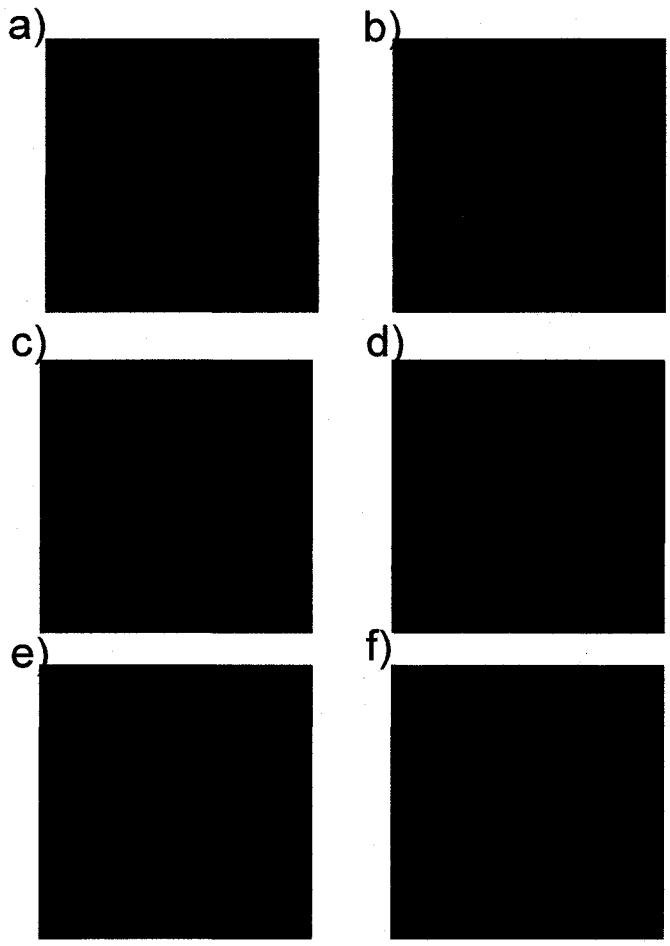


Figure 27. Sec14-bypass strains have unusual vacuoles. Cells of wild-type and isogenic *sec14^{ts}* strains indicated were grown in the presence of FM4-64 for 10 minutes followed by a 1-hour chase period. a) *sec14^{ts}* at 25°C, b) *sec14^{ts}* c) wild-type *d) sec14^{t} ckil e) sec14^{t} kesl f) sec14^{t} ckil kesl*. Images b-f are of cells grown at 37 °C

4.1.12. GFP-Snc1 RECYCLING

Snc1 is a yeast v-SNARE that participates in the fusion of exocytic vesicles with the plasma membrane (Couve and Gerst, 1994; Couve et al., 1995; Protopopov et al., 1993). Subsequent to plasma-membrane fusion, Snc1 is endocytosed into endosomes and recycled to the Golgi to be used in additional rounds of secretion. At steady state, Snc1 is found primarily at the plasma membrane of growing buds, and at the mother-bud neck in cells undergoing division. Due to the this dynamic trafficking, Snc1 localization has been used to examine several types of vesicular transport. Wild-type and isogenic *sec14*^{ts} cells were transformed with pMJL1 (expressing GFP-*SNC1*, from Hugh Pelham) and grown to early-logarithmic phase in SC-uracil medium at 25°C. At 25°C GFP-Snc1 was primarily localized to the plasma membrane in wild-type, *sec14*^{ts} and *sec14*^{ts}-bypass cells. Similar to previous results, in *sec14*^{ts} cells a shift to the non-permissive temperature of 37°C resulted in Golgi-localized GFP-Snc1 (Lewis et al., 2000), consistent with an inability to transport vesicles from the Golgi (Figure 28). Consistent with the decrease in invertase-secretion efficiency, GFP-Snc1 localized to both the plasma membrane as well as internal structures in *sec14*^{ts} *ckil* cells, indicating a delay in GFP-Snc1 transport within these cells. However, *sec14*^{ts} *kes1* and *sec14*^{ts} *ckil kes1* cells displayed plasma-membrane localization of Snc1 the same to what is seen in wild-type cells (Figure 28). Thus, it appears that the presence or absence of the CDP-choline pathway has no effect on the *kes1*-mediated bypass of Sec14 function. The observations suggest that *KES1* is a more prominent regulator of secretion.

4.1.13. VIABILITY OF QUADRUPLE MUTANTS

SGA analysis identified 37 genes which, when inactivated, resulted in a loss of viability by one or both of the Sec14-bypass strains. Because inactivating the CDP-choline pathway or Kes1 can circumvent the essential nature of Sec14, Sec14 can be viewed as acting in opposition to the negative regulation of secretion exerted by the CDP-choline pathway and Kes1. In this sense, lethality associated with the loss of *SEC14* activity is because of the unchecked opposition from CDP-choline pathway and Kes1. The *sec14 CKI1 KES1* cells are not viable, whereas *sec14 ckil KES1 or sec14 CKI1 kes1* are viable. The *sec14 ckil KES1* strain requires 33 genes for viability and *sec14 CKI1*

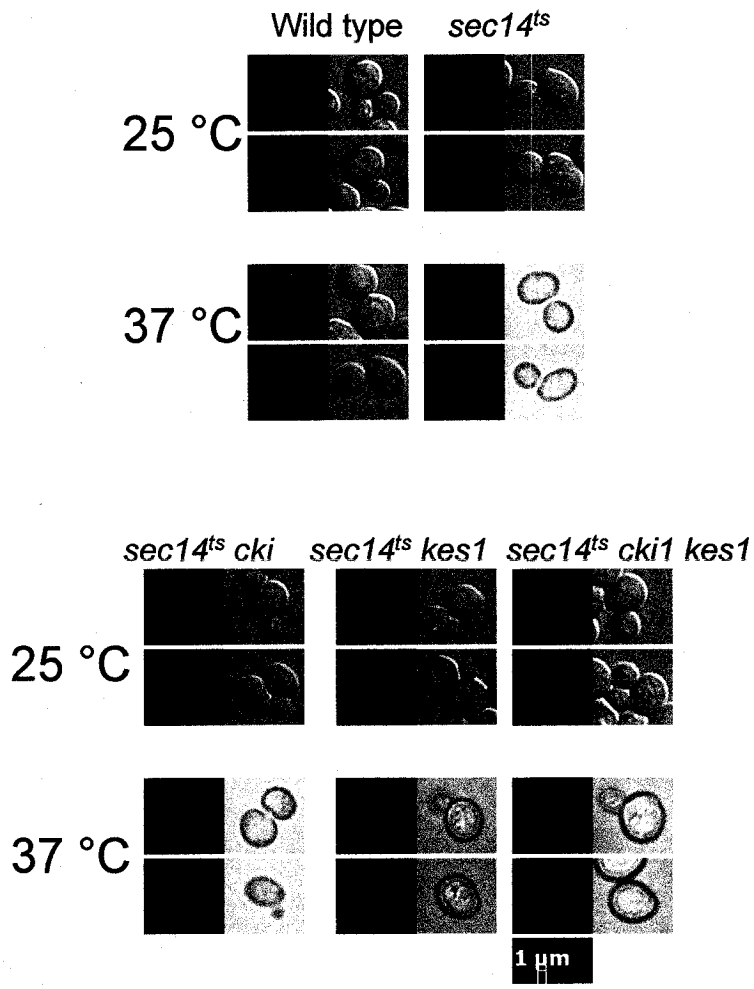


Figure 28. Ablation of *KES1* restores trafficking of Snc1-GFP. Cells of wild-type and isogenic *sec14*^{ts} strains were transformed with pSnc1-GFP and grown to early-log phase in minimal medium lacking uracil to maintain plasmid maintenance. Images were taken of live cells growing at 25°C or after incubation at 37°C for 30 min.

kes1 requires 16, but does a *sec14 ckil kes1* strain require any of these? To find out, the CMY512 strain (*sec14^{ts} ckil kes1*) was mated with the 37 deletion-collection mutants, the diploids were sporulated and random-spore analysis was performed. Only 20% of the theoretical number of spores was recovered for the *sec14^{ts} ckil kes1 vps4* quadruple mutant, suggesting a synthetic sickness/lethal interaction. The other 36 quadruple mutants examined in this way produced the appropriate number of viable spores, indicative of no synthetic lethal interaction. This suggests that the double bypass alleviates the heightened genetic requirement for these 36 normally non-essential genes. The EM and FM4-64 staining presented above suggests that Sec14 function alters vacuolar structure/dynamics and that this function is not subject to regulation by Kes1 or the CDP-choline pathway. As the *sec14^{ts} vps4* interaction is the only one not bypassed by the inactivation of *KES1* and *CKI1,* this suggests that Sec14 may have a role in endosomal sorting or transport to the vacuole, possibly upstream or downstream of Vps4.

4.1.14. INVERTASE SECRETION BY *sec14* QUADRUPLE MUTANTS

The observation that "Sec14 double bypass" alleviates nearly all of the additional genetic requirements was investigated further using the TRAPP II mutants and *YPT31*. In the triple mutants used previously, *sec14^{ts} ckil* along with *trs33, 65, 85* or *ypt31*, the *KES1* gene was targeted for replacement. The resulting cells were grown at 25°C, harvested, serially diluted, spotted on YEPD solid medium and incubated at 25°C or 37°C (Figure 29). While the *sec14^{ts}* cells died at the non-permissive temperature the other strains were viable. Consistent with this observation, *sec14^{ts} ckil kes1 trapp II* and *ypt31* cells secreted invertase at normal rates (Figure 30).

4.1.15. GLYCOSYLATION STATUS OF INVERTASE

Invertase is a 60-kDa protein that becomes heavily glycosylated as it transits from through the ER to the Golgi and is secreted. Mutations that block ER-to-Golgi transport, like inactivating mutations in *SEC18*, lead to the accumulation of an ER core-glycosylated protein. Mutations acting at the *trans*-Golgi lead to the accumulation of a high-molecular-weight fully glycosylated invertase (Stevens et al., 1982). To verify that the block in secretion occurs at the *trans*-Golgi in *sec14^{ts}* mutants the glycosylation status

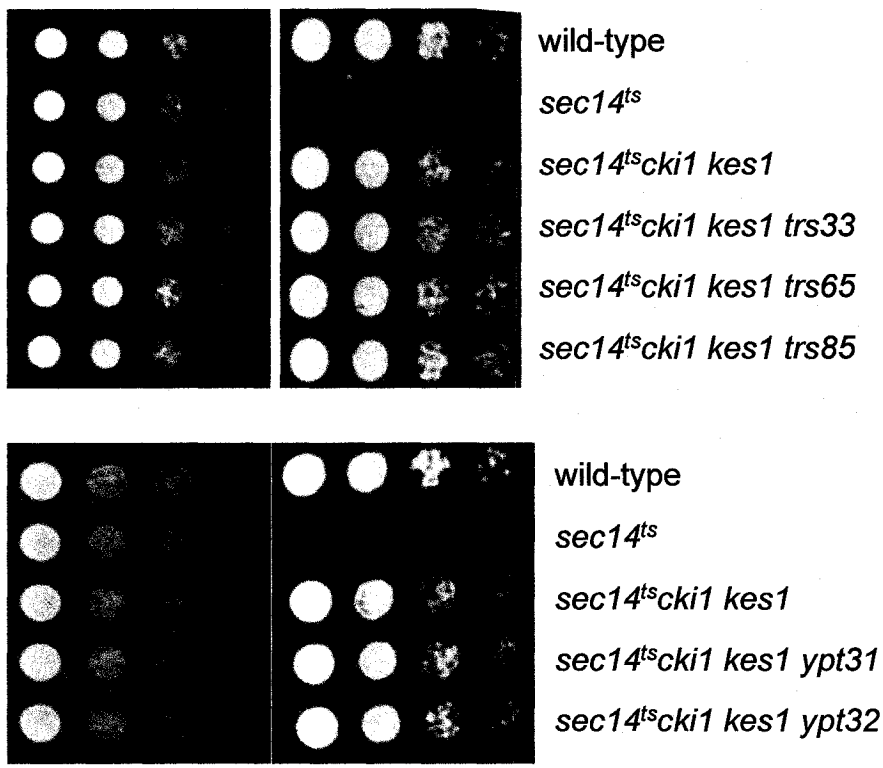


Figure 29. The nonessential TRAPP II components and Ypt31 are not required for the viability of the "Sec14p double bypass" strain. Cells of the isogenic *sec14^{ts}* strains indicated were grown to mid-logarithmic phase and harvested, and 10-fold serial dilutions were spotted on YEPD solid medium and incubated for 72 h at the indicated temperatures.

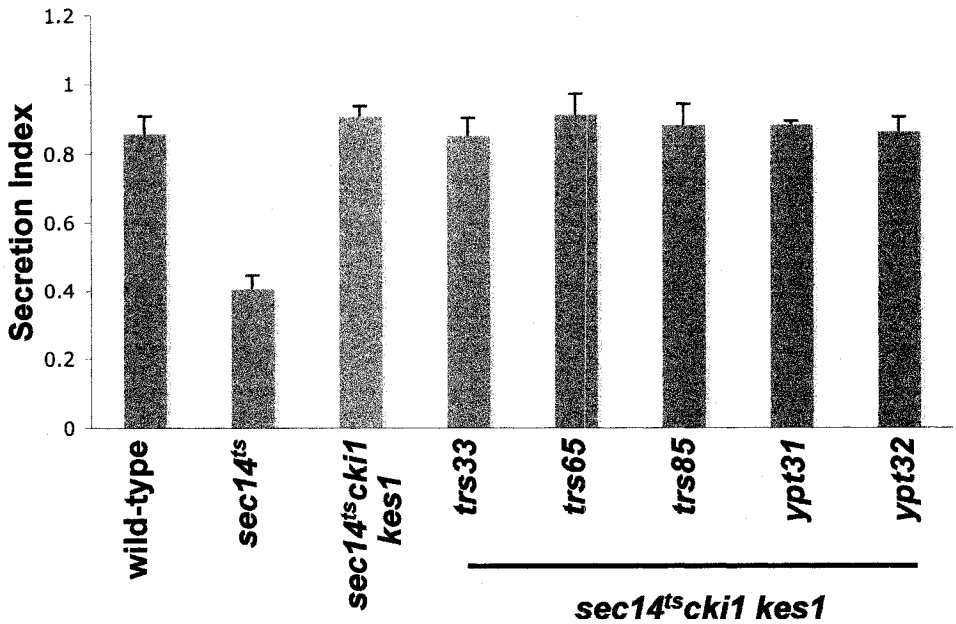


Figure 30. Ablation of *KES1* restores secretion of invertase in situations in which *cki1*-mediated "bypass of Sec14" is insufficient. Cells of the isogenic *sec14^{ts}* strains indicated were grown to mid-logarithmic phase at 25°C, harvested and washed in low-glucose (0.1%) YEPD to induce expression of the *SUC2* gene, and grown for an additional 2 hours at the indicated temperature. The secretion index was determined by measuring the activity of the extracellular invertase compared to the total. Data are the average of at least three independent experiments ± S.E.M.

of invertase was examined. Cells of wild-type and isogenic *sec14*^{ts} strains were grown to mid-logarithmic phase in YEPD, harvested, washed and transferred to 0.1% glucose YEPD. Invertase was then isolated, resolved and detected as described above. In these *sec14*^{ts} cells, all the invertase was fully glycosylated, even though in the *sec14*^{ts} and *sec14*^{ts} *ckil ypt31* cells the majority of the invertase was intracellular (I) at the non-permissive temperature (Figure 31). As a control, cells carrying a *sec18*^{ts} allele were also processed, with the majority of the invertase found in the intracellular fraction and in an underglycosylated form, reflected in its faster migration. This result demonstrates that invertase accumulates in its fully glycosylated form, consistent with the block in secretion at the *trans*-Golgi or in post-Golgi vesicles..

4.1.16. CPY SECRETION

Cells with aberrant Sec14 function have a mild defect in CPY maturation (Bankaitis et al., 1989). CPY undergoes glycosylation and proteolytic processing on its way from the ER to the Golgi and finally the vacuole (Stevens et al., 1982). In addition, cells defective for retrieval of Vps10 (a transmembrane protein that serves as a cargo receptor for transport to the vacuole) from the endosomes secrete the Golgi-modified form of CPY. To investigate CPY trafficking, cells of wild-type and *sec14*^{ts} isogenic strains were grown at the non-permissive temperature, and the medium was probed for the presence of CPY. Wild type and isogenic *sec14*^{ts} cells did not secrete CPY to any large degree (Figure 32a); for comparison, two mutants, *vps4* and *vps1,* are included (Babst et al., 1997; Vater et al., 1992). The little CPY secreted was in the P2 form, which corresponds to the Golgi-modified form. Consistent with this observation, Vps10 half-life is the same in *sec14*^{ts} cells at the non-permissive temperature as it is in wild-type cells. As a control, a strain lacking Vps5, a component of the retromer complex required for transport from endosomes to Golgi transport, is included (Horazdovsky et al., 1997) (Figure 32b). The results demonstrate that CPY is sorted properly at the *trans*-Golgi in *sec14* mutants and that the CPY receptor is properly recycled from the endosomes. The slight delay in CPY maturation described elsewhere for *sec14* mutants might be a defect in endosomes to vacuole trafficking.

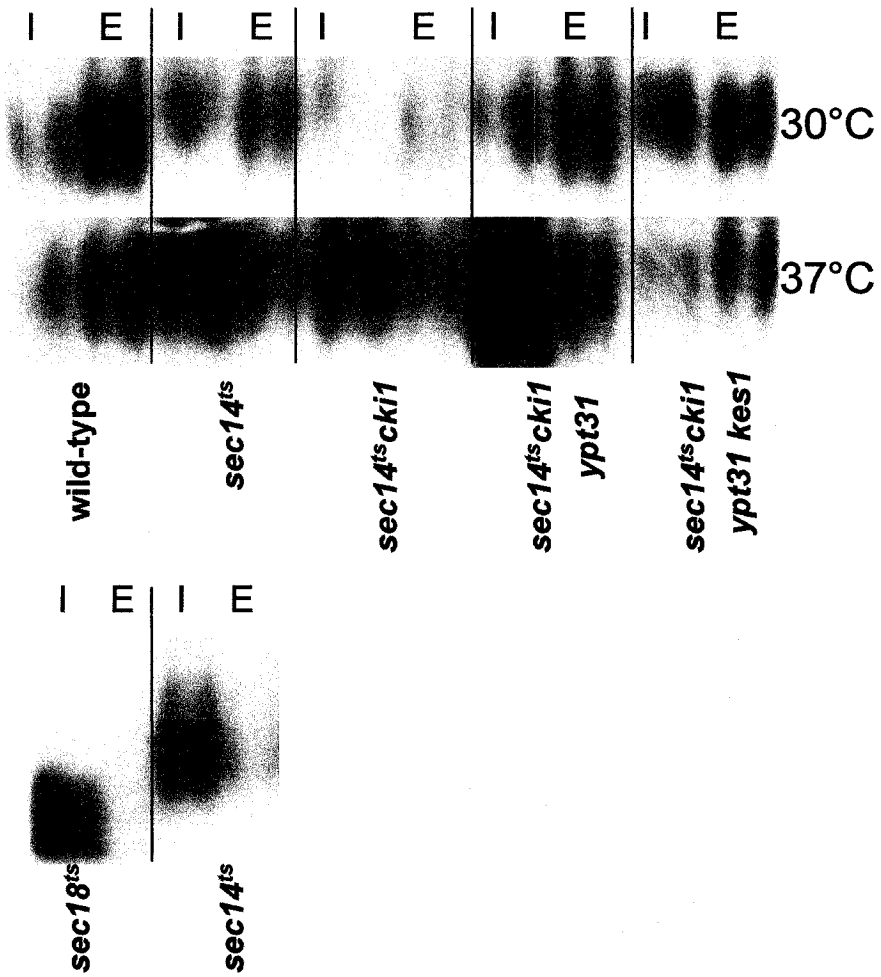


Figure 31. Analysis of Invertase Glycosylation in *sec14^{ts}* strains. Cells of wild-type or indicated mutants were grown to log phase at 30°C, washed in low-glucose YEPD and partitioned into two samples, and which were incubated at either 30°C or 37°C for two hours. Cells were then harvested and spheroplasts were generated by incubating cells with zymolyase for 45 min at 30°C or 37°C. Spheroplasts were separated from supernatant by centrifugation and resuspended in a volume equal to that of the supernatant. Extracellular (E) and intracellular (I) invertase were resolved by SDS-PAGE without heating the samples, and detected by an in-gel assay. For comparison *sec18^{ts}* cells were also included.

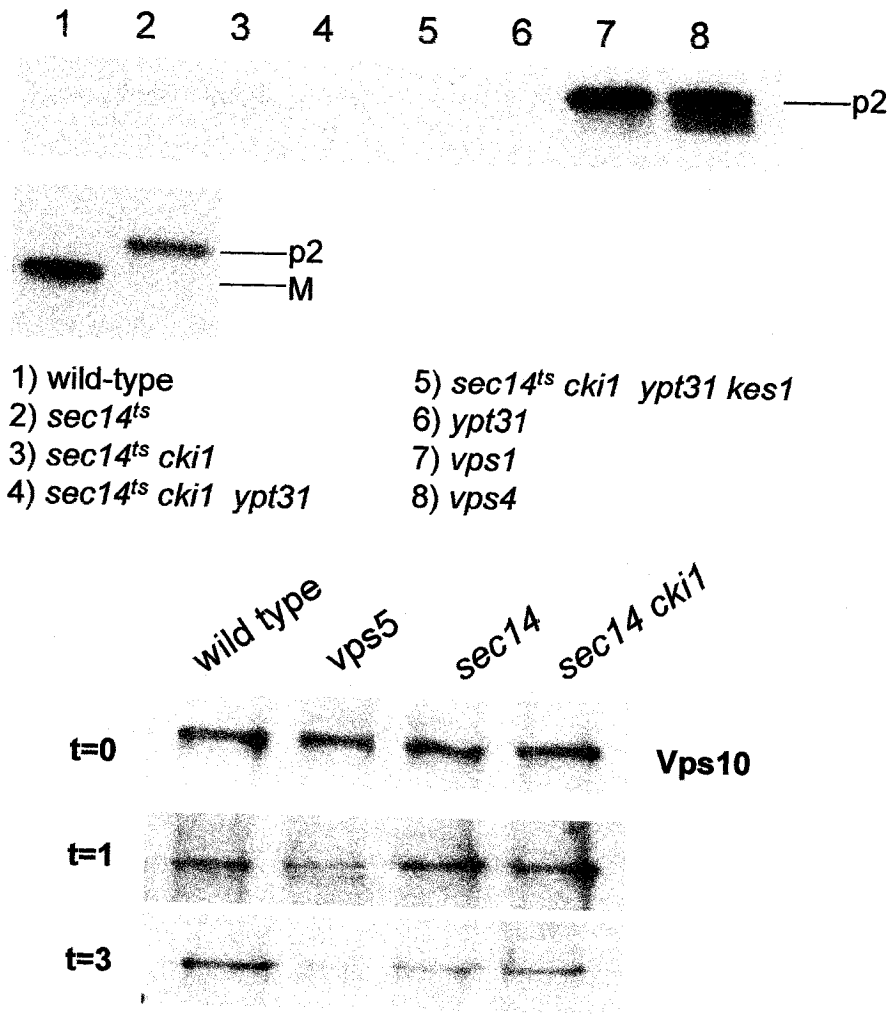


Figure 32. Analysis of CPY trafficking in *sec14^{ts}* cells. Mutants and "Sec14 bypass" strains do not secrete CPY. Cells were pelleted by centrifugation and proteins contained in the supernatant were precipitated by adding trichloroacetic acid, collected by centrifugation, washed with 100% acetone, dried, and resuspended in SDS/PAGE sample buffer. Samples were resolved by SDS/PAGE and processed for CPY immunoblots. Mutants *vps1* and *vps4* were included for comparison. b) Cells were grown to mid-log phase and treated with cycloheximde. At 0, 1 and 3 hours thereafter, cells were harvested, processed and resolved by SDS-PAGE and processed for Vps10 immunoblots.

4.1.17. SPO14 IS NOT ESSENTIAL FOR SEC14-INDEPENDENT LIFE

Previously it was reported that *SPO14* was required for Sec14 bypass, an especially attractive finding because Spo14 consumes PC and produces PA that can be dephosphorylated to produce DAG (Xie et al., 1998). While *SPO14* was identified by SGA analysis, the interaction was classified as growth inhibition rather than lethality. Figure 33 demonstrates that *SPO14* is not required for Sec14-independent growth, as the *sec14*^{ts} *ckil kesl* double-bypass strain does not require it for viability, and confirming the results from the random-spore analysis of the quadruple mutants. Furthermore, the same figure a shows that cells of the *sec14*^{ts} *kesl spol4* strain were viable and able to grow at the non-permissive temperature of 37°C. This raises the question of how important *SPO14* activity is for Sec14 bypass in genetic backgrounds other than that of the original Bankaitis strain (a meiotic segregant of SF292-A and YP54).

4.1.18. PLD2 ACTIVITY

Examination of the Sec14 synthetic lethal/sick interaction network reveals specific genetic interactions for *sec14, spo14,* and *swfl* (Figure 34). Swf1 has been recently characterized as being responsible for the palmitolylation of SNARE proteins, and cells containing a genomic deletion of *SWF1* have decreased steady-state levels of SNARE proteins (Valdez-Taubas and Pelham, 2005). Recent studies with yeast demonstrate that the phosphatidic acid produced by Spo14, is important for SNARE-mediated vesicle fusion (Nakanishi et al., 2006). Not surprisingly, *spo14* and *swfl* constitute a synthetic lethal pair (Tong et al., 2004), likely due to a decrease in vesicle-fusion rates. Cells lacking *sec14* also have a heightened requirement for Spo14 activity, but Spo14 is not the only PLD activity in yeast. Yeast cell extracts also contain a Ca^{++}-dependant, PE/PS PLD activity (Waksman et al., 1997). This activity is increased in Sec14-bypass strains (Tang et al., 2002). In Figure 12 and highlighted in Figure 34a, the only interactions common to *sec14* and *swfl* are with *spo14* and *ydl133w*. This raises the possibility that *YDL133w* encodes a PE/PS PLD. To investigate this possibility extracts were produced from wild-type and *ydl133w::*KAN^R cells and assayed for PE PLD activity. Figure 34b shows the relative positions of NDB-PE and NDB-PA, the substrate

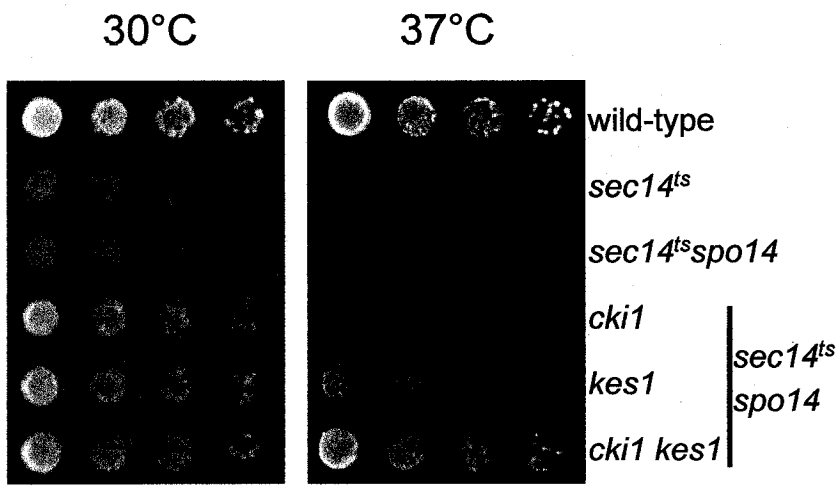


Figure 33. Loss of *SPO14* impairs *ckl1*-mediated "Sec14 bypass" Cells of the isogenic *sec14*^{ts} strains indicated were grown to mid-logarithmic phase and harvested, and 10-fold serial dilutions were spotted on YEPD solid medium and incubated for 72 h at the indicated temperatures.

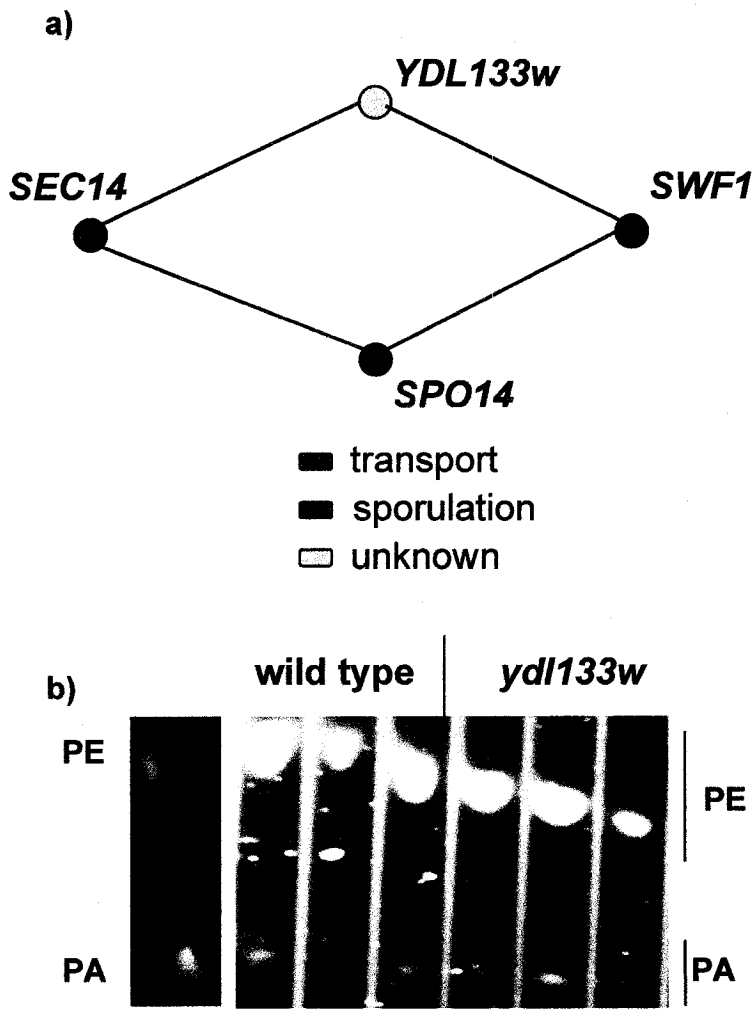


Figure 34. The *spo14* and *ydl133w* gene deletions form the same synthetic lethal interactions, but *YDL133w* does not encode the yeast PE-PLD activity. a) Synthetic lethal interactions for *spo14* and *ydl133w* are shown. b) Whole cell extracts from wild-type or *ydl133w* cells were assayed for PE-PLD activity using NDB-PE as a substrate. Included are NDB-PE and NDB-PA standards for comparison.

and product of this reaction. Compared to wild type extracts, *ydl133w* extracts contain the same relative PE PLD activity, suggesting Ydl133w is not the yeast PE PLD enzyme.

4.1.19. *SEC14, PIK1* AND *FRQ1* CO-ORDINATE PI-4P SIGNALING AT THE GOLGI

Previous reports demonstrate that upon a shift to the non-permissive temperature *sec14*^{ts} cells have a 50% decrease in cellular levels of PI-4P while PI-3P levels remain unchanged (Hama et al., 1999). Consistent with this observation, during the course of this work it was reported that *sec14 cki1* cells show a 60% reduction in PI-4P and PI-4,5P (Routt et al., 2005). However, in that report PI-3P suffered a 50% reduction as well. Perhaps this difference in PI-3P levels is a result of (i) acute versus chronic loss of Sec14, (ii) differences in genetic background, or (iii) differences in phosphoinositide production at 30°C versus 37°C. In any event, this raises the possibility that gene deletions found to be synthetically lethal with *sec14 cki1* could actually have this effect be due to a decrease in the levels of one or more of the phosphoinositides. For example, decreased PI-3P levels could be the underlying cause of the aberrant vacuoles in *sec14*^{ts} cells and also the reason for the synthetic lethal interaction between *sec14* and *vps4*. Recently, SGA analysis has been performed using partial loss-of-function alleles of *PIK1, STT4 & MSS4* (Audhya et al., 2004; Sciorra et al., 2005; Tabuchi et al., 2006). Several of the synthetic interacting partners for *sec14* were identified in those three screens. *STT4* shares two genes in common, *SPO14* and *GAS1; MSS4* shares one, *ROM*; *PIK1* shares at least seven, *YPT31, TRS33, TRS65, GCS1, VPS51, VPS4,* and *ARL1*. While *sec14 cki1* strain has pleiothropic alterations in phosphoinositide metabolism, the genetic interactions suggest that levels of PI-4P generated by Pik1 appear to be the most compromised.

4.1.20. CO-EXPRESSION OF *PIK1* AND *FRQ1* IN *sec14*^{ts}

Pik1 localizes to the Golgi via by the small calcium-binding protein Frq1 (Strahl et al., 2005). The *FRQ1* gene is essential, but its essential nature can be bypassed by increased expression of *PIK1,* suggesting Pik1 has other intrinsic cues for targeting to the Golgi (Hendricks et al., 1999). Previously it was reported that increased expression of *PIK1* could partially suppress defects associated with *sec14*^{ts} and allow growth at a semi-

permissive temperature of 33°C (Hama et al., 1999). With the observation that Pik1 functions in concert with another protein, Frq1, the effects of simultaneous over-expression of *PIK1* and *FRQ1* were examined in *sec14*^{ts} cells. Cells were transformed with YEp352 and YEp351 empty vectors or *PIK1* and/or *FRQ1* multicopy plasmids or a plasmid containing *SEC14,* then grown to mid-logarithmic phase, harvested, serially diluted and spotted on SC-ura-leu solid medium for 3 days of growth at 30°C, 35°C or 37°C. As shown in Figure 35, only restoration of *SEC14* function restored growth at all temperatures. However, simultaneous over-expression of *PIK1* and *FRQ1* supported robust growth at 35°C, whereas over-expression of either on its own did not. This result suggests that elevated production of PI-4P at the Golgi can suppress *sec14*^{ts} defects at 35°C. Upon shifting *sec14*^{ts} to a non-permissive temperature, the viability of the cells quickly decreased. However, as shown in Figure 36, *sec14*^{ts} cells co-over-expressing *PIK1* and *FRQ1* remained viable after 3 days at 37°C, whereas vector controls or cells with increased expression of *PIK1* or *FRQ1* did not.

In describing my SGA analysis, it was noted that certain interactions (e.g. *GCS1* and *ARF1*) were only deleterious when cells were grown at elevated temperatures. This suggests that under this stressful condition (high temperature) those additional genetic effectors of Sec14 were required. This raises the question of whether the inability of increased Pik1/Frq1 activity to rescue Sec14-deficient cells is a consequence of the elevated temperatures used for the *sec14*^{ts} allele. To address this, a *SEC14/sec14::*KAN^{R} diploid cells were transformed with *PIK1, FRQ1,* both or empty vectors, sporulated and haploid spores isolated at 25°C. This procedure produced viable Kan-resistant haploids (*sec14*::KAN^{R}), suggesting that at 25°C the simultaneous increase in Pik1 and Frq1 levels can bypass the essential requirement of Sec14. However, to investigate this observation more closely tetrad analysis must be employed.

Although available *in vitro* data are limited, it would appear that Sec14 either directly (acting as a co-enzyme) or indirectly (by influencing PI distribution) increases the production of phosphoinositides. (Cockcroft, 1997; Cunningham et al., 1996; Jones et al., 1998). The primary defect in *sec14* cells appears to be a block in secretion from the Golgi. Pik1 and Frq1 are two essential proteins that localize to the Golgi. Pik1 catalyzes the conversion of PI to PI-4P, and Frq1 both activates Pik1 activity *in vitro* and aids in

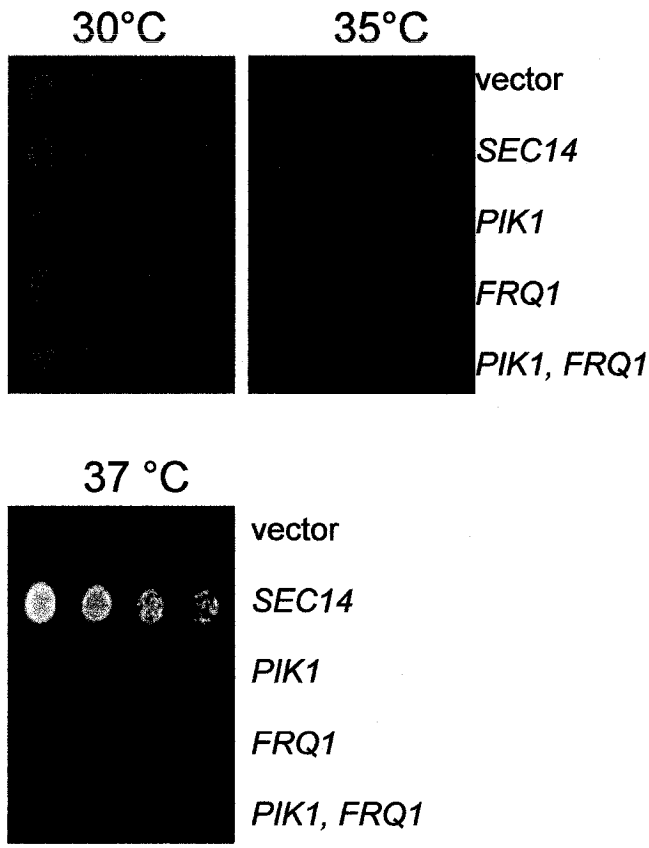


Figure 35. Increased expression of *PIK1* and *FRQ1* can partially suppress defects associated with *sec14^{ts}* cells. Cells of the CMY503 (*sec14^{ts}*:natR) strain were transformed with plasmids encoding *PIK1* or *FRQ1,* or with empty vectors as indicated. Cells were then grown to mid-logarithmic phase in minimal medium lacking leucine and uracil and harvested, and 10-fold serial dilutions were spotted on minimal medium solid lacking uracil and leucine and incubated for 72 h at the indicated temperatures.

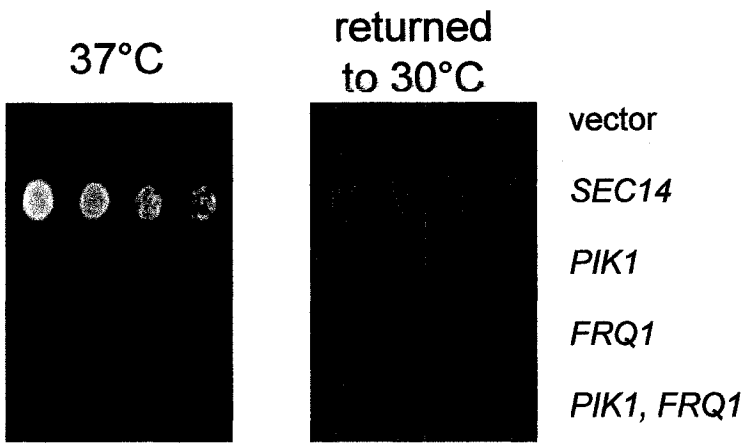


Figure 36. Increased expression of *PIK1* and *FRQ1* prevents the death of *sec14*^{ts} cells. Cells of the CMY503 (*sec14*^{ts}:natR) strain were transformed with plasmids encoding *PIK1 or FRQ1* or with empty vectors as indicated. Cells were then grown to mid-logarithmic phase in minimal medium lacking leucine and uracil and harvested, and 10-fold serial dilutions were spotted on minimal solid medium lacking uracil and leucine and incubated for 72 h at 37°C. Plates were then place at 30°C and incubated for another 48 h.

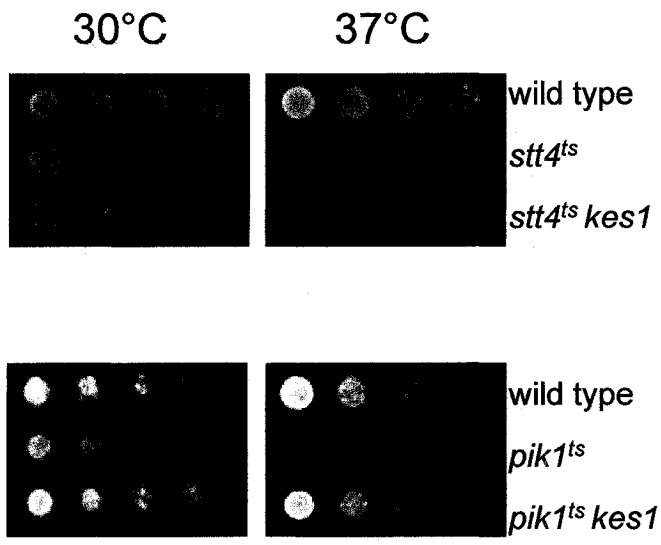


Figure 37. Genomic replacement of *KES1* ameliorates the defects in *pik1-83*^{ts} but not *stt4-4*^{ts} mutants. Cells of strain AAY104 (*pik1-83*), GFY201 (*pik1-83 kes1::*kanR), AAY102 (*stt4-4*) and GFY207 (*stt4-4 kes1::*kanR) were grown to mid-logarithmic phase in YEPD medium and harvested, and 10-fold serial dilutions were spotted on YEPD solid medium and incubated for 72 h at the indicated temperatures.

targeting Pik1 to the Golgi *in vivo*. Cells lacking *PIK1* or *FRQ1* also have defects in secretion and accumulate the same aberrant structures, Berkeley bodies, that *sec14* cells accumulate (Audhya et al., 2000; Hendricks et al., 1999; Strahl et al., 2005; Walch-Solimena and Novick, 1999). Recent crystallographic data suggest that the TRAPP II component Bet3 has a positively charged flat face which is crucial for its binding to the Golgi (Kim et al., 2005). This positively charged surface would be suitable for binding to the negatively charged PI-4P produced by Pik1 in collaboration with Sec14 and Frq1. The mechanism of assembly for the ten-component containing TRAPPII complex is unknown, but likely relies on the proper lipid environment of the *trans*-Golgi. In any event, increasing evidence suggests that the complex can acts as an exchange factor for Ypt31 and Ypt32. Cells lacking *YPT31/32* have defects in secretion from the Golgi and accumulate Berkeley bodies, as do cells lacking any of the other essential components described here. It appears that *SEC14, FRQ1, PIK1, TRAPPII and YPT31/32* all function in the regulation of vesicular transport from the *trans*-Golgi network. The mounting genetic data suggest that they all function in the PI-4P-dependent secretion pathway at the Golgi.

4.2. *KES1* ATTENUATES PI-4P SIGNALING AT THE GOLGI

Evidence was shown above demonstrating that the modes of Sec14 bypass are indeed additive. The triple-mutant *sec14^{ts} ckil ypt31* cells do not grow or secrete at the non-permissive temperature of 37°C, while genomic replacement of *KES1* rescues these cells and restores secretion. Due to the fact that *sec14 ckil* demonstrate a reduction in Golgi PI-4P, this suggests that Kes1 attenuates some portion of the PI-4P signaling cascade localized to the Golgi. To investigate this further a series of mutants was created to try to pinpoint the action of Kes1.

4.2.1. *KES1* IS A NEGATIVE REGULATOR OF *PIK1*

The *S. cerevisiae* genome encodes three PI-4-kinases, two essential ones Pik1 and Stt4, and the non-essential Lsb6 (Audhya et al., 2000; Han et al., 2002). Previously published results suggest that inactivation of *KES1* in cells carrying the temperature-sensitive *pik1-101* allele allowed an approximate 10-fold increase in growth at the non-

permissive temperature of 35°C (Li et al., 2002). To investigate the genetic interaction between *PIK1* and *KES1* further, *KES1* was inactivated in cells carrying the temperature-sensitive *pik1-83* allele. While *pik1-83* cells die at the non-permissive temperature of 32°C, when *KES1* was inactivated in these cells they continued to grow up to a temperature of 39°C (Figure 37). Kes1 does not appear to be a promiscuous regulator of PI-4P signaling, as deletion of *KES1* in cells carrying the temperature-sensitive *stt4-4* allele had no effects on its growth rate. This suggests Kes1 is a regulator of Golgi Pik1/PI-4P.

4.2.2. AVAILABILITY OF GOLGI PI-4P

Pik1 synthesizes the Golgi PI-4P pool (Audhya et al., 2000; Roy and Levine, 2004). During the course of my study, it was reported that strains with decreased Pik1 activity also require Ypt31 and members of the TRAPP II complex for viability (Sciorra et al., 2005). Kes1 can bind PI-4P (and PI-4,5P_2) and this ability to bind PI-4P was required for the translocation of Kes1 to the Golgi (Li et al., 2002). Combined with the results from the study by Sciorra et al, I predicted that Kes1 and Pik1 influence the same regulatory process with respect to vesicular transport from the *trans*-Golgi. As Kes1 acts as a negative regulator of Pik1 Golgi- derived vesicular transport, I postulated that the PI-4P-binding ability of Kes1 could sequester Golgi PI-4P, resulting in titration of the effective concentration of available Golgi PI-4P. To directly test this prediction, GFY201 (*pik1-83^{ts} kes1*), AAY104 (*pik1-83^{ts}*) or wild-type cells were transformed with a GFP-tagged PH domain from Osh2 (pTL511 plasmid from, Tim Levine). Recent characterization of GFP-PH^{Osh2} by the Levine lab demonstrated that this fusion protein was a useful probe for PI-4P (Roy and Levine, 2004). In cells with an enfeebled Stt4, the GFP-PH^{Osh2} fusion protein localized to internal punctate structures consistent with the Golgi. In cells with an enfeebled Pik1 activity, the GFP-PH^{Osh2} fusion protein was detected only at the plasma membrane. As seen in Figure 38, in *pik1-83^{ts}* cells even at the permissive temperature of 25°C, there was a reduction in punctate/Golgi staining and this staining was completely abolished after a 15-minute incubation at to the non-permissive temperature. However, in *pik1-83^{ts} kes1* cells at both 25°C and 37°C the GFP-PH^{Osh2} PI-4P-reporter protein was still visible on the Golgi.

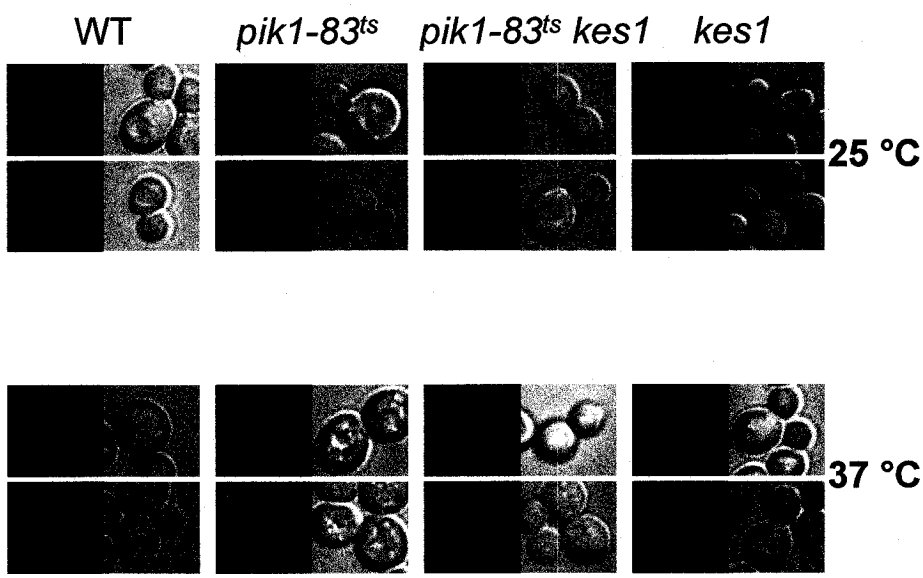


Figure 38. GFP-PH^{Osh2} localizes to the Golgi and plasma membrane in *pik1-83 kes1* cells. Cells of the strains indicated were transformed with pTL511 that encodes the GFP-PH^{Osh2} chimeric protein. Cells were then grown to early-log phase in minimal medium lacking uracil at 25°C. Images were taken of live cells growing at 25°C or after incubation at 37°C for 15 min.

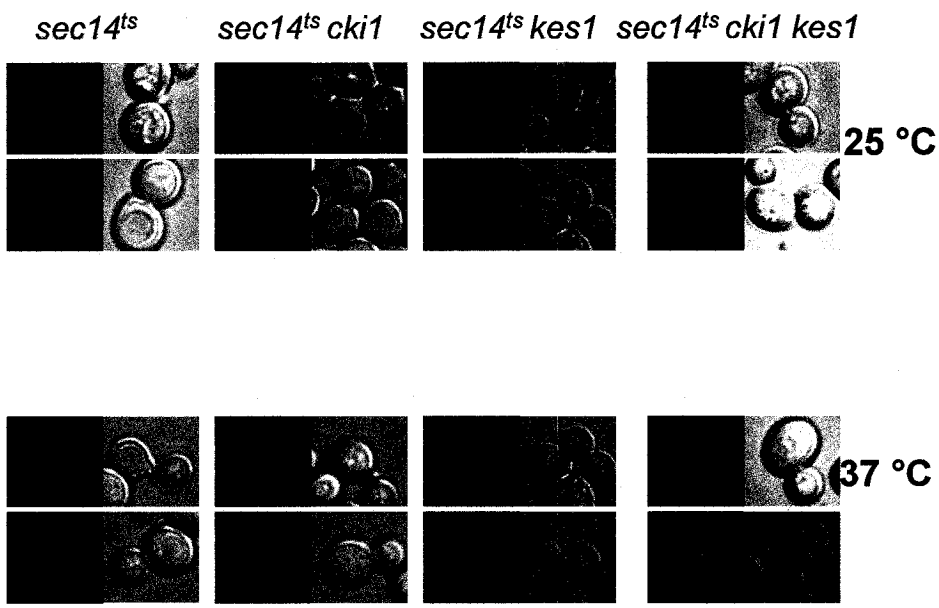


Figure 39. GFP-PH^{Osh2} localizes to the Golgi and plasma membrane in Sec14 Bypass cells. Cells of the strains indicated were transformed with pTL511 that encodes the GFP-PH^{Osh2} chimeric protein. Cells were then grown to early-log phase in minimal medium lacking uracil at 25°C. Images were taken of live cells growing at 25°C or after incubation at 37°C for 60 min.

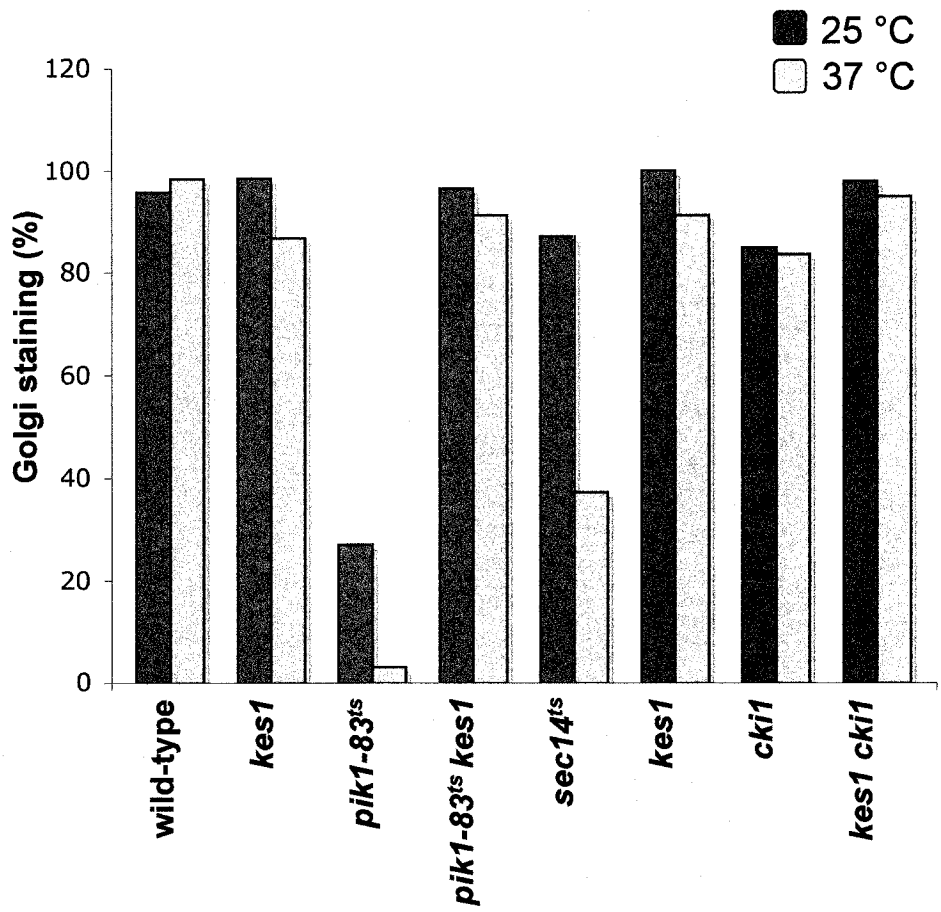


Figure 40. Quantification of GFP-PH^{Osh2} distribution in *pik1* and *sec14* mutant cells. The presence of Golgi staining by the PI-4P reporter was assessed from 6 random fields from at least two separate experiments. At least 120 different cells were documented for each strain and growth condition. Values are represented as percentage of cells with Golgi (punctate) staining.

My SGA results imply that Kes1 and the CDP-choline pathway bypass the essential function of Sec14 through different pathways that eventually converge to regulate *trans*-Golgi function and vesicular transport. It is believed that the CDP-choline pathway modulates PC/DAG ratios within the Golgi and in my current work, I indicate that Kes1 regulates Golgi PI-4P availability. To further test this we monitored PI-4P distribution in *sec14*^{ts} cells and in bypass cellss. Isogenic *sec14*^{ts} strains of interest were transformed with the GFP-PH^{Osh2} PI-4P-reporter plasmid and visualized by microscopy at 25°C and 37°C (Figure 39). Figure 40 summarizes the results of >100 cells analyzed under each condition. At the permissive temperature for the *sec14*^{ts} allele all cells possessed both plasma-membrane and Golgi PI-4P (*sec14*^{ts} *kes1* cells displayed a slight increase in GFP intensity on Golgi membranes). At the non-permissive temperature, *sec14*^{ts} cells showed a reduction in punctate staining. All of the bypass strains, *sec14*^{ts} *ckl1 sec14*^{ts} *kes1* and *sec14*^{ts} *ckl1 kes1* displayed both plasma-membrane and Golgi staining at the non-permissive temperature. While less then 10% of the *pik1-83*^{ts} cells displayed Golgi staining at 37°C, nearly 50% of *sec14*^{ts} cells still had some degree of Golgi staining. Previously, it was noted that *sec14 ckl1* cells have across-the-board decreases in PIP levels. Consistent with this, *SEC14* shares synthetic lethal partners with *PIK1, STT4,* and *MSS4*. Given the localization data for the GFP-PH^{Osh2} fusion protein, cells containing *sec14*^{ts} grown at the non-permissive temperature may maintain a normal distribution of PI-4P, with the global reduction of PI-4P a result in reduction in both the Stt4 and Pik1 pools.

4.2.3. PI-4P BINDING DOMAINS INHIBIT CELL GROWTH

Kes1 is an abundant protein, estimated at 32,000 molecules/cell, and in several experimental systems can bind PI-4P or PI-4,5P_2 (Ghaemmaghami et al., 2003). One simple explanation for Kes1 function would be that Kes1 acts as an insulator and effectively competes for available PI-4P on the Golgi membranes. To investigate this further, plasmids expressing the PH domains from human FAPP1 and yeast Osh2 were transformed into cells of a strain that is sensitive to increased levels of *KES1*, CMY510 (*sec14*^{ts} *ckl1*). Cells were grown to mid-logarithmic phase, harvested, serially diluted and

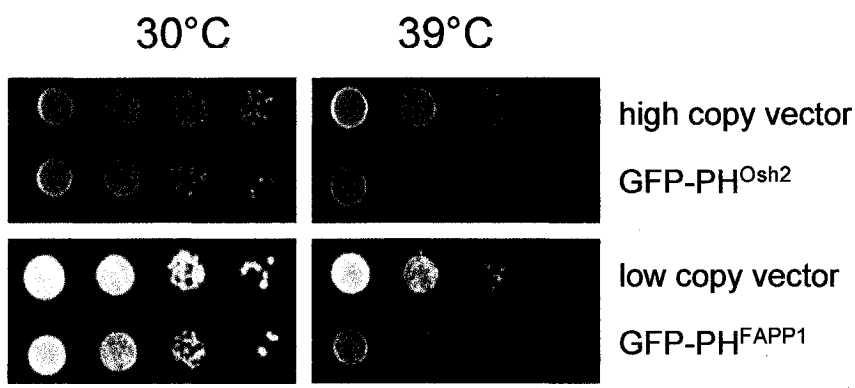


Figure 41. Availability of Golgi PI-4P is a requirement for *ckl1* mediated Sec14 bypass. Cells of the strain CMY510 (*sec14^{ts}*:natR *ckl1::LEU2*) were transformed with either pRS416 (low copy) or pRS426 (high copy) empty vector or the plasmids pRS416-PH^{FAPP}or pRS426-PH^{Osh2}. Cells were then grown to logarithmic phase, harvested, and washed, and equal concentrations were 10-fold serially diluted and spotted on minimal solid medium lacking uracil and incubated at the indicated temperature for 48-72 h.

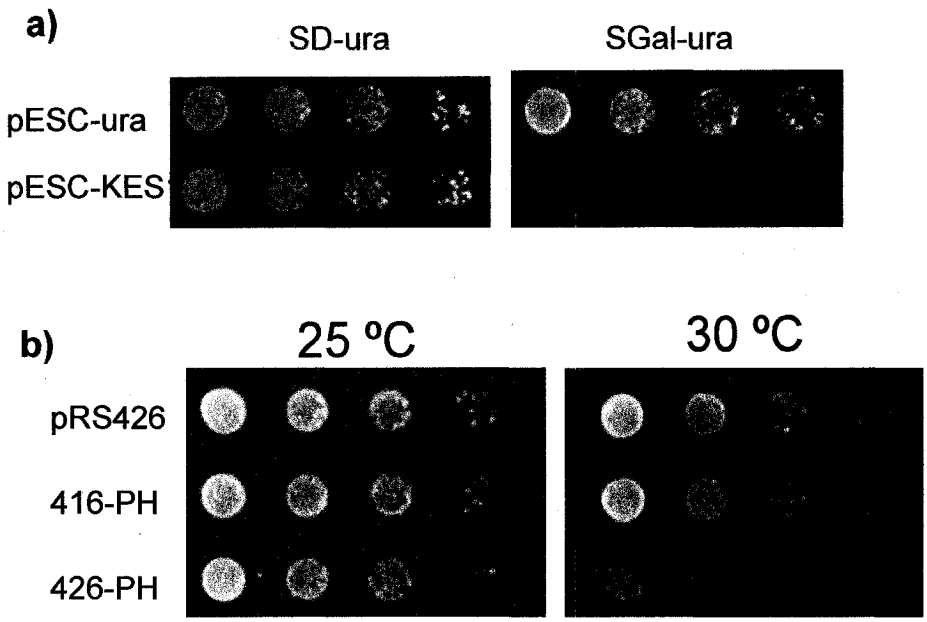


Figure 42. Increased dosage of *KES1* inhibits growth of *pik1-139* cells. a) Cells of the strain AAY1139 (*pik1-139^{ts}*) were transformed with pESC-KES1 or with empty vector as indicated. Cells were then grown to mid-logarithmic phase in minimal medium lacking uracil. Cells were then harvested and equal concentrations were 10-fold serially diluted and spotted on synthetic solid medium containing dextrose and lacking uracil or synthetic solid medium containing galactose and lacking uracil. Plates were incubated at 25°C for 72 h. b) Cells of strain AAY1139 were transformed with the PH^{Osh2} gene on a high-copy (pRS426 vector) or low-copy (pRS416) plasmids. Transformants were grown to mid-log phase in selective medium and harvested, and serial dilutions were spotted on selective solid medium and incubated at 25°C or 30°C.

spotted on SC-ura solid medium. Figure 41 shows that high levels of PH^{Osh2} and PH^{FAPP1} impaired the growth of *sec14^{ts} ckil* cells at 39°C.

The above result demonstrates that in a condition in which the levels of Golgi PI-4P are sub-optimal that expression of Golgi-localized PI-4P-binding proteins can impair growth. To investigate this finding further, I postulated that increased expression of Kes1 or PH^{Osh2} would inhibit the growth of a partial-loss-of-function allele of *PIK1*, *pik1-139^{ts}* (Sciorra et al., 2005). *Pik1-139^{ts}* cells were transformed with pESC-ura or pESC-KES1, grown in selective medium, harvested, serially diluted and spotted on SC-ura or Sgal-ura solid agar and incubated at 25°C or 30°C. Figure 42a shows that increased levels of Kes1 prevented growth even at 25°C, prevented growth at 30°C. In contrast, vector controls showed robust growth at 25°C and 30°C.

As well, vector control, pRS416-GFP-PH^{Osh2} (low copy) or pRS426-GFP-PH^{Osh2} (high copy) were transformed into *pik1-139^{ts}* cells, and the resulting transformants were grown at 25°C serially diluted and spotted on minimal solid medium. Figure 42b shows that cells grew normally at 25°C; however, at 30°C the cells containing the high-copy pRS426-GFP-PH^{Osh2} plasmid were inhibited for growth.

Previous results demonstrated that Pik1, Frq1 and Kes1 all localize to the Golgi, with Kes1 localization dependent upon PI-4P production on the Golgi (Li et al., 2002; Strahl et al., 2005). While Kes1 binding to the Golgi is dependent upon Pik1 activity, it may be possible that Kes1 recruitment to the Golgi involves interactions with other proteins.

4.2.4. THEORETICAL ESTIMATION OF PHOSPHOLIPID NUMBER IN YEAST

As noted earlier, Kes1 is an abundant protein (32,000 molecules/cell) that can bind to PI-4P, and whose Golgi localization is dependant upon PI-4P production in the Golgi (Ghaemmaghami et al., 2003; Li et al., 2002). The levels of Pik1 generated PI-4P were estimated at 25-50,000 molecules/cell (Table 7), based on reported values (Guthrie and Fink, 1991; Waechter and Lester, 1971). This supports the possibility that Kes1 competes for available PI-4P on the Golgi with other pro-secretory factors. In wild-type cells an equilibrium is reached and secretion is able to proceed, but in cells with limited

Table 7. Molecules of phospholipid per cell

Phospholipid composition in *S.cerevisiae* (molecules /cell)

growth phase	Total	PC	PE	PI	PIP
early	321,468,000	168,560,000	69,109,600	55,263,600	132,440
mid	316,652,000	161,336,000	63,210,000	60,681,600	252,840
stationary	302,204,000	161,336,000	80,186,400	36,120,000	180,600

Phosphatidylinositol species distribution in *S. cerevisiae* (molecules/cell)

growth phase	PI	PI-3P	PI-4P	PI-3,5P	PI-4,5P
early	55,263,600	43,705	55,625	3,973	29,137
mid	60,681,600	83,437	106,193	7,585	55,625
stationary	36,120,000	59,598	75,852	5,418	39,732

Phosphotidylinositol 4-phosphate produced by each enzyme (molecules/cell)

growth phase	Pik1	Stt4	Lsb6
early	26,422	26,422	2,781
mid	50,442	50,442	5,310
stationary	36,030	36,030	3,793

levels of PI-4P production in the Golgi (i.e. *sec14*^{ts}, *pik1*^{ts} or *frq1*^{ts}mutants) Kes1 out-competes other factors that require PI-4P binding to facilitate secretion, resulting in a secretion block and death under these situations.

Much of my discussion on the mode of Kes1-mediated inhibition of secretion is based upon PI-4P. Published evidence suggests that Pik1 and Stt4 produce equal amounts of PI-4P and that the resultant pools are non-overlapping, with the Pik1 pool in the Golgi and the Stt4 pool in the plasma membrane (Audhya et al., 2000). To illuminate the situation further we estimated the number of phospholipid molecules/cell. Table 7 summarizes the phospholipid composition of *S. cerevisiae* cells grown to early-, mid-logarithmic and stationary phase. The μmoles of lipid phosphorus/gram of cells was determined by Waetcher and Lester (Guthrie and Fink, 1991; Waechter and Lester, 1971), and was converted to molecules/cell by multiplying by Avogadro's number (6.02 x 10^{23}) and by the mass/yeast cell of 2.0 x 10^{-11} grams/cell (Guthrie and Fink, 1991; Waechter and Lester, 1971). The results suggest that a single yeast cell contains ~ 310 million phospholipids, with PC representing ~155 million (50%) of the total phospholipid pool. As noted in the Introduction, PIPs are not very abundant with about 200,000 present per cell, which is <0.08% of the total phospholipid pool. Of the PIPs, PI-4P represents ~40%, or slightly less than 100,000 molecules per cell (Audhya et al., 2000; Sciorra et al., 2005). As Lsb6 produces 5% of the total PI-4P pool, and Stt4 and Pik1 equally produce the remainder, the Pik1-produced pool of PI-4P varies between 25,000 – 50,000 molecules/cell (Audhya et al., 2000; Han et al., 2002). Kes1 has been estimated to bind PI-4,5P2 with an affinity of 2.5 μM. In a simple one-to-one binding scheme, Kes1 would be able to bind the majority of PI-4P under normal conditions.

4.2.5. SEPARATION OF FUNCTION MUTANT OF KES1

Recently Prinz, Hurley and coworkers demonstrated that Kes1 can transfer ergosterol, cholesterol and to a lesser extent phospholipids *in vitro* (Raychaudhuri et al., 2006). Their hypothesis is that the Osh proteins are sterol transporters *in vivo*. That lab also made a series of point mutants which could not suppress *kes1*-mediated Sec14 bypass (Im et al., 2005). If the point mutants could not suppress *kes1*-mediated bypass of Sec14, and Sec14 functions by positively regulating Pik1, the point mutants should not

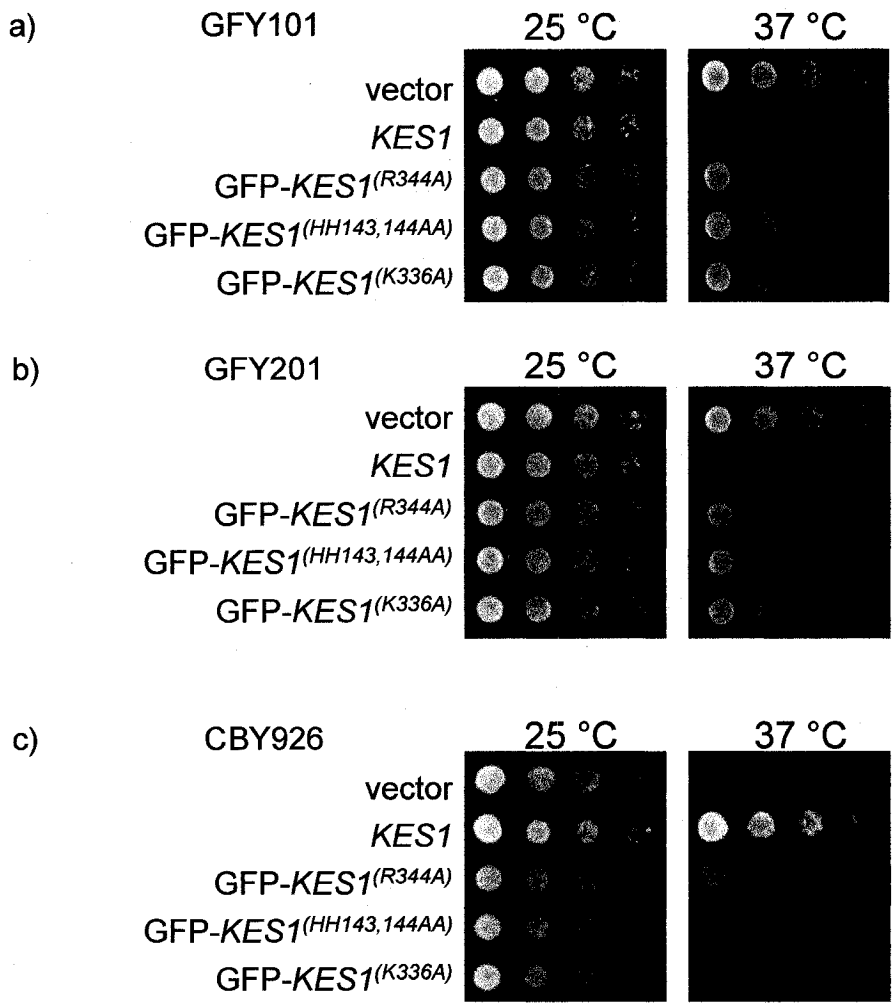


Figure 43. Mutational analysis of Kes1 function in the regulation of secretion and sterol distribution. Cells of the strains GFY101 *(sec14^{ts} kes1),* GFY201 *(pik1-83^{ts} kes1)* and CBY926 *(osh1-osh7 osh4-1^{ts})* were transformed with plasmids expressing wild-type Kes1 or the GFP-tagged mutants indicated. Cells were then grown to logarithmic phase, harvested, and washed, and equal concentrations were 10-fold serially diluted, spotted on minimal medium lacking uracil and incubated at the indicated temperature for 48-72 h.

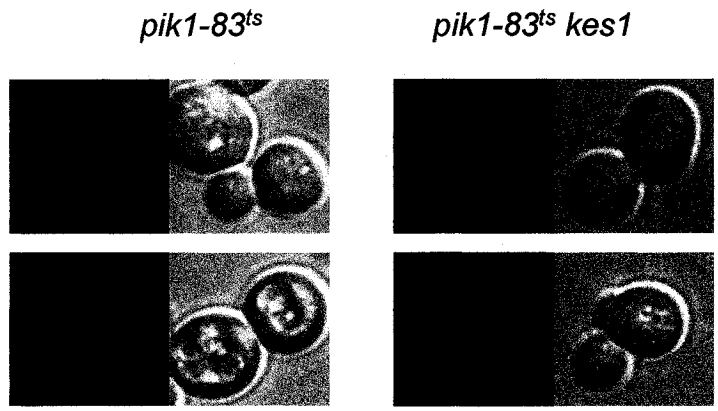


Figure 44. Genomic replacement of *KES1* in *pik1-83* mutants restores trafficking of GFP-Snc1p. Wild-type or the isogenic *pik1-83*^{ts} cells indicated were transformed with pMJL1 (Snc1-GFP) and grown to early-log phase in minimal medium lacking uracil to ensure plasmid maintenance. Images were taken of live cells growing at 25°C.

re-establish a temperature defect in *pik1-83^{ts}* cells. To test this idea, cells of strains GFY101 (*sec14^{ts} kes1*), GFY201 (*pik1-83^{ts} kes1*) and CBY926 (*osh1Δ-7Δ osh4-1^{ts}*) were transformed with plasmids pJH1 (carrying *URA3* and GFP-*kes1$^{(H143A,H144A)}$*), pJH2 (*URA3* GFP-*kes1$^{(K336A)}$*), pJH3 (*URA3 kes1$^{(R344A)}$*) or pSLS-KES1. The Kes1$^{(H143A,H144A)}$ mutant protein was one of the original mutants identified in the Sec14-bypass screen. It is unable to suppress an Osh-deficient strain and it is unable to complement *kes1*-mediated sec14 bypass suppression (Figure 43). Quantitative immunoblotting, shows that its steady-state levels of the Kes1 (H143A,H144A) protein is about 60% of wild-type Kes1 protein (Im et al., 2005). However, *in vitro* sterol-transfer assays suggest that can function as well as the wild-type Kes1 protein. The Kes1(K336A) mutant protein was unable to provide either Kes1 function *in vivo. In vitro,* Kes1(K336A) protein is unable to extract ergosterol from liposomes and its ability to transfer ergosterol is greatly diminished (80-90%) (Raychaudhuri et al., 2006). The Kes1(R344A) protein is potentially the most informative mutant, as it is able to support the viability of an Osh deficient strain, but, it could not suppress *kes1*-mediated Sec14 bypass. While it has been demonstrated that this mutant can bind to sterols (Im et al., 2005), its ability to transfer sterol *in vitro* has not been tested (Raychaudhuri et al., 2006). As a result it is unknown if this mutant is able to extract or transfer sterols between liposomes. GFY101 cells carrying the pSLS-KES1 vector die at 37°C; however, cells carrying any of the other three *kes1* alleles grew similarly to the vector control. The same results were seen for the GFY201 transformants. The CBY926 transformants show the opposite situation: they require Kes1 function for viability as only pSLS-KES1 or pJH3 support growth at the non-permissive temperature for the *osh4-1^{ts}* allele (Figure 43). Due to the fact that Kes1$^{(H143,144A)}$ and Kes1$^{(K336A)}$ cannot provide either of the functions attributed to Kes1, it is difficult to know if they have any residual Kes1 function *in vivo*: though they are simple point mutants they may in fact be completely non-functional. The fact that GFP-Kes1(R344A) is able to support life of the Osh-deficient cells suggests that this mutant protein is at least partially functional *in vivo,* though it is unable to inhibit secretion. This suggests that the shared function of all Osh proteins and the ability of Kes1 to affect Sec14-dependent secretion are independent activities.

4.2.6. GFP-Snc1 LOCALIZATION IN *PIK1-83* CELLS

Previously it was reported that Pik1 activity was required for the exit of GFP-Snc1 from the Golgi (Sciorra et al., 2005). Cells containing the less severe *pik1-139* mutant allele trafficked GFP-Snc1 normally at 25°C, but when shifted to 37°C, the GFP-Snc1 accumulated in internal structures. Genomic replacement of *KES1* restored growth to the *pik1-83*^{ts} cells, so it is possible that in *pik1-83 kes1* cells GFP-Snc1 trafficking is also restored. To assess this hypothesis, wild-type, *pik1-83*^{ts} and *pik1-83*^{ts} *kes1* cells were transformed with pMJL1 (from Hugh Pelham encoding GFP-Snc1) grown to early-logarithmic phase at 25°C, and viewed by fluorescent microscopy. Figure 44 shows that even at 25°C, GFP-Snc1 is localized to internal punctate structures in the *pik1-83* cells. Genomic replacement of *KES1* in these cells restored the localization of GFP-Snc1 to the sites of polarized growth (e.g the growing bud and bud neck). This observation is consistent with Kes1 negatively regulating the actions of Pik1 with regards to vesicular traffick from the *trans*-Golgi.

4.2.7. Hsp150 SECRETION IN *PIK1-83* CELLS

Hsp150 is a high-molecular weight glycoprotein that is constitutively produced and secreted by yeast, with a 7-fold increase in expression upon shift to 37°C (Russo et al., 1992). However, in *pik1-83*^{ts} cells >95% of Hsp150 is retained within the cell, predominantly in a Golgi-modified form (Audhya et al., 2000). To investigate the ability of genomic replacement of *KES1* to re-establish secretion of Hsp150 in *pik1-83*^{ts} cells, Hsp150 secretion was monitored in wild-type, *pik1-83*^{ts} and *pik1-83*^{ts} *kes1* cells. Cells were grown to mid-logarithmic phase, washed, incubated at 37°C and grown in the presence of radiolabeled methionine and cysteine for 30 minutes. Medium was harvested and proteins were precipitated by TCA and resolved on an SDS-PAGE gel. Figure 45 shows that *pik1-83* cells secrete no Hsp150, whereas *pik1-83 kes1* cells secrete Hsp150 at levels similar to wild-type cells. Previous results demonstrated that wild-type and *pik1-83* cells incorporate radiolabel equally, and that the production of Hsp150 is similar (Audhya et al., 2000). These results are consistent with Kes1 acting as a negative regulator of Pik1.

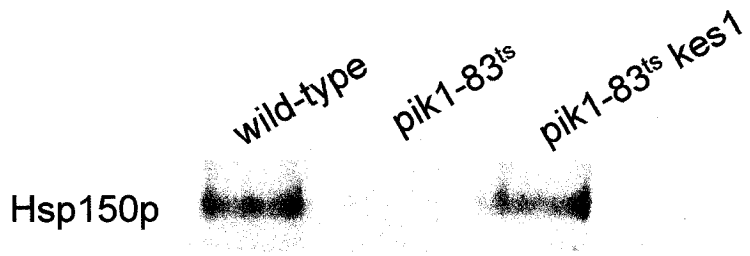


Figure 45. Genomic replacement of *KES1* restores secretion of Hsp150 in *pik1-83* mutant cells. Wild-type or cells of the isogenic *pik1-83*^{ts} strains indicated were grown to early-log phase in YEPD medium. Wild-type and mutant yeast cells were pre-incubated at 37°C for 10 min, metabolically labeled with a ^{35}S-protein labeling mix during a 10-min pulse, and then chased in the presence of excess unlabeled methionine and cysteine for 30 min. Cells and medium were separated by centrifugation, medium was removed and precipitated with 10% TCA. Proteins were washed twice with acetone, air dried, and resuspended in equal volumes of SDS/PAGE loading buffer. Protein extracts were resolved by SDS/PAGE on a 10% acrylamide gel. Gels were dried, exposed to a phosphorus screen and captured with a phosphoimager (Bio-Rad).

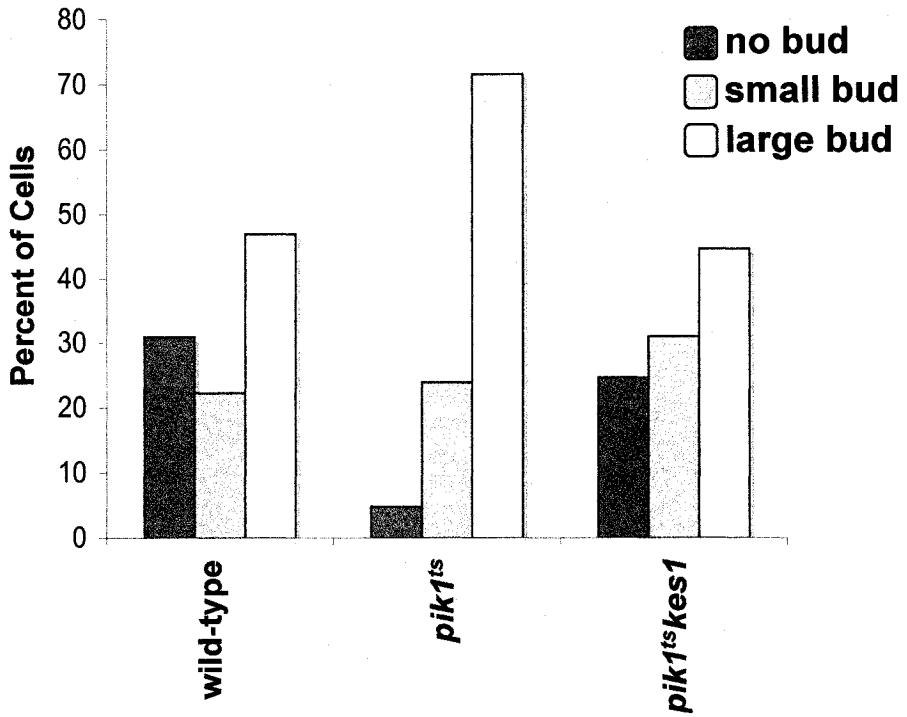


Figure 46. Genomic replacement of *KES1* in *pik1-83^{ts}* cells reduces the number of large budded cells. Wild-type or the isogenic *pik1-83^{ts}* cells indicated were grown to early-log phase in YEPD medium. Wild-type and mutant yeast cells were incubated at 37°C for 6 h. Cells were immobilized on an agaorse pad on a glass slide, cover slips were added and sealed. Cells were viewed under 100X oil objective lens, at least 100 cells were classified for each strain.

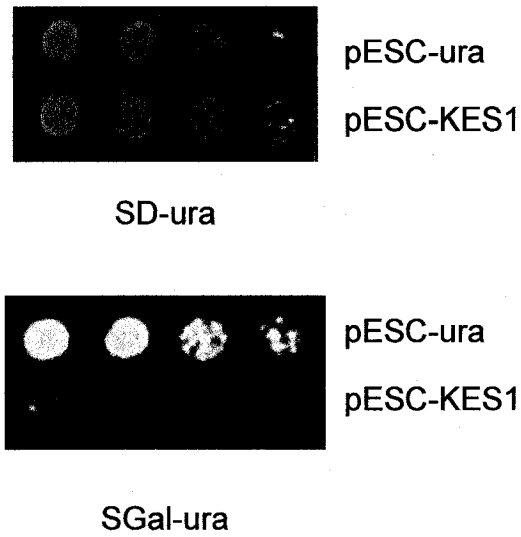


Figure 47. Genomic replacement of *KES1* bypasses the essential requirement of *FRQ1*. Cells of the strain GFY218 (*kes1::*natR *frq1::*hybR) were transformed with the plasmid pESC-KES1 or empty vector as indicated. Transformants were then grown to mid-logarithmic phase in minimal medium lacking uracil. Cells were harvested, and equal cell concentrations were 10-fold serially diluted and spotted on synthetic solid medium containing dextrose and lacking uracil or synthetic solid medium containing galactose and lacking uracil. Plates were incubated at 30°C for 72 h.

4.2.8. INACTIVATION OF *KES1* BYPASSES THE CYTOKINESIS DEFECT IN PIK1-83 CELLS

The original characterization of a temperature sensitive *PIK1* allele demonstrated that at the non-permissive temperature cells carrying the *pik1-12* allele arrested and subsequently died with large buds (Garcia-Bustos et al., 1994). This suggests that a cytokinesis defect may be the cause of the fatality for this allele. Wild-type, *pik1-83^{ts} and pik1-83^{ts} kes1* cells were grown to early-logarithmic phase and shifted to the non-permissive temperature of 37°C for 6 hours. Cells were classified as having no buds, small buds or larger buds. Figure 46 shows that after 6 hours the percentage of large budded cells increases to 70% for the *pik1-83^{ts}* cells, at the consequence of the number of unbudded cells. However, the *pik1-83^{ts} kes1* cells has a similar bud size distribution profile to that of wild-type cells. This demonstrates a need for Pik1 produced PI-4P for the completion of cytokinesis and that removal of Kes1 can restore this signal in cells with a partial-loss-of-function of *PIK1*.

4.2.9. GENETIC REPLACEMENT OF *KES1* BYPASSES THE ESSENTIAL REQUIREMENT OF *FRQ1*

Recently, Pik1 has been characterized as producing at least two pools of PI-4P, one nuclear and the other Golgi-localized, with both of them being essential for viability (Strahl et al., 2005). In the same study it was determined that a direct protein-protein interaction between Frq1 and Pik1 aids in targeting Pik1 to the Golgi. This lead to the hypothesis that removal of *KES1*, a potential negative regulator of Pik1/PI-4P, might bypass the essential requirement of Frq1. The strain GFY217 (*kes1::KAN ^{R}*) was transformed with a linear DNA fragments to selectively target and replace the *FRQ1* gene (as described in the Methods section). The resulting transformation resulted in dozens of colonies with the genotype *kes1::KAN^{R} frq1::NAT^{R}* suggesting replacement of *KES1* does indeed bypass the essential requirement for Frq1. The bypass was abolished by the re-introduction on a plasmid carrying a galactose-inducible *KES1* when the cells were grown in the presence of galactose Figure 47. This observation suggests Kes1 can attenuate the Golgi-localized Pik1 produced PI-4P-signaling.

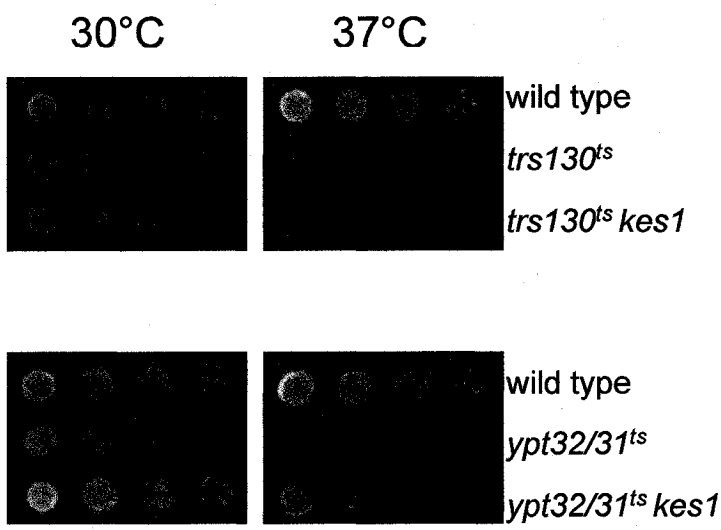


Figure 48. Genomic replacement of *KES1* in *trs130*^{ts} or *ypt31*^{ts}*/32* mutants has minor effects. Cells of the strain VSY446 (*trs130*^{ts}),, GFY209 (*trs130 kes1*::kanR), VSY468 (*ypt32 ypt31-101*) and GFY211 (*ypt32 ypt31-101 kes1*::kanR) were grown to mid-logarithmic phase YEPD medium, harvested, and 10-fold serial dilutions were spotted on YEPD solid medium and incubated for 72 h at the indicated temperatures.

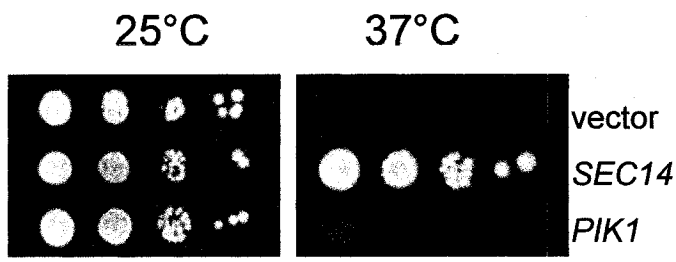


Figure 49. Increased expression of *PIK1* can mildly suppress defects associated with *sec14^{ts} ckil ypt31* cells. Cells of the GFY309 (*sec14^{ts}*:natR *ckil::LEU2 ypt31::*kanR) strain were transformed with plasmids encoding *PIK1, SEC14* or empty vector as indicated. Transformants were grown to mid-logarithmic phase in minimal medium lacking uracil, harvested, and 10-fold serial dilutions were spotted on minimal solid medium lacking uracil and incubated for 72 h at the indicated temperatures.

4.2.10. GENETIC REPLACEMENT OF *KES1* DOES NOT RESCUE GROWTH DEFECTS OF *trs130^{ts}* CELLS

The data suggests that the TRAPPII complex functions downstream of PI-4P, and may in fact be a direct effector of PI-4P. Perhaps Kes1 functions downstream of Pik1, if this was the situation Kes1 may inhibit some function of the TRAPPII complex. To investigate this further, *KES1* was replaced in cells containing a *trs130^{ts}* allele (Sciorra et al., 2005). Wild-type, *trs130^{ts}* and *trs130^{ts} kes1* cells were grown to mid-logarithmic phase, serially diluted and spotted on YEPD solid medium. Genomic replacement of *KES1* had no effect on the viability of *trs130^{ts}* mutants at a non-permissive temperature (Figure 48).

4.2.11. GENETIC REPLACEMENT OF *KES1* HAS MINOR EFFECTS ON THE GROWTH RATE OF *ypt32 ypt31^{ts}*CELLS

The same experiment was performed in an *ypt32 ypt31-101^{ts}* strain. Here ablation of *KES1* had minor effects on the viability of the double mutant (Figure 48). This minor restoration in growth is likely not significant since disruption of *GYP2,* the RabGAP for Ypt31/32, restores growth of *ypt32 ypt31^{ts}* to wild-type levels (Sciorra et al., 2005). The results suggest that Kes1 attenuates PI-4P signaling above TRAPPII and Ypt31/32.

4.2.12. INCREASED *PIK1* DOSAGE PHENOCOPIES INACTIVATION OF *KES1*

The evidence suggests Kes1 inhibits secretion by regulating the Golgi-localized PI-4P signaling cascade. Genetic replacement of *KES1* alleviates cellular defects associated with reduction of Pik1 activity, and is able to bypass the essential requirement of Sec14 and Frq1. However, replacement of *KES1* is unable to suppress defects associated with potential downstream effectors of the PI-4P signal (e.g TRAPPII-Ypt31). Some phenotypes associated with genomic replacement of *KES1* are similar to phenotypes associated with increased Pik1 levels (e.g. Frq1 bypass). As genomic replacement of *KES1* rescues cells of the *sec14^{ts} ckl1 ypt31* strain, *sec14^{ts} ckl1 ypt31* these cells were transformed with high-copy number plasmids containing *PIK1.* Figure 49 shows that increased expression of *PIK1* prevented death and restored mild growth to

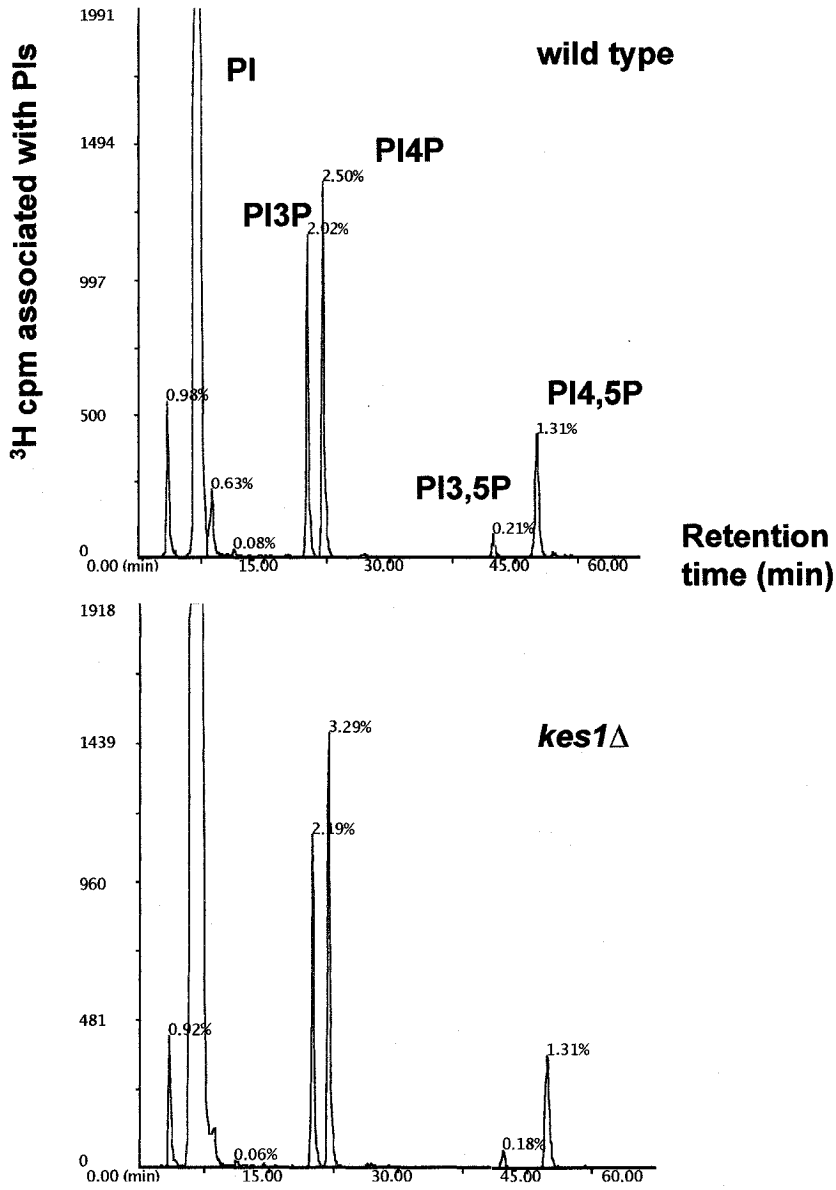


Figure 50. Levels of PI-4P are increased in *kes1* cells. Cells of the strains Y2454 and CMY210 were labelled with myo-[2-^{3}H]inositol for 10 min at 26°C, and chased with excess unlabeled myo-inositol for 30 min, and lysed with glass beads. Lipids were extracted, deacylated, and separated by HPLC (see Methods). Peaks corresponding to glycero-Ins(3)P, glycero-Ins(4)P, glycero-Ins(3,5)P2, and glycero-Ins(4,5)P2 are indicated.

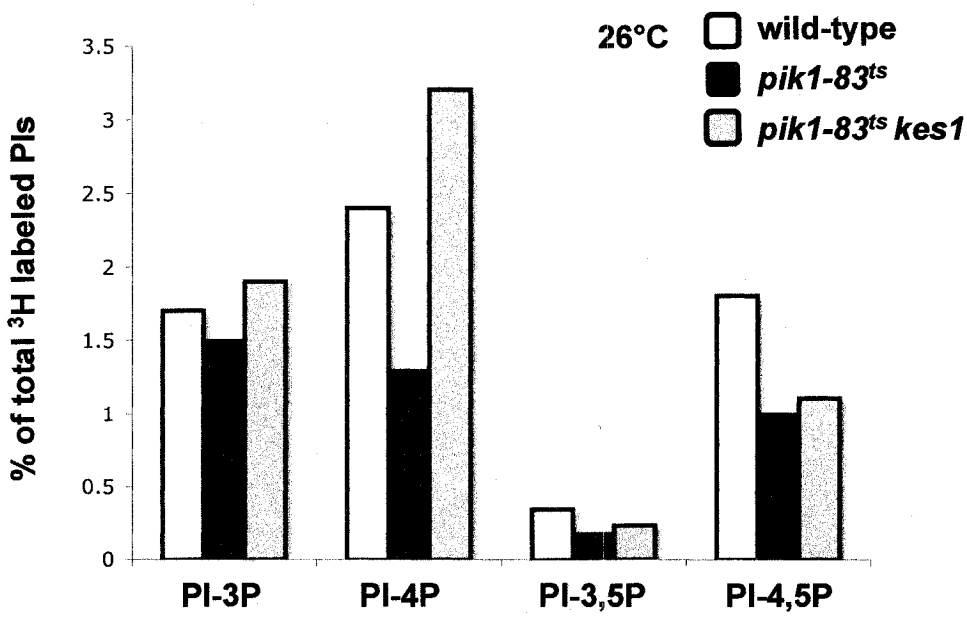
sec14^{ts} ckil ypt31 cells at the non-permissive temperature. Due to the lack of selectable markers the co-overexpression of *PIK1 and FRQ1* in these cells have not yet been examined. However, based on the results in figure 35, that show that co-overexpression of *PIK1* and *FRQ1* suppressed the *sec14^{ts}* defects more strongly than *PIK1* on its own, it is possible that the growth rate of *sec14^{ts} ckil ypt31* cells would be even higher.

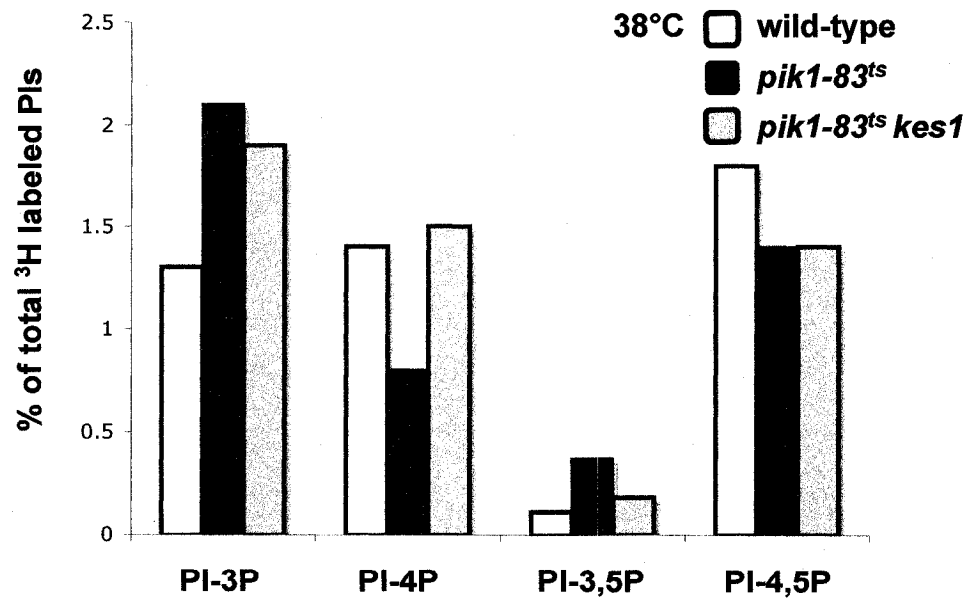
4.2.13. *in vivo* ANALYSIS OF PHOSPHOINOSITIDES

The genetic, phenotypic and cellular evidence suggests that Kes1 attenuates PI-4P signaling on Golgi membranes. To investigate if Kes1 influences phosphoinositide homeostasis metabolic labeling was performed. Levels of phosphoinositides were determined in wild-type cells and in cells lacking *KES1*. Figure 50 shows two high-performance liquid chromatography (HPLC) chromatographs from run sthat shows the distribution and levels of the different glycerolphosphoinositolphosphates from the two strains after chemical deacylation. In wild-type cells the total PI-4P levels represent 2.5% of the total GPI (with ~50% produced by Pik1 and ~50% produce by Stt4) (Audhya et al., 2000). In *kes1* cells the PI-4P levels is 3.3% of the GPI, a modest 34% increase. While this value is small, it should be noted that cells carrying *PIK1* on high-copy vectors only display a 100% increase in PI-4P levels (Schorr et al., 2001).

The phenotypic observations and the localization of the GFP-PH^{Osh2} fusion protein suggests that Kes1 regulates the Golgi PI-4P pool, therefore this would mean that the Golgi PI-4P increases by 68%. PI-4P is the only lipid significantly altered in *kes1* strains. This suggests that very little if any of the Golgi produced PI-4P is converted to PI-4,5P_2 consistent with previous observations. To extend this analysis further, PIP levels were measured in *pik1-83^{ts} and pik1-83^{ts} kes1* cells at 26°C and 38°C. Figure 51 shows that wild-type, AAY104 and GFY201 all have similar levels of PI-3P at 26°C, however, PI-4P levels are varied with 2.4%, 1.4% and 3.3% for wild-type, *pik1-83^{ts}* and *pik1-83^{ts} kes1* cells at 26 °C. Genomic replacement of *KES1* in the *pik1-83* cells results in a 2.5-fold increase in PI-4P in the cell at 38°C. While replacement of *KES1* leads to an increase in PI-4P, it does not restore the production of PI-4,5P_2, as both AAY104 (*pik1-83^{ts}*) and GFY201 (*pik1-83^{ts} kes1*) cells have levels of PI-4,5P_2 around 1.1-1.2% whereas wild-type cells have levels around 1.7%. At the non-permissive temperature, wild-type and

Figure 51. Genomic replacement of *KES1* restores the levels of PI-4P in *pik1-83^{ts}* cells. Quantitative comparisons of glycero-phosphoinositols generated by wild-type, *pik1-83^{ts}* and *pik1-83^{ts} kes1* cells at 26°C or 38°C are shown below the elution profile. These data represent means of three independent experiments.


26°C
wild-type
pik1-83ts
pik1-83ts kes1
% of total 3H labeled PIs
3.5
3
2.5
2
1.5
1
0.5
0
PI-3P
PI-4P
PI-3,5P
PI-4,5P


38°C
wild-type
pik1-83ts
pik1-83ts kes1
% of total 3H labeled PIs
2.5
2
1.5
1
0.5
0
PI-3P
PI-4P
PI-3,5P
PI-4,5P

GFY201 cells have the same levels of PI-4P (~1.4%), while the levels drop in the AAY104 strain is further reduced (~0.7%).

4.2.14. Pik1 INTERACTS WITH IMMOBILIZED GST-Kes1

As the levels of PI-4P are restored to wild-type levels in *pik1-83^{ts} kes1* cells, it is possible that Kes1 inhibits Pik1 activity through direct protein-protein interactions. This is an attractive model, where the production of PI-4P is fine-tuned in the Golgi. As levels of PI-4P increase more Kes1 would translocate to the Golgi, which in turn inhibits production the production of PI-4P. When the levels of PI-4P decrease, Kes1 releases into the cytosol allowing the production of PI-4P to increase.

To determine if Kes1 and Pik1 could interact directly, GST-Kes1 was purified from *E. coli* and immobilized on glutathione Sepharose. Pik1 was produced using *in vitro* transcription-coupled translation in the presence of [^{35}S]-methionine and incubated with immobilized GST-Kes1 in the presence of 100 μM DTBP (dimethyl 3,3′-dithiobispropionimidate, a cleavable, bifunctional, imidoester crosslinker) for 30 minutes. The excess DTBP was quenched by the addition of Tris-HCl buffer. After washing with Tris-buffered saline the resin was incubated with SDS-loading buffer supplemented with 100 μM DTT and incubated at 37°C for 30 minutes to cleave the DTBP. Figure 52a shows Pik1 binding to GST-Kes1 but not GST alone. As a control, firefly luciferase was also produced by *in vitro* transcription-translation and incubated with GST or GST-Kes1 and shown not to bind. Although the interaction appears weak (~1% bound), Kes1 and Pik1 can directly interact *in vitro*.

Previous work demonstrated that Pik1 can interact with Frq1 and Importin β, which raises the possibility that Kes1 binding to Pik1 could be strengthened in the presence of other proteins (Strahl et al., 2005). To investigate this further, purified GST-Kes1 was immobilized on glutathionine sepharose. (Previous reports demonstrated that Pik1 has weak affinity for resin on its own, so the resin was incubated with 3% BSA/PBS prior to the addition of the yeast cell extract). Cell extracts were produced from *PIK1:TAP:HIS3 kes1::*KAN^{R} cells as described in the methods and incubated with GST or GST-Kes1 for 1 hour at room temperature with or without 100 μM DTBP. Figure 52b is a western blot using the anti-TAP antibody. Pik1-TAP interacted with GST-Kes1 when

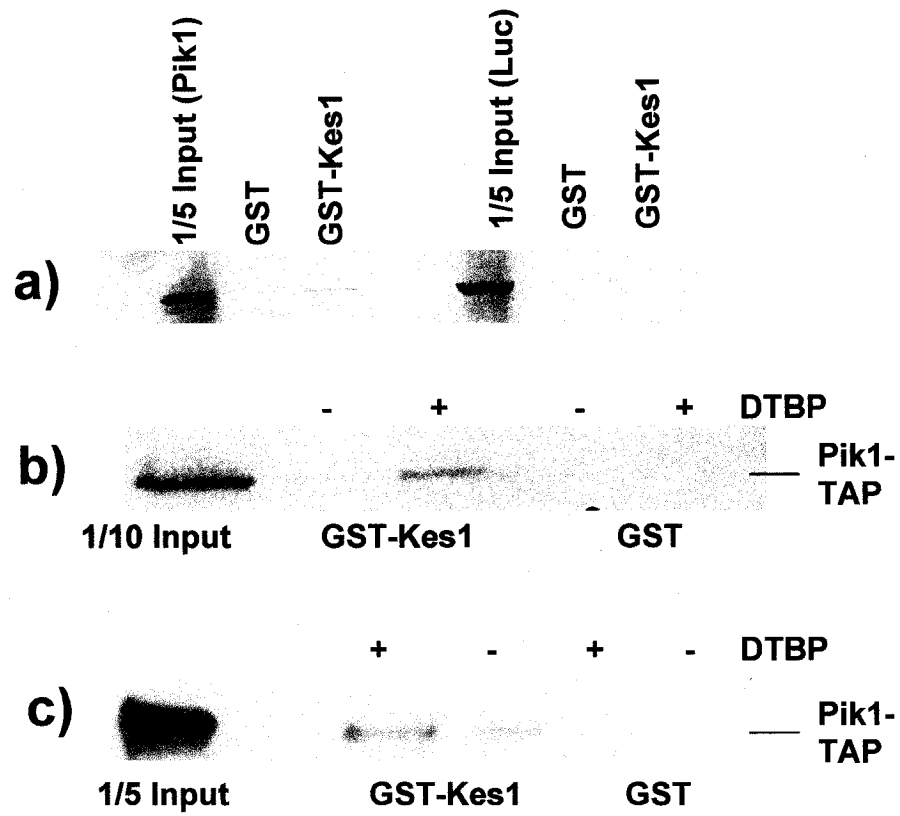


Figure 52. Kes1 binds to Pik1 directly and binding is influenced by ergosterol. a) autoradiograph of binding assay samples where [^{35}S]methionine, cysteine-labeled Pik1 of firefly Luciferase (Luc) prepared by *in vitro* transcription and translation was incubated with GST-Kes1 or GST control in the pressence of the 100 µM DTBP. b+c) GST-Kes1 and GST were immobilized on glutathione sepharose and preincubated with b) 1% ethanol (vehicle) or c) 10 µM ergosterol for 30 min at 30°C, washed and incubated with whole cell lysates derived from strains expressing TAP-tagged Pik1 in the presence or absence of 100µM DTBP for 1 h at 30°C. Whole cell lysates (input) and bound fraction were resolved by SDS-PAGE and Pik1-TAP chimera was detected by Western blotting.

incubated with the cross-linker, but Pik1-TAP but not with GST alone with or without DTBP.

Recent work involving Osh6 and Osh7 demonstrated that the interaction with Vps4 that was abolished when Osh6 and Osh7 were pre-loaded with ergosterol (Wang et al., 2005a). To investigate if ergosterol altered the interaction between Kes1 and Pik1, purified GST-Kes1 was immobilized on glutathione Sepharose and blocked with 3% BSA. GST-Kes1 was incubated for 30 minutes in the presence of 10 μM ergosterol (dissoleved in 10% ethanol). Cell extracts were produced from the GFY220 (*PIK1:TAP:HIS3 kes1::*KAN^R) and incubated with GST or GST-Kes1 for 1 hour at room temperature with or without 100 μM DTBP. Figure 52c is a western blot using the anti-TAP antibody. Pik1-TAP interacting with GST-Kes1, both in the presence and absence of cross-linker, but Pik1-TAP did not interact with GST (Figure 52c). It appears that ergosterol-binding by Kes1 strengthens the interaction between Kes1 and Pik1.

4.2.15. *In vitro* Pik1 ASSAY

Pik1 produced by an *in vitro* transcription-translation reaction was tested for *in vitro* Pik1 kinase activty. Pik1 activity was easily detected using PI-Triton X-100 mixed-micelles and increasing amounts of *in vitro* transcription-translation reaction product increased the rate of radiolabel incorporation (the linear range being found when using 1 to 2 μL of reaction product) Figure 53. Assuming that Pik1 purified from yeast (Flanagan and Thorner, 1992) and produced by *in vitro* transcription/translation have the same kinetics then 2μL of reaction mix contained ~50 picomoles of Pik1. The addition of 400 picomoles (30μg) of GST-Kes1 to the Pik1 assay had no effect on product formation (Figure 53).

An alternative way to determine if Kes1 can inhibit Pik1 activity was to produce cells extracts from wild type and *kes1* strains and assay for Pik1 activity. Vps34 (the yeast PI 3-kinase) activity is inhibited by Triton X-100 (Schu et al., 1993). PI-Triton X-100 mixed micelles are a poor substrate for Stt4 (Cutler et al., 1997). Wild-type extract and *kes1* protein extracts (34 μg) were assayed for Pik1 activity. Figure 54 shows that extracts from the *kes1* cells have 100% of the activity of a wild-type extract. If Kes1 is able to inhibit Pik1, the extracts from the *kes1* strain should have more product formation

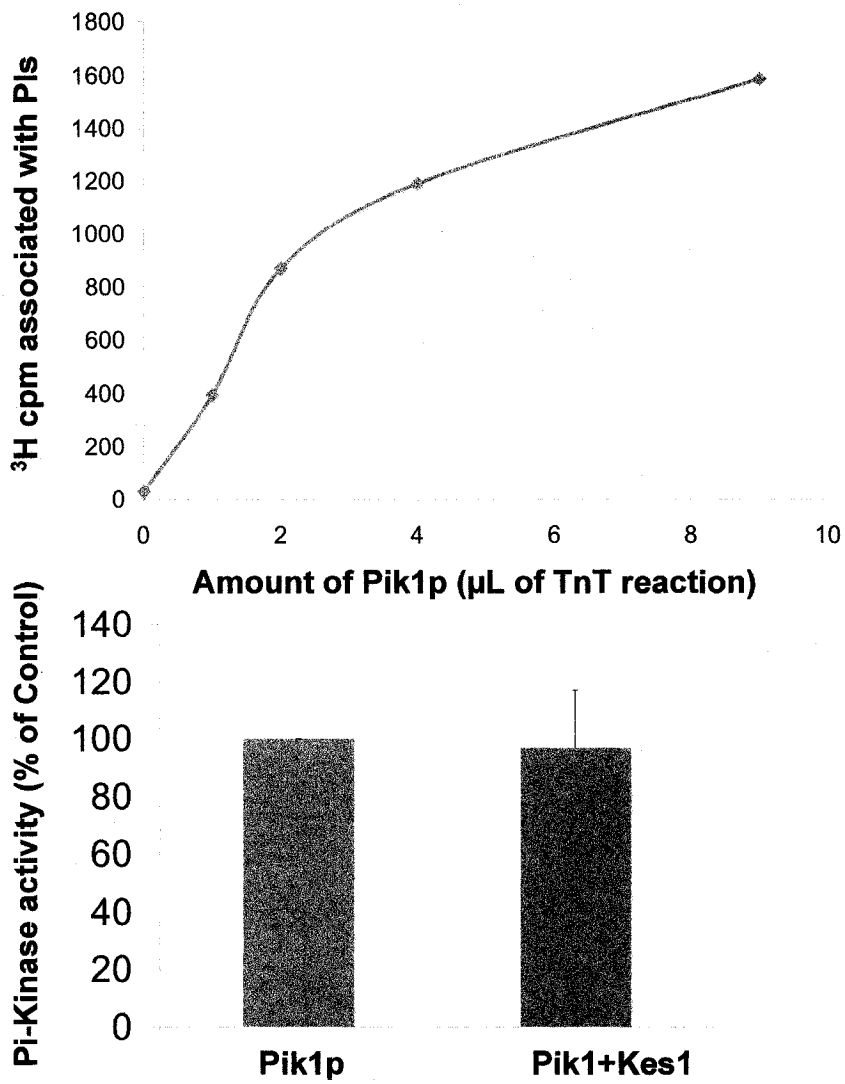


Figure 53. Pik1 is not inhibited by recombinant Kes1 *in vitro*. a) Pik1 was produced by *in vitro* transcription coupled translation. Increasing amounts of transcription/translation mix were added to the PI-4 kianse assay mix and incubated for 20 min at 34°C. Reactions were terminated and the organic phase was subjected to thin layer chromatography and counts determined by TLC plate scanner. b) *in vitro* activity of Pik1 in response to the addition of GST-Kes1. Basal level was set to 100%, and activity upon the addition of GST-Kes1p was measured. Each condition represents averaged data from three independent experiments (mean ± S.E.M.)

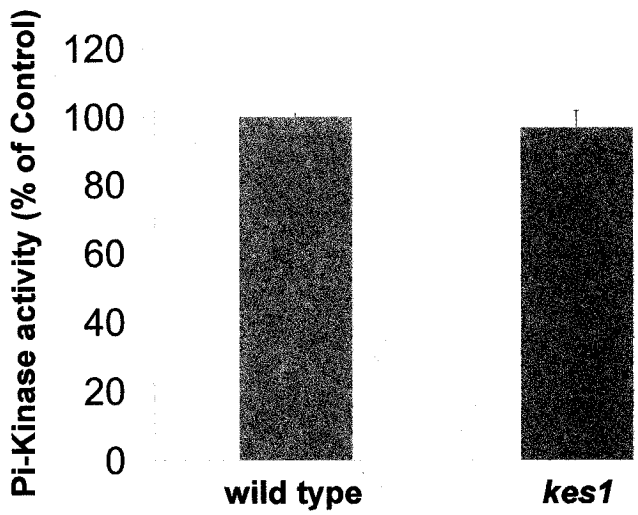


Figure 54. The presence of Kes1 has no effects on the PI 4-kinase activity in *S. cerevisiae.* Cell extracts from the indicated cells were prepared from BY4741 or GFY217 (*kes1*) at the exponential phase of growth and assayed for PI 4-Kinase activity using Triton X-100/Phosphatidylinositol mixed micelles as substrates. Note Triton X-100 inhibits the yeast PI 3-Kinase, Vps34, and the activity measured above is not inhibited by wortmannin, an inhibitor of the other prominent yeast PI 4-kinase Stt4. The data is the average of three separate experiments ± S.E.M.

which is does not. *In vivo* analysis of phosphoinositide metabolism suggests Kes1 inhibits Pik1, however this is not reconstituted using PI-Triton X-100 mixed micelles.

4.2.16. MODEL OF Kes1 INHIBITORY ACTION (Figure 55)

Kes1 is recruited to the Golgi at least in part by PI-4P, and *in vitro* Kes1 can bind to PI-4P (or PI-4,5P_2) by a number of different assays (Fairn and McMaster, 2005a; Knodler and Mayinger, 2005; Li et al., 2002). *In vivo* analysis of phosphoinositides demonstrates that Kes1 can negatively regulate Pik1 activity. As such, due to its ability to bind PI-4P, Kes1 may be a diffusible sensor and feedback inhibitor of Pik1. Kes1 is also an abundant protein with ~32,000 molecules/cell, and the estimated Golgi localized PI-4P pool in the cells is in the range of 25,000-50,000 molecules/cell. Therefore, with Kes1 localization dependant upon PI-4P, at any given time a significant portion of the Golgi resident PI-4P could be bound by Kes1. In situations where Golgi PI-4P is limiting (i.e. *pik1^{ts}, frq1^{ts} or sec14^{ts}* cells) pro-secretory factors may not be recruited/activated to initiate secretion from the *trans*-Golgi. Genetic evidence suggest that the TRAPPII complex and Ypt31 are downstream of PI-4P, with TRAPPII possibly being a direct effector of PI-4P. Figure 55 highlights the co-ordinated efforts of Pik1, Frq1 and Sec14 in the production of PI-4P, with Kes1 inhibiting Pik1 and binding and restricting the availability to PI-4P.

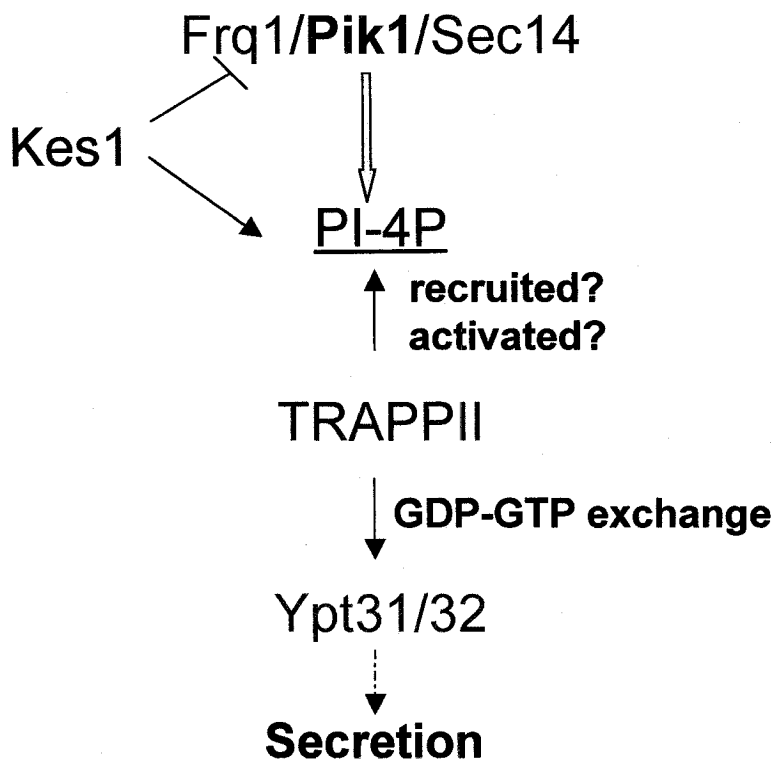


Figure 55. Kes1 is a negative regulator of Pik1. Pik1 and components of the TRAPPII complex are truly essential genes (coloured green). The TRAPPII complex is a GEF for the Rab-like proteins Ypt31 and Ypt32, whose activity is required for secretion. Sec14 and Frq1 are quasi-essential proteins (coloured blue) and are both positive regulators of Pik1 activity. The essential nature of *FRQ1* and *SEC14* is bypassed by inactivation of *KES1*. Kes1 is able to bind PI-4P and in cells lacking a functional Kes1, PI-4P levels are increased.

4.3 ArfGAPs ARE DOWNSTREAM OF DIACYLGLYCEROL

As previously described, the ArfGAPs (Gcs1 and Age2) are downstream of Sec14 and appear to be effectors of DAG based signaling. Gcs1 functions in retrograde transport from the Golgi to the ER along with Glo3 (Poon et al., 1999). Gcs1 also functions in anterograde transport from the Golgi along with Age2 (Poon et al., 2001). Gcs1-3 is an unstable protein at elevated temperatures, while Gcs1-28 is defective in retrograde transport. The Gcs1-4 protein is defective in transport from the *trans*-Golgi or post-Golgi trafficking at the non-permissive temperature. A high-copy suppresser screen was performed by Tania Wong (Johnston Lab at Dalhousie University) and identified *SFH2* as a suppressor of *gcs1-4^{ts} age2* cells (Wong et al., 2005). As previously mentioned in the Introduction, the *SFH* family is thought to be required for the optimal Spo14 activity (Li et al., 2000; Routt et al., 2005). While increased expression of three other Sfh proteins (Sfh3-5) could partially rescue the *gcs1-4^{ts} age2* cells, Sfh2 provided a more robust rate of growth. *In vitro* analysis of Gcs1 ArfGAP activity suggests that both PA and DAG stimulate Gcs1 activity (Yanagisawa et al., 2002). This raises the possibility that alteration in lipid composition may suppress defects associated with *gcs1-4^{ts} age2* cells.

4.3.1. CELL GROWTH OF CELLS CONTAINING A REDUCTION IN ArfGAP ACTIVITY

Cells of the strains *gcs1-4^{ts} age2, gcs1-4^{ts} age2 GCS1* and *gcs1-3^{ts} age2* were grown to mid-logarithmic phase in selective medium at 25°C, harvested, serially diluted and spotted on minimal solid medium containing 50 μM di8:0 DAG. Figure 56a shows that cells contain only the *gcs1-4* gene as its only source of Gcs1 grows in the presence of di8:0 DAG. The short chain DAG is not bypassing the requirement for Gcs1 as the *gcs1-3* containing cells are not rescued by the addition of short chain DAG (Wong et al., 2005).

To determine if the Sfh proteins are suppressing the Gcs1-4 defects by increasing Spo14 activity, the ability of increased Sfh2 to suppress growth defects in *gcs1-4 age2* cells was assessed by disrupting *SPO14*, the sole PC-PLD in yeast. The *gcs1-4 age2 spo14, gcs1-4 age2 spo14 GCS1* and *gcs1-4 age2 spo14 SFH2* cells were grown to mid-

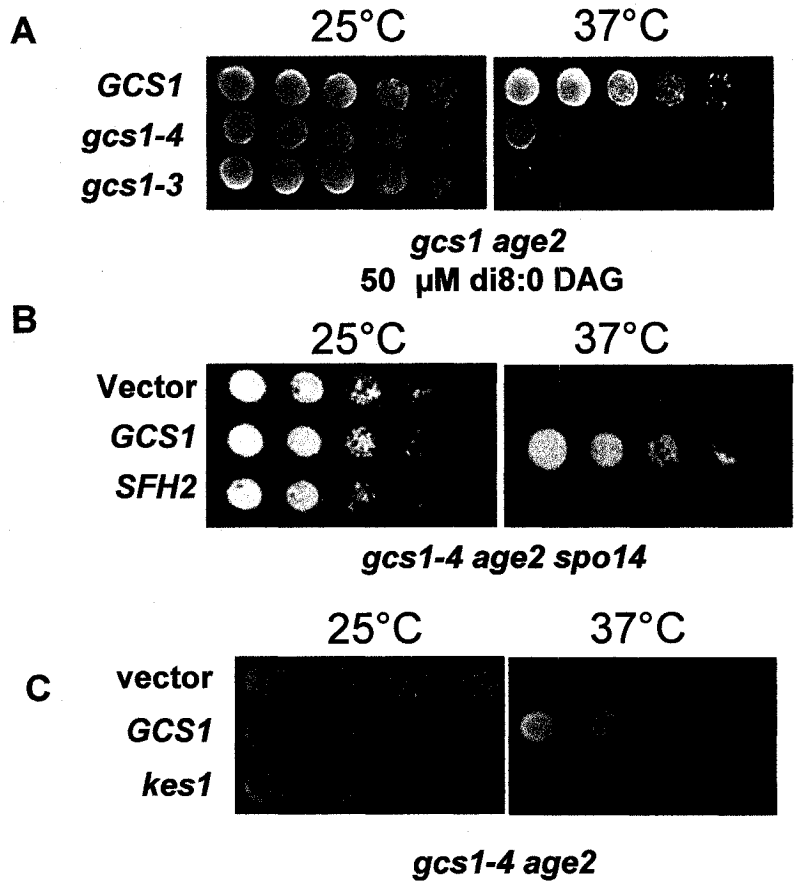


Figure 56. Gcs1-4 temperature sensitivity is relieved by short-chain DAG and increased Spo14 activity, but not by inactivation of *KES1*. a) Ten-fold serial dilutions with the genotypes below the panel and carrying the plasmids indicated on the left side were grown in the presence of 50 µM di8:0 DAG. b) the *ade2 gcs1-4 spo14* strain was transformed with the indicated plasmid, and 10-fold serial dilutions were spotted solid medium, and were incubated at either the indicated temperatures. c) 10-fold serial dilutions of *gcs1-4 age2* cells transformed with a plasmid carrying a wild-type *GCS1* or empty vector were used for comparison to the a *gcs1-4 age2 kes1* strain.

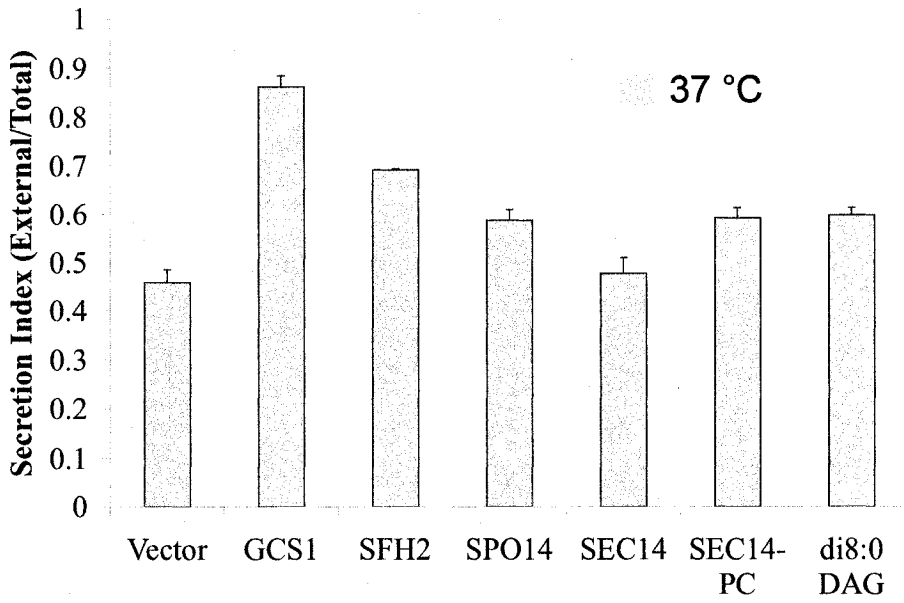


Figure 57. Exogenous DAG or increased expression of *SFH2, SPO14,* or *sec14-PC* alleviates transport defects in *gcs1-4 age2* cells. Ratios of secreted invertase to total invertase after 2 h at 37°C were determined for *gcs1-4 age2* cells harboring the indicated genes on high-copy plasmids and also for *gcs1-4 age2* cells growing in the presence of di8:0 DAG. Data are mean +/- S.E.M. of three separate experiments, each performed in triplicate.

logarithmic phase in selective medium, harvested, serially diluted, spotted on selective solid medium and incubated at 25°C or 37°C (Figure 56b). Genomic replacement of *SPO14* did not impair growth at the permissive temperature, however, increased levels of Sfh2 at the non-permissive temperature no longer suppressed *gcs1-4^{ts}* cells lacking *SPO14* (Wong et al., 2005). In addition to increased levels of Sfh proteins, and di8:0 DAG, increased expression of *SPO14* also suppressed growth defects associated with *gcs1-4^{ts} age2* cells. The results are consistent with increases in PA and/or DAG suppress the growth defects associated with cells containing enfeebled ArfGAP activity at the *trans*-Golgi.

Previous reports have suggested that Kes1 a) acts as an inhibitor of Spo14, and; b) may influence the Arf cycle possibly by regulating ArfGAPs (Li et al., 2002). Earlier evidence was presented suggesting that the target of Kes1 was Pik1 and PI-4P. To investigate the ability of Kes1 to regulate Spo14 and the Arf cycle, the genomic copy of *KES1* was replaced in the *gcs1-4^{ts} age2* strain. Cells were grown to mid-logarithmic phase, harvested, serially diluted, and spotted on minimal solid medium and placed at 25°C or 37°C. Figure 56c shows that replacement of *KES1* does not suppress the growth defects of *gcs1-4^{ts} age2* at the non-permissive temperature.

4.3.2. INVERTASE SECRETION

Increased expression of *SFHs, sec14$^{(K66,239A)}$, SPO14* and the addition of di8:0 DAG were able to suppress the growth defects associated with *gcs1-4^{ts} age2* strains (Figure 56 and Wong et al., 2005). Figure 57 shows that situations that suppress the growth defects of *gcs1-4^{ts} age2* also restore the secretion efficiency of invertase at the non-permissive temperature for the *gcs1-4^{ts}* allele (Wong et al., 2005).

4.3.3. RANDOM SPORE ANALYSIS

Previously it was mentioned, the intentional construction of Sec14 bypass strains (*sec14Δ*) lacking *GCS1, AGE2* or *ARF1* were viable at 30°C but not 37°C. Flux through the CDP-choline pathway is increased approximately 2-fold when cells are shifted from 30°C to 37°C. Previously, it was reported that Sec14 bypass strains (*sec14^{ts}*) lacking *GCS1, AGE2* and *ARF1* were not viable at 37°C (Yanagisawa et al., 2002). To

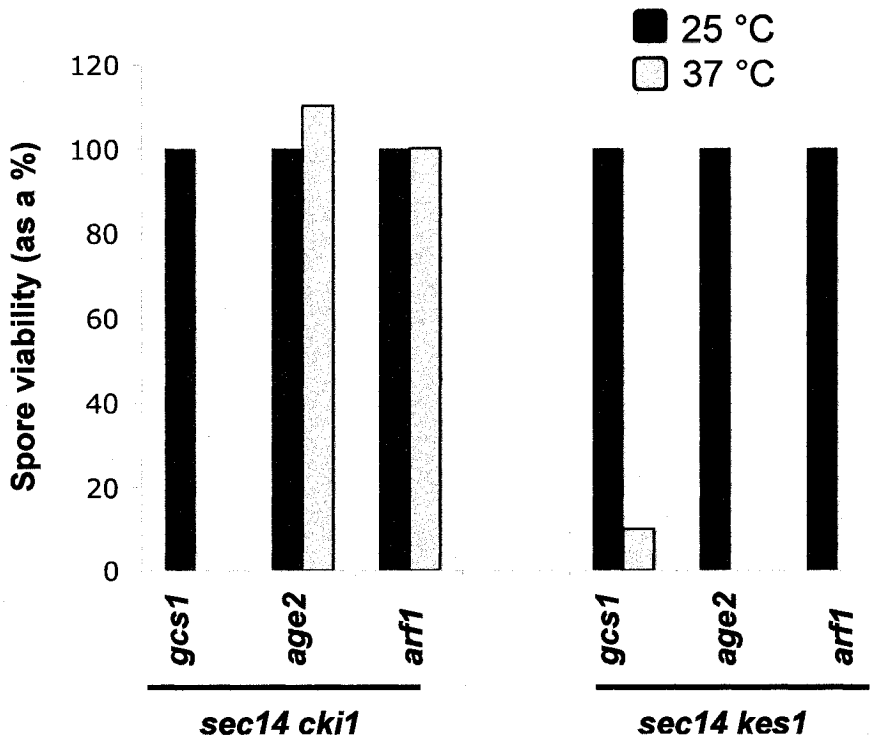


Figure 58. Arf1 and ArfGAP activity are required by Sec14-bypass cells at elevated temperatures. Cells of strains CMY212 (*sec14 cki1)* and CMY213 (*sec14 kes1*) were mated with the single deletion mutants indicated. Random spore analysis was performed, with spore plates incubated at either 25°C or 37 °C for 72 hours.

investigate further, random spore analysis was performed at 30°C and 37°C using the following heterozygous diploids cells; *SEC14/sec14::LEU2 CKI1/cki1::*NAT^R *GCS1/gcs1::*KAN^R, *SEC14/sec14::LEU2 KES1/kes1::*NAT^R *GCS1/gcs1::*KAN^R, *SEC14/sec14::LEU2 CKI1/cki1::*NAT^R *AGE2/age2::*KAN^R, *SEC14/sec14::LEU2 KES1/kes1::*NAT^R *AGE2/age2::*KAN^R, *SEC14/sec14::LEU2 CKI1/cki1::*NAT^R *ARF1/arf1::*KAN^R, and *SEC14/sec14::LEU2 CKI1/cki1::*NAT^R *ARF1/arf1::*KAN^R. The results are displayed in Figure 58 and show that Arf1 and ArfGAPs are required for the viability of Sec14 bypass strains, but only at elevated temperatures. This suggests that DAG may not be limiting in *sec14* cells at 30°C, but that it becomes limiting at elevated temperatures. If ablation of the CDP-choline pathway leads to the preservation of DAG pools in the Golgi, this may further stimulate secretion. Although previous results demonstrate that ablation of the CDP-choline pathway does not lead to an increase in total cellular DAG levels.

4.3.4. MODEL OF PC METABOLISM AND GOLGI-DERIVED SECRETION

Figure 59 is schematic representation of DAG consumption and the effects on ArfGAP activity. Phosphatidic acid is converted to DAG, and both DAG and PA have been shown to stimulate ArfGAP activity *in vitro* and increasing DAG appears to rescue an enfeebled ArfGAP, Gcs1-4. DAG is consumed in the production of PC through the CDP-choline pathway. The rate of synthesis through the CDP-choline increases two-fold when cells are growing at 37°C. Sec14 is thought to inhibit the rate-limiting step of the CDP-choline pathway when bound to PC. In this model, Sec14 is a diffusible sensor of PC content. In cells with a functional Sec14, Nte1 converts the majority of PC produced through the CDP-choline pathway to GPC and two fatty acids, whereas cells lacking a functional *SEC14,* Spo14 converts the PC to PA and choline. To date, the ability of free fatty acids in membranes to stimulate ArfGAP activity has not been assessed. Spo14, originally characterized as being essential for Sec14-independent growth is largely associated with the endosomes and partially with the plasma-membrane. Spo14 activity may function downstream of the Golgi and support *sec14* deficient cells by an indirect mechanism.

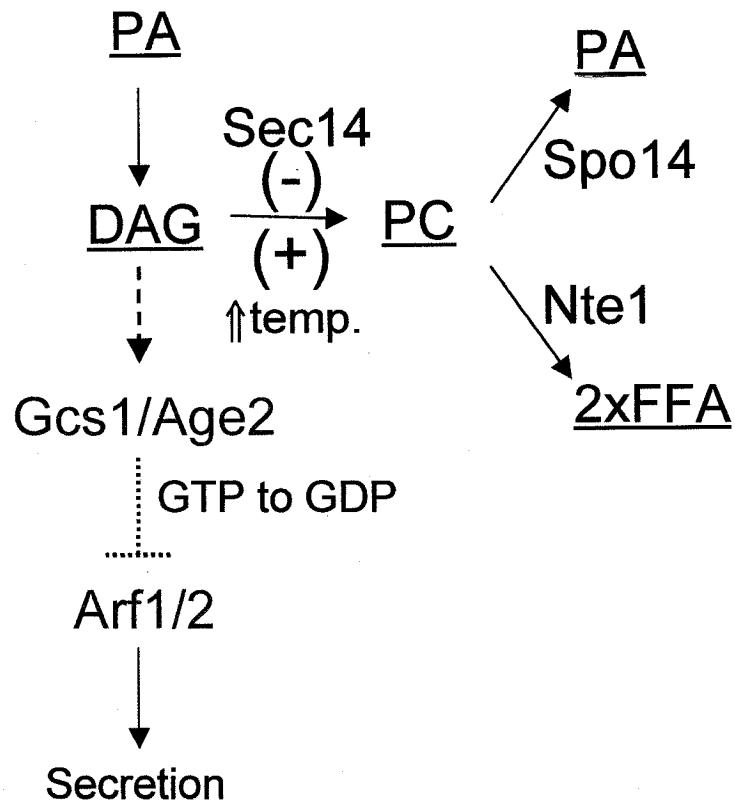


Figure 59. ArfGAPs are downstream from DAG in the Sec14 pathway. Lipids are underlined, note that DAG is consumed to a greater extent at elevated temperatures, while the presence of Sec14 inhibits the CDP-choline pathway. Both DAG and PA are known to stimulate the ArfGAP activity of Gcs1 and Age2. To date it is unknown if fatty acids have any effect on ArfGAP activity. Essential pairs are shown in purple. ArfGAP activity converts GTP-bound Arf1 to a GDP-bound, which appears to inactivate Arf1, is essential for secretion from the *trans*-Golgi.

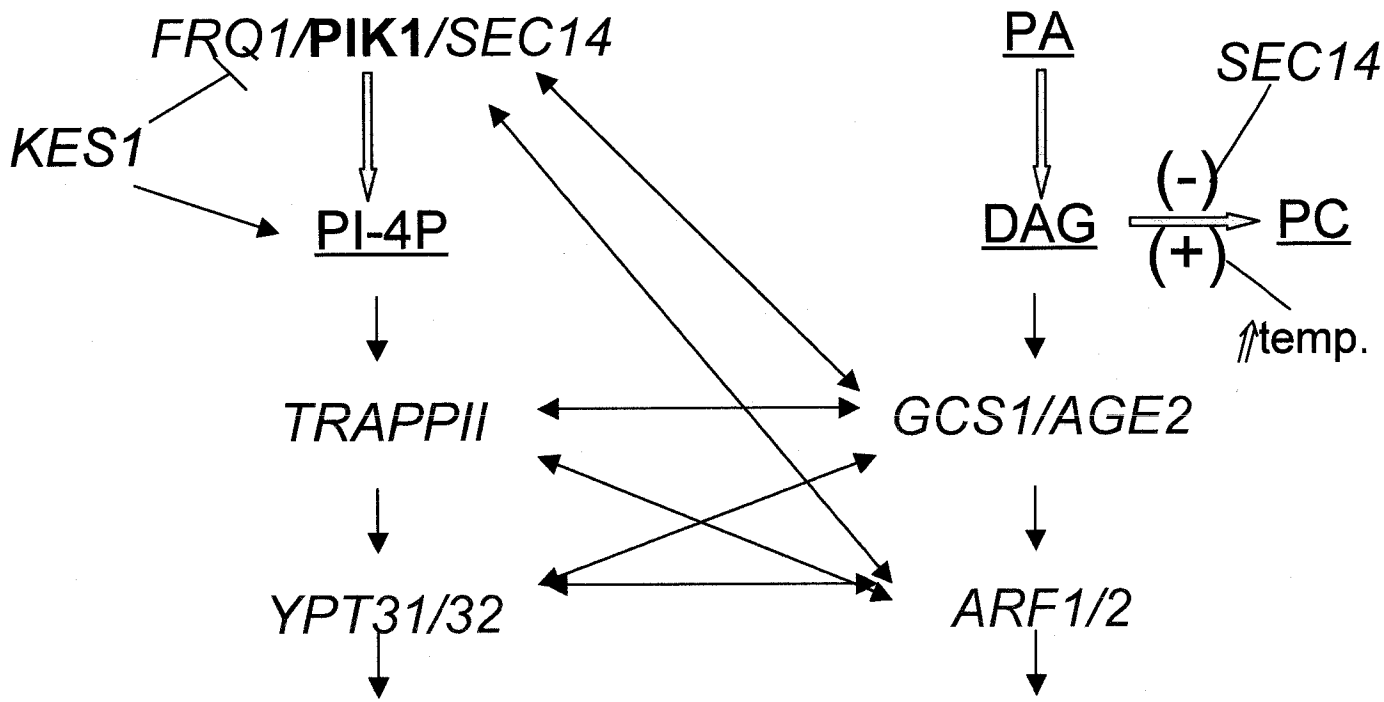


Figure 60. Genetic interaction map for *SEC14* in transport from the *trans*-Golgi. Genes in green represent true essential genes, genes in purple represent essential pairs and genes in blue represent quasi-essential genes. Double headed arrows represent synthetic lethal interactions. Lipids relevant to the Sec14 pathway are underlined, with metabolic steps represented by the blue arrow. Single arrow heads represents stimulatory action or recruitment. Kes1 appears to be a sensor of Golgi PI-4P and feedback inhibits its production.

CHAPTER 5. RESULTS - CHARACTERIZATION OF INDIVIDUAL *OSH* MUTANTS

5.1.1. METABOLIC ANALYSIS OF PC SYNTHESIS THROUGH THE CDP-CHOLINE PATHWAY

Wild-type and *osh1Δ-6Δ* cells were grown to mid-logarithmic phase in 1 μM choline, grown in the presence of [^{14}C]choline for 1 hour at 30°C, and lipids were extracted and counted. To correct for any differences in the total cellular phospholipid content, lipid phosphorous was used to normalize the data. Figure 60 shows that none of the single *osh* mutants tested had any significant alterations in choline incorporation into PC.

5.1.2. METABOLIC ANALYSIS OF SPHINGOLIPID SYNTHESIS

The synthesis of sphingolipids in yeast starts with the formation of ceramide in the ER from serine and fatty acids in the form of acyl-CoA. Ceramide must be transported from the ER to the Golgi for the additional steps in sphingolipid synthesis. Once in the Golgi, ceramide and PI are used as substrates by the enzyme, IPC synthase, to produce inositolphosphoceramide (IPC) and DAG. The IPC and GDP-mannose can be used as substrates by a family of enzymes to produce mannose-inositol-phosphoceramide (MIPC). Finally, MIPC and PI can be used as substrates by Ipt1 (inositolphosphotransferase) to produce mannose-(inositol-P)$_2$-ceramide (M(IP)$_2$C) (Dickson et al., 2006).

Sphingolipid metabolism depends upon the non-vesicular transport of ceramide from the ER to the Golgi apparatus (Funakoshi et al., 2000; Funato and Riezman, 2001). Recently, in mammalian cells the protein responsible for this transport was identified and termed CERT (ceramide transport) (Hanada et al., 2003). CERT is largely comprised of two modular protein domains, a PH domain and a START domain, along with a small protein motif used for targeting protein-protein interactions, the FFAT motif. The yeast genome may contain one START domain containing protein, however to date has no described role in sphingolipid metabolism (Barros et al., 2005). Osh1-3 proteins contain a lipid binding domain (the OSBP related domain), a PH domain, and a FFAT motif and may be candidates for transferring ceramide *in vivo*. To investigate further wild-type and

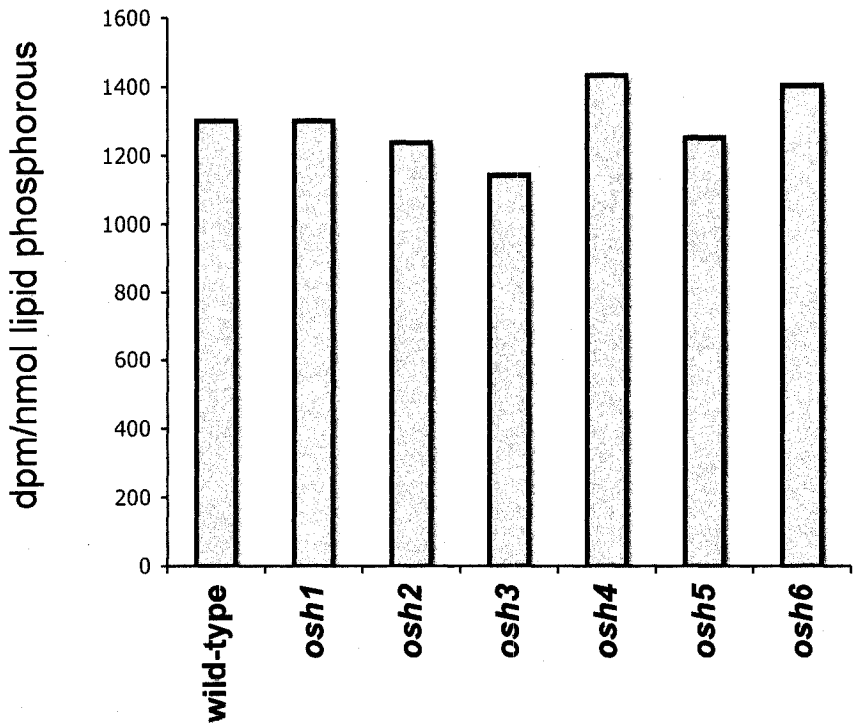


Figure 61. PC synthesis through the CDP-choline pathway in *osh* mutants. Wild-type and *osh* mutant cells were grown to log phase at 30°C in minimal medium supplemented with 10 μM choline, harvested and incubated in the presence of [^{14}C]choline for 1 h. Lipids were then extracted and quantified by scintillation counting. Values represents the incorporation of radiolabelled choline corrected for the total amount of phospholipid.

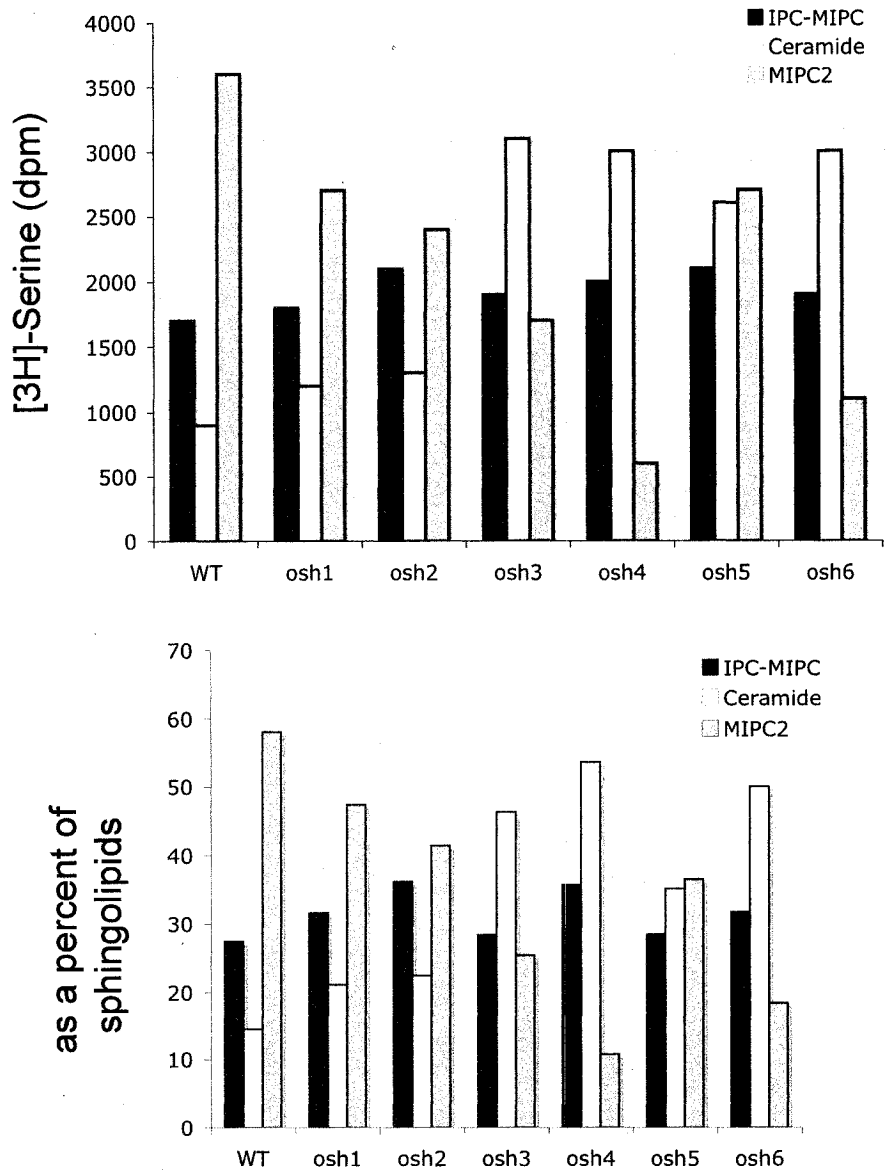


Figure 62. Serine labeling of yeast cells. Wild-type and *osh* mutant cells were grown to log phase at 30°C in minimal medium supplemented with [^{3}H]serine as described in the methods. Lipids were extracted, and base hydrolyzed to separate the sphingolipids. Sphingolipids were resolved by TLC, scraped and quantified by scintillation counting. Values are shown as total radiolabel, and a a percent of radiolabel associated with the sphingolipid pool.

osh1, osh2, osh3, kes1/osh4, osh5, osh6 cells were labeled with [^{3}H] serine for 30 minutes to monitor the incorporation into sphingolipids. Sphingolipids were separated from phospholipids by base hydrolysis (NaOH), sphingolipids were separated by TLC, lipids were scraped into vials and radioactivity determined by scintillation counting. The results are presented in Figure 61. In wild type cells ~ 60% of the label was associated with $M(IP)_2C$, ~15% with ceramide and ~25% in IPC-MIPC. The *osh1* and *osh2* strains showed only a minor alteration in the distribution of label, while *osh3, osh4, osh5* and *osh6* cells showed increased ceramide labelling, mainly at the expensive of $M(IP)_2C$. While the conversion of ceramide to IPC is not completely blocked in any of the single *osh* mutants tested, the synthesis of complex sphingolipids appears to be altered in cells lacking certain Osh proteins. These results are preliminary in nature and the significance of these results are still unclear.

5.1.3. SUSCEPTIBILITY TO LATRUNCULIN B

Characterization of a yeast cells lacking all the Osh proteins genes indicated a defect in endocytosis and sterol distribution (Beh and Rine, 2004). During the course of my investigations it was published that Osh proteins were also involved in promoting cell polarity established by the small Rho-like G-protein Cdc42 (Kozminski et al., 2006b). Due to the previous observation of defects in endocytosis in the Osh deficient strain, the sensitivity of single *osh* mutants to latrunculin B (Lat-B) was investigated. Wild-type and *osh1Δ - 6Δ* single mutant cells were grown to mid-logarithmic phase, mixed with liquefied agar and poured onto YEPD agar medium. Round disks containing increasing concentrations of Lat-B was placed on the cells, and the plates were incubated until a lawn of cells appeared. The width of the halo was measured and is displayed in Figure 62. The results show that *osh3* and *osh5* mutant cells are more sensitive to Lat-B then wild-type cells or the other *osh* single mutant cells. Thus, the function of Osh3 and Osh5 appears to directly or indirectly effect actin polymerization.

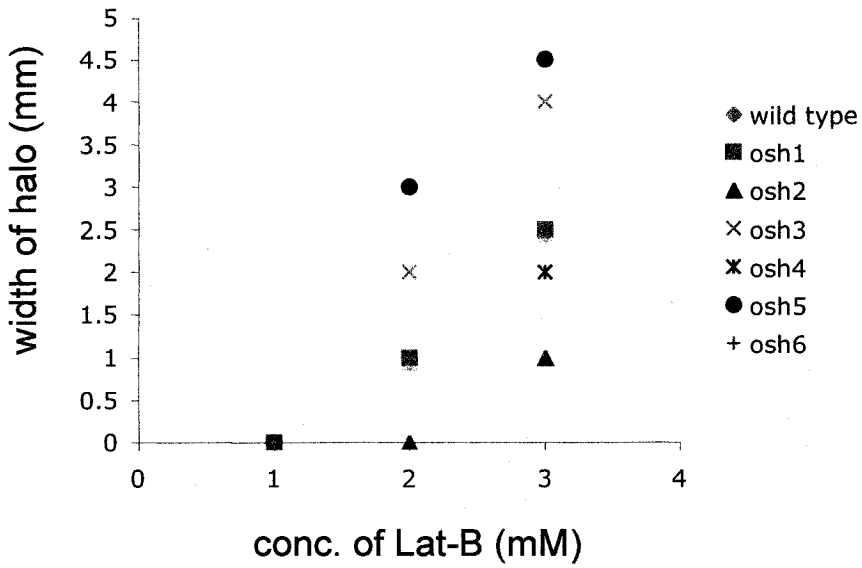


Figure 63. Sensitivity of *osh* mutants to Latrunculin B. Halo assays were used to access the sensitivity of wild-type and *osh* mutant cells to the indicated concentration of Lat-B. Briefly, 10 μL of mid-logarithmic phase cells were added to 2 ml of 2xYPD medium after which 2 ml of 2% of warm low-melting point agarose was added to the cells and the mixture was poured onto the surface of YPD solid medium. Lat-B was diluted in DMSO and 11μL of vehicle or the indicated concentration of Lat-B was spotted onto 10 mm filter disks, which were placed onto the top agar. Plates were incubated at 30°C until a lawn appeared.

CHAPTER 6. RESULTS - CHARACTERIZATION OF SELECT HUMAN HOMOLOGUES OF *KES1*

Previous research in the McMaster lab demonstrated that human ORP1S could phenocopy *KES1* and inhibit *kes1*-mediated Sec14 bypass (Xu et al., 2001). Expression of ORP1S in *sec14^{ts} kes1* cells restored inhibition of CPY trafficking to the vacuole and prevented cell growth at the non-permissive temperature for the *sec14^{ts}* allele.

6.1.1. ORP9S BUT NOT ORP10S CAN PHENOCOPY *KES1* (FAIRN AND MCMASTER, 2005b)

CMY136 (*sec14^{ts} kes1::HIS3*) were transformed with pESC-ura, pESC-ORP9S or pESC-ORP10S, grown in minimal medium containing galactose to mid-logarithmic phase, harvested and cell lysates were produced. Equal protein mass was resolved by SDS-PAGE, transferred to PVDF and probe with an anti-myc antibody. Figure 63a shows human ORP9S and ORP10S are expressed at the predicted size in yeast with minimal degradation.

To investigate the ability of ORP9S and ORP10S to phenocopy *KES1*, CMY136 (*sec14^{ts} kes1*) cells transformed with pESC-ura, pESC-ORP9S or pESC-ORP10S, were grown in minimal medium containing galactose to mid-logarithmic phase, harvested, serially diluted and spotted on minimal solid medium containing galactose as the sole carbon source and where incubated at 25°C or 37°C. Figure 63b shows that ORP9S inhibits *kes1*-mediated Sec14 bypass, whereas ORP10S does not. Expression of ORP9S is not toxic to yeast as the re-introduction of *SEC14* on a plasmid is able to restore growth (figure 63b).

The inhibition of growth of *sec14^{ts} kes1* cells at the non-permissive temperature by ORP9S is also evident in the secretion efficiency of invertase. CMY136 cells were transformed with pDB31 (from Randy Schelman, containing *pTPI-SUC2*) and either pESC-trp, pESC-trp-ORP9S or pESC-trp-ORP10S, grown to mid-logarithmic phase in selective medium containing galactose as the sole carbon source, washed and shifted to 37°C. ORP9S expression reduced the secretion efficiency of *sec14^{ts} kes1* cells to 72% from 85%. In this assay the baseline for *sec14^{ts}* cells at 37°C is 65%. Because the cells were grown in galactose, I used the constutively expressed plasmid-borne *SUC2*, this

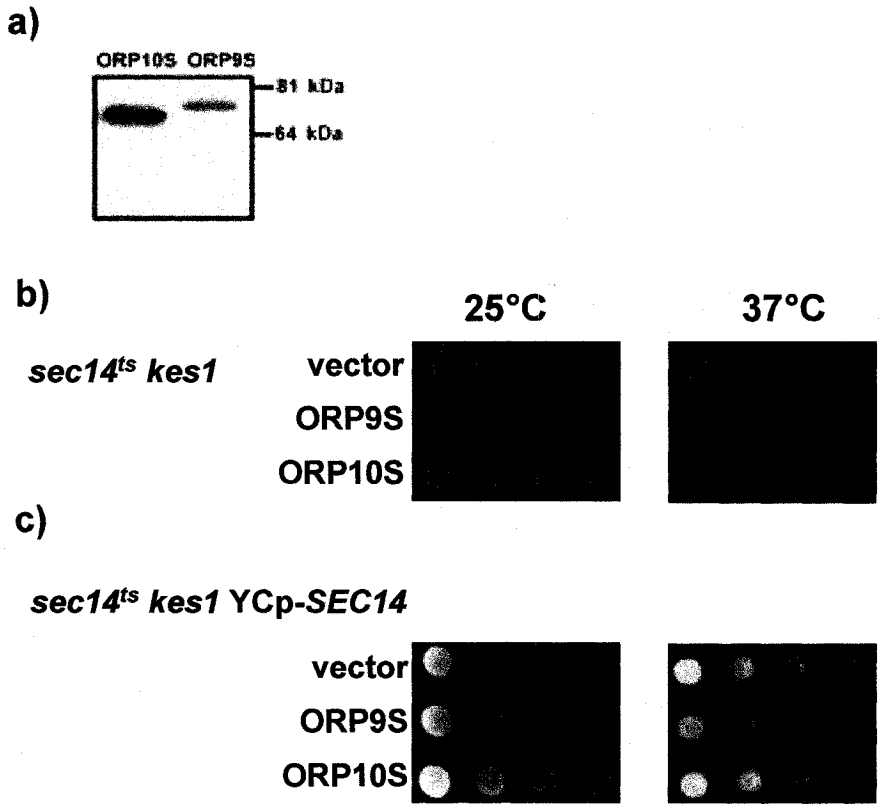


Figure 64. Human ORP9S can suppress *kes1*-mediated Sec14 bypass. Cells of the strain CMY136 (*sec14^{ts} kes1::HIS3*) was transformed with plasmids pESC-ura, or pESC-ORP9S, or pESC-ORP10S. Cells were grown in minimal medium containing raffinose and lacking uracil to mid-log phase. Cells were harvested, washed in minimal medium containing galactose, and equal cell concentrations were 10-fold serially diluted and spotted on minimal solid medium containing galactose and lacking uracil. Plates were incubated for 72 h at either 25°C or 37°C.

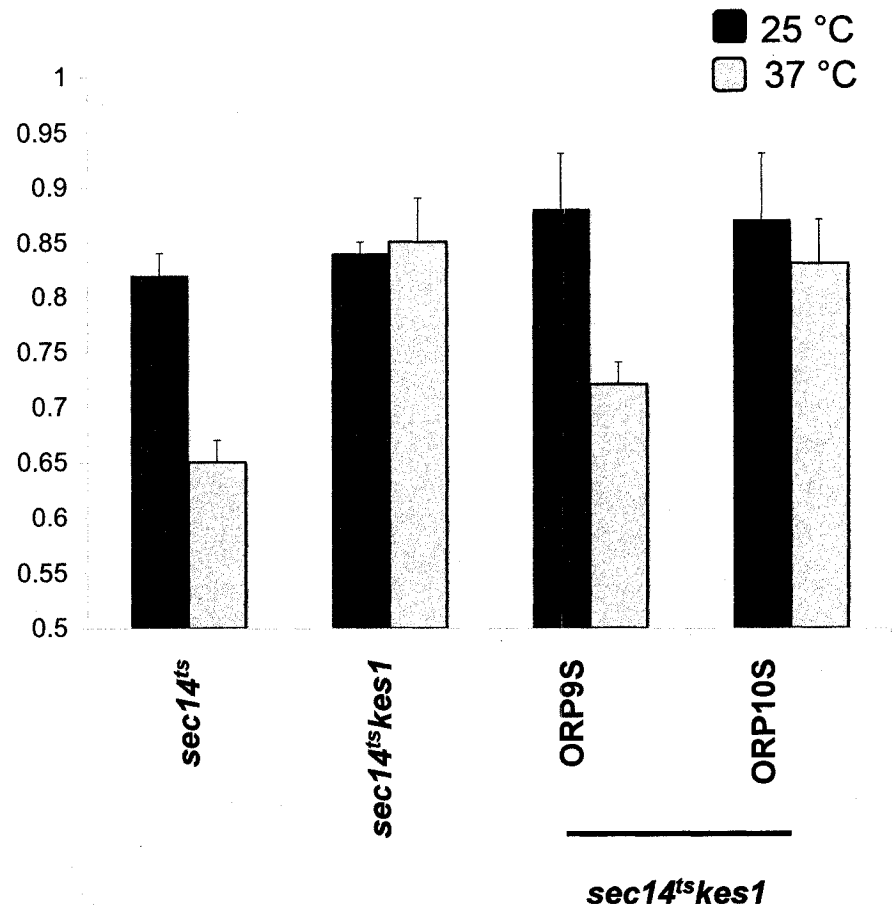


Figure 65. Human ORP9S inhibits the secretion of invertase in *sec14^{ts} kes1* cells. Cells of strain CMY136 (*sec14^{ts} kes1::HIS3*) were transformed with either plasmid pESC-trp, or pESC-ORP9S, or pESC-ORP10S as well as pDB31 (*SUC2*). Cells were grown to mid-logarithmic phase in minimal medium lacking uracil and tryptophan, harvested and washed. Cells were partitioned and incubated for 2 h at 25°C or 37°C. The secretion index was determined by measuring the activity of the extracellular invertase compared to the total.

caused a shift in the baseline for our secretion index. Cells expressing ORP10S had no significant decrease in invertase secretion efficiency (Figure 64).

6.1.2. ORP9S AND ORP10S BIND TO IMMOBILIZED PHOSPHOLIPIDS

BL21 (DE3) *E. coli* were used to express a 6xHIS-tagged ORP9S, which was solubilized from inclusion bodies by 8 M urea, bound to metal-affinity resin (Talon, Clontech) and the urea was washed away in step-wise fashion (8M to 0M in a 1M step-gradient). Figure 65a shows a Coomassie stained protein gel of the ORP9S from bacteria. ORP9S was greater than 95% pure. Purified ORP9S was incubated with immobilized phospholipids and subsequently detected using anti-T7 HRP conjugated antibody ECL detection. ORP9S appears to bind to monophosphorylated PIPs, PI-3,5P_2 and PI-4,5P_2.

BL21 DE3 *E. coli* were used to express a GST-ORP10S fusion protein, which was soluble and was purified using a glutathione-affinity resin. Figure 65b, shows a protein gel stained with Coomassie of the appropriate size of the predicted GST-ORP10S fusion. Purified GST-ORP10S was incubated with immobilized phospholipids and found to bind only to PI-3P.

6.1.3. EXPRESSION OF ORP1S AND ORP9S PHENOCOPY *KES1* IN *PIK1-83* CELLS

Previously it was demonstrated that ORP1S, but not ORP2, could phenocopy *KES1* in *sec14^{ts} kes1* cells, and I also demonstrated that ORP9S could also phenocopy *KES1*. To determine if human ORPs could also influence PI-4P signaling or Pik1 activity, human ORP1S, ORP2, ORP9S and ORP10S were transformed into cells of the GFY201 (*pik1-83^{ts} kes1*) strain. Cells were grown to mid-logarithmic phase, harvested, serially diluted, spotted on minimal solid medium and incubated at 25°C or 37°C. Figure 66 shows that Kes1 inhibited growth of cells of the GFY201 strain even at 25°C. ORP1S inhibited growth strongly at 37°C, while ORP9S inhibits to a lesser degree. Consistent with results seen with the cells of the CMY136 (*sec14^{ts} kes1*) strain, expression of ORP2 and ORP10S did not inhibit growth of cells of the GFY201 strain. The results suggest that the ability to regulate secretion is maintained in at least some of the human ORPs.

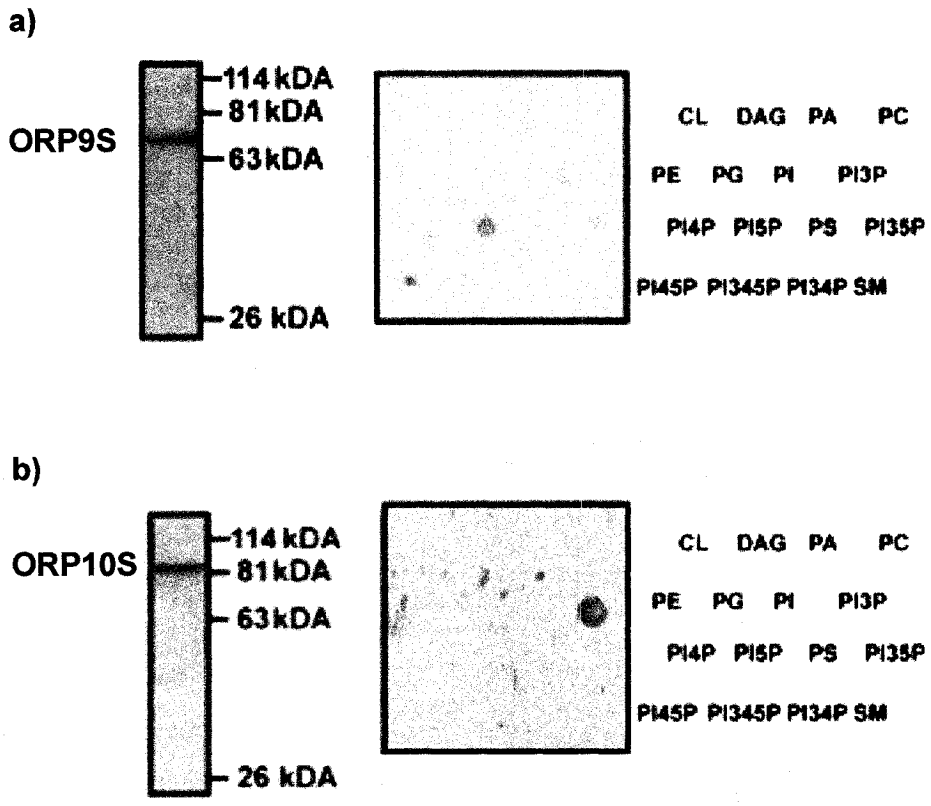


Figure 66. Purification and phospholipid binding of ORP9S and ORP10S. a) Recombinant T7-ORP9S-6xHIS fusion protein was expressed and purified from BL21(DE3) *E.coli.* SDS-PAGE and Coomassie staining reveal a single protein band correlating to ORP9S. Purified T7-ORP9S-6xHIS was incubated with phospholipids immobilzed on nitrocellulose and detected using an Anti-T7 antibody HRP conjugate. ORP9S binds to several of the phosphoinositides b) Recombinant GST-ORP10S fusion protein was expressed and purified from BL21 DE3 *E.coli.* SDS-PAGE and Coomassie staining reveal a single protein band correlating to ORP10S. Purified GST-ORP10S was incubated with phospholipids immobilzed on nitrocellulose and detected using an Anti-GST antibody HRP conjugate. ORP10S appears to only bind PI-3P.

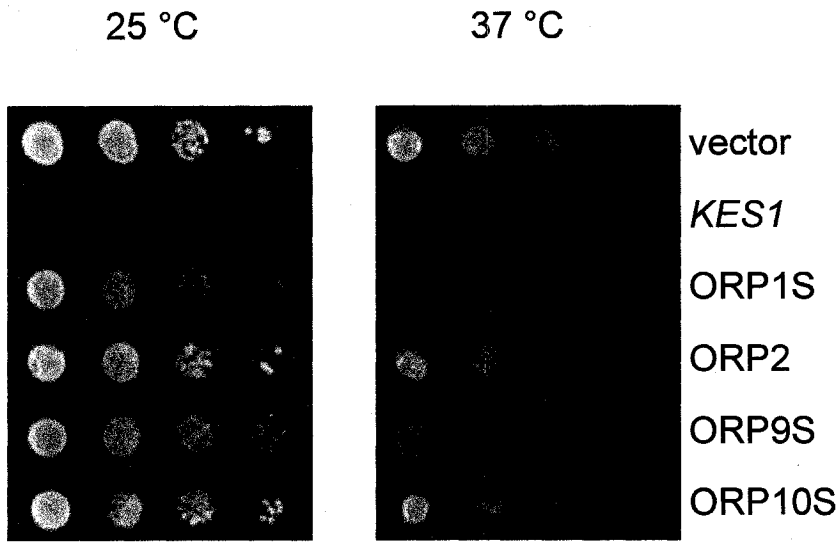


Figure 67. Human ORP1S and ORP9S can inhibit the growth of *pik1-83 kes1* cells. Cells of the strain GFY201 were transformed with plasmids pESC-ura or, pESC-KES1 or, pESC-ORP1S, 2, 9S, or 10S. Transformants were grown in minimal medium containing raffinose and lacking uracil to mid-log phase and harvested, and washed in minimal medium containing galactose, and equal cell concentrations were 10-fold serially diluted and spotted on minimal solid medium containing galactose and lacking uracil. Plates were incubated for 72 h at either 25°C or 37°C.

6.1.4. ORP1S CAN PROVIDE ESSENTIAL FUNCTION TO *OSH1Δ-OSH7Δ* CELLS

In addition to its role in regulating secretion, Kes1 can also provide an essential function that any of the Osh proteins can provide (Beh et al., 2001). Some of the human ORPs were transformed into cells of the CBY926 (*osh1Δ-osh7Δ, osh4-1^{ts}*) strain and transformants were grown at the non-permissive temperature for the *osh4-1^{ts}* allele. Figure 67 shows that of the ORPs only human ORP1S could provide this essential function to the Osh-deficient strain. This suggests that the overlapping essential function of the yeast Osh proteins may not be conserved by all their mammalian counterparts. The ability of ORP1S to correct specific cellular defects associated with the Osh-deficient yeast have yet to be investigated.

6.1.5. A PHOSPHOLIPID-BINDING FRAGMENT FROM ORP1S CAN INFLUENCE SECRETION (FAIRN and MCMASTER, 2005a)

The initial characterization of human ORP1S suggested that it bound to phospholipids and could phenocopy Kes1. To determine if phospholipid binding could be sufficient for phenocoping Kes1 the minimal phospholipid binding region of ORP1S was identified though the creation of deletion contructs. Figure 68 is a schematic representation of the 15 ORP1S constructs created to try and isolate the PA binding region. Subsequent studies have shown that Kes1 is a globular protein and it is likely that ORP1S has the same general shape. Furthermore, as seen in Figure 69, ORP1S binds to several phospholipids in addition to PA.

The sixteen constructs in Figure 69, were expressed as His6x-fusion proteins in BL21 DE3 *E. coli.* Any construct containing the amino acids 192-273 retained the ability to bind to anionic phospholipids whereas 1-176 and 324-437 had no or little ability to bind to phospholipids (Figure 70 a and b). GST-ORP1S was compared to GST-KES1 and GST-PH^{OSBP} in binding ability using immobilized phospholipids. ORP1S can phenocopy *KES1* in yeast as previously described, furthermore both proteins can bind to monophosphorylated PIPs and PI3,4-P (Figure 70).

Studies using the PH domains of FAPP1 and Osh2 showed that the ability to bind PI-4P located at the Golgi could inhibit grow of strains with compromised PI-4P

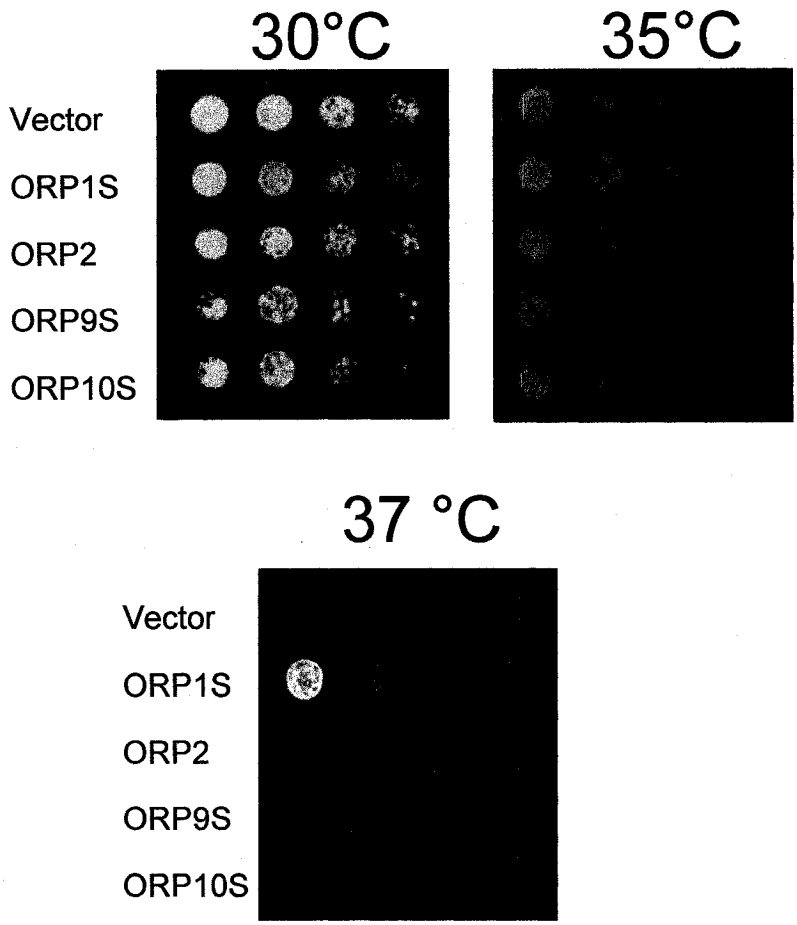


Figure 68. Expression of Human ORP1S suppresses growth defects associated with loss of Osh function. Cells of the strain CBY926 (*osh1*Δ-*7*Δ *osh4-1*^{ts}) were transformed with plasmids pESC-ura, or pESC-ORP1S or, ORP 2 or, ORP9S or, ORP10S. Transformants were grown in minimal medium containing galactose and lacking uracil to mid-log phase. Cells were harvested, washed in minimal medium containing galactose, and equal cell concentrations were 10-fold serially diluted and spotted on minimal solid medium containing galactose and lacking uracil. Cells were incubated for 72 h at the indicated temperature.

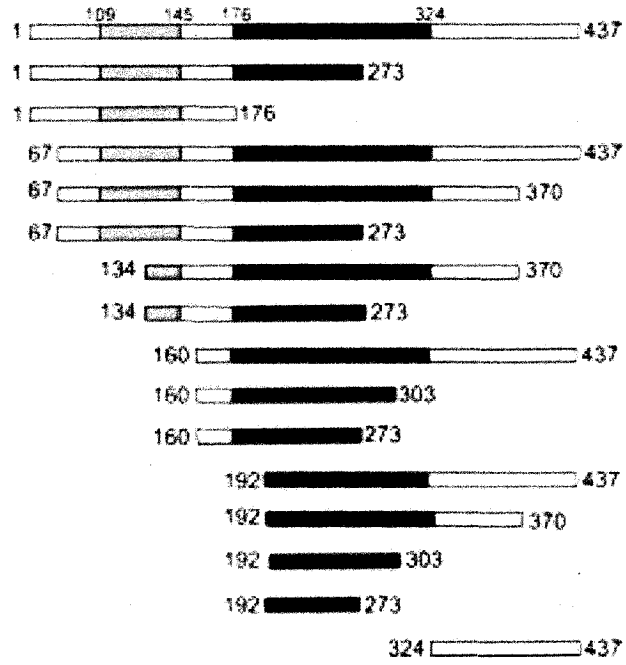


Figure 69. A schematic representation of the deletion constructs used to map a minimal phospholipid binding domain in ORP1S. Based on the primary sequence of ORP1S two regions of presumed importance have been highlighted. In grey, amino acids 109-145, encompasses the ORP signature motif. In black, 176-324 is the region of ORP1S that corresponds to the proposed PH domain of Kes1 (Li et al).

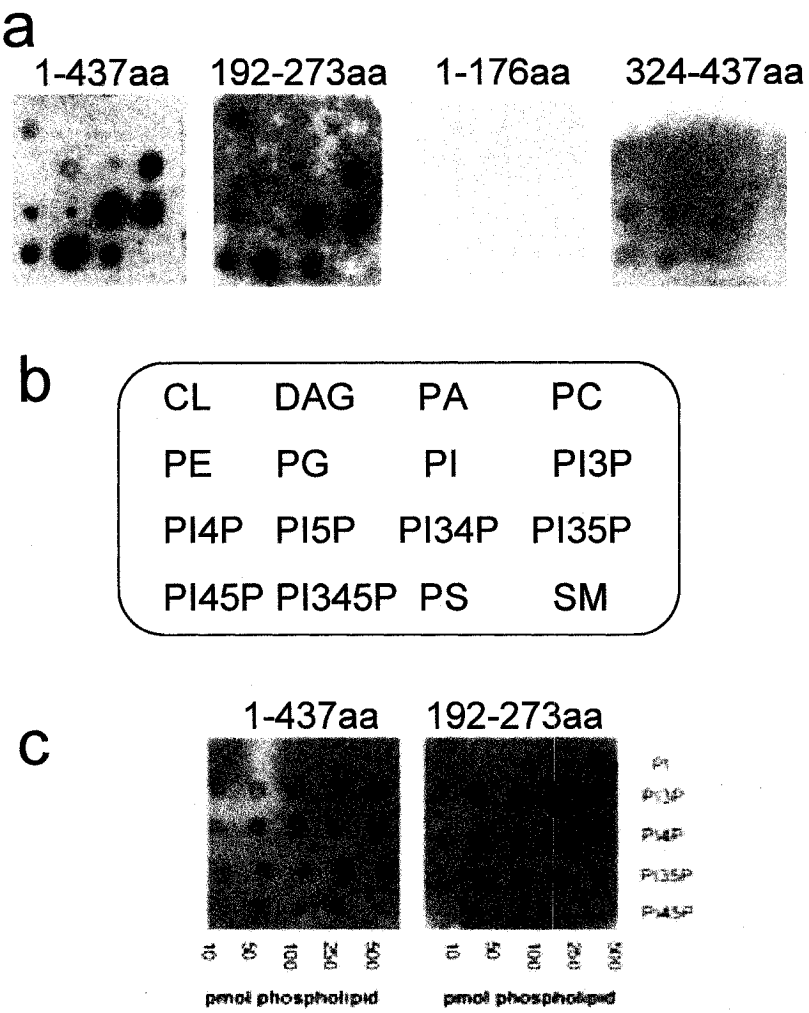
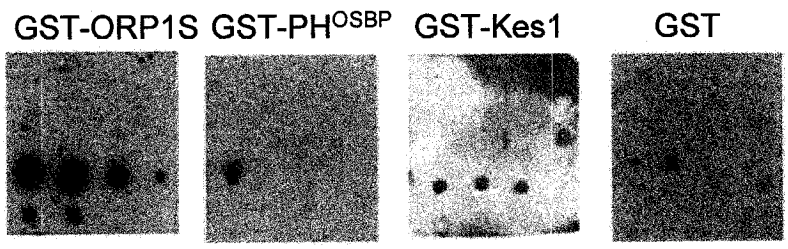


Figure 70. Full length ORP1S and amino acids 192-273 bind to phosphorylated phosphophatidylinositol. a) Purification of 6xHIS-tagged ORP1S fragments from their open reading frames in *E. coli* was by metal chelate resin. Purified ORPs were dialysed overnight against Tris-buffered saline at 4 °C, and fat Western lipid overlays were performed. b) A schematic of the position of phospholipids included on the membranes for this assay. c) Titration of binding for ORP1S to selected PIPs.

a



b

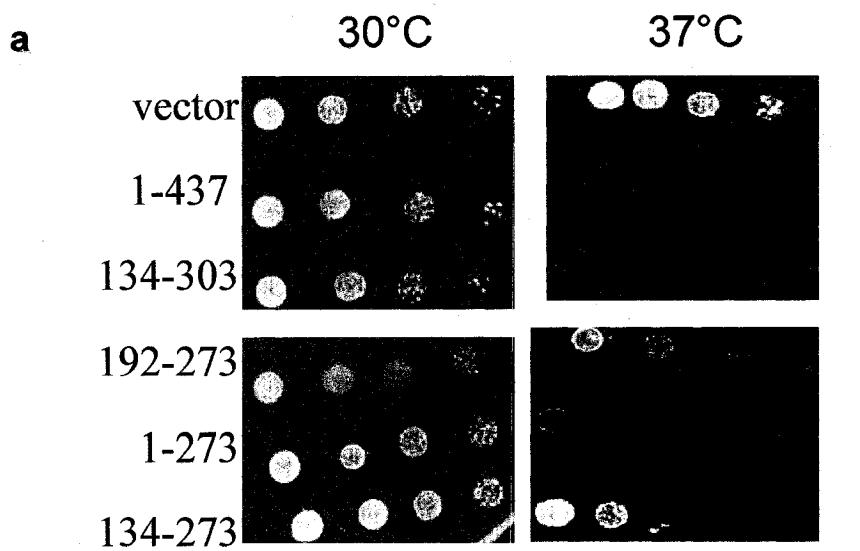
CL	DAG	PA	PC
PE	PG	PI	PI3P
PI4P	PI5P	PI34P	PI35P
PI45P	PI345P	PS	SM

Figure 71. GST-ORP1S binds to phosphoinositides. a) The GST constructs indicated were expressed and purified from *E.coli*. Purified chimeras were incubated with immobilized phospholipids and detected using an anti-GST HRP-conjugated antibody. For comparison the PH domain of OSBP and Kes1 are included. b) a schematic of the position of the phospholipids used in the assay.

production. The ability of the 192-273 fragment and others of ORP1S was investigated. Figure 72a shows that the expression of ORP1S fragments can partially impair the growth of *sec14*^{ts} *kes1* at elevated temperatures. The table in Figure 72b summarizes the results. The ORP1S fragments used in subsequent experiments were 192-273 and 134-273, both of which are expressed to similar levels in yeast as full length (Figure 72c).

To investigate the ability of the fragments of ORP1S to inhibit *sec14* independent secretion, CMY136 was transformed with pDB31 and pESC-trp vectors containing the ORP1S construct of interest. Cells expressing full length ORP1S secrete 65% of their invertase, cells expressing 134-273 secrete about 70% of their invertase whereas vector control or 192-273 expressing cells express 85% of their invertase. These results are consistent with the growth rate of these cells at the non-permissive temperature.

From the results ORP1S (192-273) the smallest fragment identified as having PIP binding activity could not inhibit growth or regulate invertase secretion. However, the fragment 134-273, could partly inhibit invertase secretion and cell growth. Studies using PI-4P binding PH domains suggest that the ability to bind PI-4P and localize to the Golgi is sufficient to inhibit cell growth in *sec14*^{ts} and *pik1*^{ts} cells.



b

vector	++
1-437	-
1-273	+/-
67-370	+/-
67-273	+/-
134-273	+/-
134-370	+/-
192-273	++
160-273	++
160-437	++

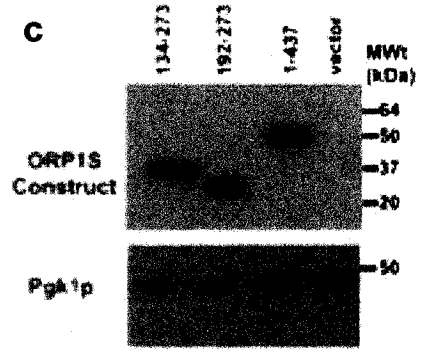


Figure 72. Human ORP1S can suppress *kes1*-mediated Sec14 bypass. a) Cells of strain CMY136 (*sec14^{ts} kes1::HIS3*) were transformed with plasmid pESC-ura or, pESC-ORP1S, or the indicated pESC-ORP1S deletion constructs. Transformants were grown in minimal medium containing galactose and lacking uracil to mid-log phase. Cells were harvested, washed in minimal medium containing galactose, and equal cells concentrations were 10-fold serially diluted and spotted on minimal solid medium containing galactose and lacking uracil. Cells were incubated for 72 h at either 30°C or 37°C.. Respesentitive plates are shown. b) Table summarizing the constructs tested, ++ (full growth) +/- (impaired growth) - (death). c) Full length and ORP1S deletion constructs of interest express have similar expression levels.

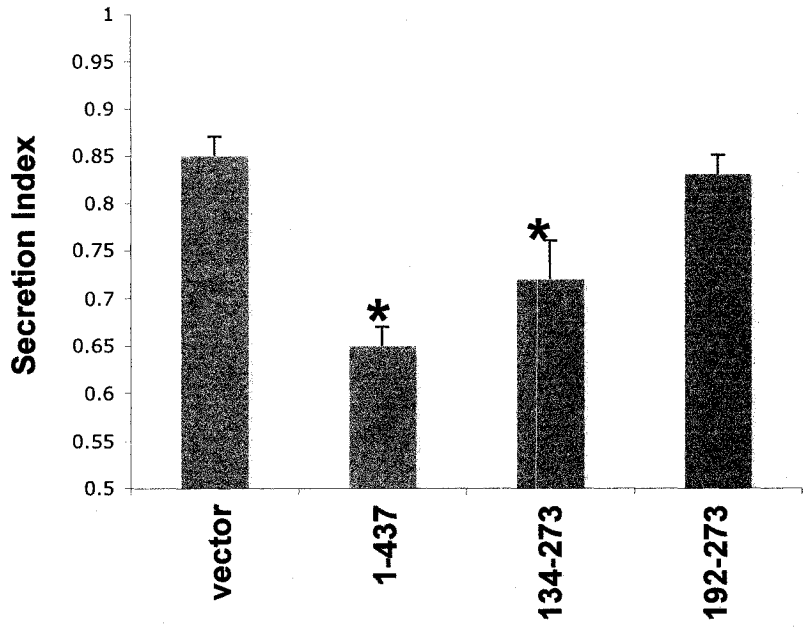


Figure 73. Human ORP1S impairs the secretion of invertase in *sec14^{ts} kes1* cells. Cells of the strain CMY136 (*sec14^{ts} kes1::HIS3*) were transformed with plasmids pESC-trp, or pESC-trp-ORP1S, or the indicated deletion constructs as well as pDB31 (*SUC2*). Transformants were grown to mid-logarithmic phase in minimal medium lacking uracil and tryptophan, harvested and washed. Cells were partitioned and incubated for 2 h at 25°C or 37°C. The secretion index was determined by measuring the activity of the extracellular invertase compared to the total. (* $p<0.05$)

CHAPTER 7. DISCUSSION

This study identified two characteristics of Kes1 that likely contribute to its ability to negatively regulate secretion from the *trans*-Golgi. The PI-4P synthesized by Pik1 in the Golgi is crucial for secretion from the Golgi, and the presence of Kes1 appears to attenuate this production. Furthermore, as demonstrated before in yeast and in mammalian cell culture, the expression of Golgi localized PI-4P binding domains can attenuate secretion and cell growth (Godi et al., 2004; Levine and Munro, 1998). Kes1 is an abundant (32,000 molecules/cell) Golgi-localized PI-4P binding protein possible of limiting the accessibility of the Golgi PI-4P pool (estimated to be 25,000-50,000 molecules in the Golgi). Consistent with these observations genetic replacement of *KES1* is able to bypass two normally-essential genes that stimulate PI-4P production in the Golgi, Sec14 and Frq1.

Previous experimentation suggested that DAG is a pivatol molecule involved in secretion at the Golgi and is deficient in Sec14-lacking cells. My results largely support this previous work, however, suggests that DAG is limiting at elevated temperatures and inactivation of the CDP-choline pathway may provide an artifical or indirect means of Sec14 bypass.

Finally, the thesis demonstrates that functions associated with Kes1 are conserved in some, but not all of the human homologues tested. This suggests that select human OSBP related proteins (ORPs) may regulate secretion and possibly PI-4P production.

7.1.1. SGA ANALYSIS OF DOUBLE MUTANTS

In this study, we examined the genetic requirements for the survival of two separate viable double-mutants. To date, this remains the only time SGA analysis has been performed using a double-mutant as the query strain. Our double-mutants of interest happen to be mutants that bypass the essential requirement for a given gene, namely *SEC14*. This gives us the opportunity to examine the genetic interaction spectra for an essential gene in its complete absence. This method is complementary to the methods currently used to examine essential genes; using of hypomophic alleles, mRNA destabilization or repressible promoters (Budd et al., 2005; Davierwala et al., 2005; Mnaimneh et al., 2004; Schuldiner et al., 2005). This study also had the benefit of using

two different bypass mutations; that revealed information about the modes of bypass. The ability to use double (or even perhaps triple) mutants as a query strain should also aid in the examination of gene families or complex genetic interactions by SGA analysis.

7.1.2. SPECIFIC REQUIREMENTS FOR THE RAB-LIKE GTPASE YPT31 AND THE TRAPPII COMPLEX IN ANTEROGRADE GOLGI TRAFFIC IN *sec14 cki1* CELLS

Prior to this study, the major known defect in cells with deficient-Sec14 function was an inability to effectively transport vesicles out of the *trans*-Golgi (Bankaitis et al., 1989). Results from my SGA analysis confirmed that this is a major role for Sec14, and I now connect Sec14 to proteins that regulate distinct processes in this regard. To further assess the new interactions I constructed a set of isogenic strains through integration of a *sec14*^{ts} allele into the wild-type *SEC14* locus to allow for phenotypic analysis of cells subsequent to loss of function of Sec14 by temperature shift. From the current SGA analysis several genes were identified that were specific to preventing bypass of *sec14* by *cki1* versus *kes1*. These include the Rab GTPase encoding *YPT31* as well as *TRS33, TRS65/KRE11,* and *TRS85/GSG1* that code for the three non-essential components of the 10 subunit Golgi-associated TRAPPII complex (the essential subunits being Bet3, Bet5, Trs20, Trs23, Trs31, Trs120 and Trs130) (Sacher et al., 2001). The TRAPPII complex has been described as a RabGEF for Ypt31 and the highly similar Ypt32 (Jones et al., 2000; Morozova et al., 2006). The Ypt31/32 GTPases function as an essential pair and control exit from the *trans*-Golgi as well the cycling of the v-SNARE Snc1 between the Golgi and plasma-membrane (Benli et al., 1996; Jedd et al., 1997).

I confirmed the specificity of the interaction as of *YPT31* as specific to modulating *cki1*-mediated bypass of *sec14* as targeted replacement of *YPT31* prevented growth of the *sec14*^{ts} *cki1* cells but not the *sec14*^{ts} *kes1* cells at 37°C, a non-permissive temperature for the *sec14*^{ts} allele. Importantly, replacement of the *YPT31* gene alone did not affect growth or secretion indicating a specific requirement for Ypt31 in *sec14 cki1* cells. Our analysis of the TRAPPII complex components revealed similar but more modest interactions when compared to *ypt31*. Genetic replacement of *TRS33, TRS65, TRS85* in *sec14*^{ts} *cki1* cells resulted in moderate growth and secretion defects at 37°C, and more pronounced growth defects at 39°C. However, loss of Trs33, Trs65 and Trs85

resulted in an obvious decrease in invertase secretion in *sec14^{ts} ckil* cells at 37°C. Replacement of *TRS33*, *TRS65* or *TRS85* alone did not affect growth or secretion indicating specificity of the interaction of the TRAPPII complex with loss of Sec14 function. Disruption of *TRS85* in some genetic backgrounds has been described as having reduced fitness at 37°C, consistent with our observation that replacement of *TRS85* decreased growth at 37°C. However, loss of Trs85 alone did not decrease secretion at 37°C, but did so in *sec14^{ts} ckil* cells indicating a specific interaction between *TRS85* and regulation of transport from the Golgi by Sec14. We consistently observed that the growth defect for inactivation of the non-essential members of the TRAPP II complex was much more obvious in cells completely lacking a functional *SEC14* gene, as per the SGA analysis and subsequent random spore analysis, than in cells containing the *sec14^{ts}* allele. This is likely due to residual Sec14 activity at 37°C for this allele. Indeed, it has been observed that over-expression of the *sec14^{ts}* allele can restore growth and secretion at 37 °C in cells carrying the genomic *sec14^{ts}* allele indicating some residual Sec14 functionality (Amy Curwin, McMaster Lab, unpublished observation). Taken together, the Rab-GTPase Ypt31 and the TRAPPII complex are positive regulators of Sec14 regulated vesicular transport from the *trans*-Golgi. However, they appear to be insensitive to the DAG/PC balance due the absence of interaction with the *sec14 kes1* strain. The cells of the *pik1-139^{ts}* strain also has a heightened genetic requirement for the TRAPPII complex and Ypt31 (Sciorra et al., 2005) suggesting that alterations in PI-4P may be a major defect in *sec14 ckil* cells.

7.1.3. SPECIFIC INTERACTION OF *YPT31* WITH *SEC14*

TRAPPII has been described as having GEF activity toward Ypt31 and the highly similar Ypt32, (Jones et al., 2000). *YPT31* and *YPT32* form an essential gene pair whose simultaneous inactivation results in an inability to secrete from the *trans*-Golgi (Benli et al., 1996; Jedd et al., 1997). As my SGA analysis only revealed a synthetic lethal interaction with *ypt31* in *sec14 ckil* cells, I constructed strains containing *sec14^{ts} ckil ypt31* and *sec14^{ts} ckil ypt32* to confirm this specificity. The *sec14^{ts} ckil ypt31* strain demonstrated severe growth and secretion defects at 37°C, while replacement of *ypt32* in *sec14^{ts} ckil* cells displayed no growth or secretion defects compared to the parental

sec14^{ts} *ckil* cells. While there is a slight excess of Ypt31 over Ypt32 in the cell (6,000 vs. 4,300 molecules/cell) these results provide evidence for a specific functional interaction between Sec14 and Ypt31 in vesicular transport from the Golgi. Perhaps Ypt31 and Ypt32 while largely redundant have aquired additional functions.

7.1.4. MODES OF SEC14-BYPASS ARE ADDITIVE

Sec14 provides an essential function, as witnessed by the fact that inactivation of *SEC14* results in lethality. The essential nature of Sec14 can be alleviated by inactivation of the CDP-choline pathway or Kes1. Therefore, Sec14 function can be viewed as acting in opposition to the negative regulation of secretion by the CDP-choline pathway and Kes1. It has been previously determined that over-expression of Kes1 prevented growth of *sec14 ckil* cells, however, increased expression of Kes1 did not affect growth or secretion in yeast that contained functional Sec14 (Fang et al., 1996; Sciorra et al., 2002). This implies that Sec14 supercedes Kes1 function, with the absence of Sec14 inhibition of Kes1 resulting in an increase in Kes1 activity, that in turn inhibits vesicular transport from the Golgi and leads to cell death.

Although we know that the CDP-choline pathway and Kes1 both oppose Golgi-derived vesicular transport, the magnitude of the regulatory effect imposed by each is not known. We recovered only four genes that were specific to synthetic lethality with *sec14 kes1* versus 21 that were specific to loss-of-function of the CDP-choline pathway. The *sec14 ckil* bypass strain can be thought of as synonymous with a strain containing unopposed Kes1. Meaning, *SEC14* genetically opposes *KES1,* and in *sec14 ckil* cells, *KES1* is unopposed. I interpret the result whereby genes are synthetic lethal only with *sec14 ckil* as being due to the loss-of-function of a positive regulator of secretion (the loss of function of each gene isolated in this screen) in combination with a cell that already possesses unopposed Kes1 function (as this strain does not contain Sec14). If this is the case, removal of Kes1 function in the *sec14 ckil* triple-mutants isolated in this study should ameliorate the synthetic defects. In almost every instance (the exception being *VPS4*) I found that genetic replacement of *kes1* allowed for restoration of growth and secretion. The examples illustrated are replacement of *KES1* in the *sec14*^{ts} *ckil ypt31* or *sec14*^{ts} *ckil trappII* cells ameliorating the growth defects and re-establishing Golgi-

derived vesicular transport (Fig. 4). The additive nature of the modes of Sec14-bypass is also seen with genes unique to the *sec14 kes1* screen, suggesting an unabated CDP-choline pathway has defects in combination with the loss of function of the four genes synthetically lethal with *sec14 kes1*. Genes common to both lists are different in that either an unregulated CDP-choline pathway or an unopposed Kes1 can cause sickness/death. Interactions within this group should fall into one of two categories (i) simultaneous inactivation of both *KES1* and the CDP-choline pathway amends the growth defect, or (ii) an additional and perhaps non-lipid Sec14 function that would not be subject to regulation by the Kes1/CDP-choline pathway. This remains to be determined.

To extend our observations with respect to Kes1 and CDP-choline pathway regulation of Golgi-derived vesicular transport, I monitored vesicular transport from the Golgi in the single Sec14-bypass cells, *sec14^{ts} cki1* and *sec14^{ts} kes1,* as well as in "double-bypass" *sec14^{ts} cki1 kes1* cells by monitoring invertase secretion and determining the localization of GFP-Snc1. Snc1 is a yeast v-SNARE that participates in the fusion of exocytic vesicles with the plasma-membrane (Couve and Gerst, 1994; Protopopov et al., 1993). Snc1 is found in Golgi-derived vesicles destined for the plasma-membrane, subsequent to membrane fusion Snc1 is endocytosed into endosomes and recycled to the Golgi to execute another round of vesicular transport (Lewis et al., 2000). Previous examination of GFP-Snc1 localization in *sec14^{ts}* cells demonstrated that at the permissive temperature GFP-Snc1 was localized mainly to the plasma-membrane, similar to wild-type cells, and upon shift to the non-permissive temperature it accumulated in the Golgi (Lewis et al., 2000).

Under permissive conditions for the *sec14^{ts}* allele, 25°C, the *sec14^{ts}, sec14^{ts} cki1, sec14^{ts} kes1, sec14^{ts} cki1 kes1* and wild-type cells all displayed similar levels of invertase secretion, while at non-permissive temperatures for the *sec14^{ts}* allele the secretion efficiency of *sec14^{ts}* and *sec14^{ts} cki1* cells decreased to 40% and 22%, and 70% and 55%, at 37°C and 39°C, respectively. Secretion at 37°C and 39°C in the *sec14^{ts} kes1* and *sec14^{ts} cki1 kes1* strains remained similar to wild-type. To further investigate the differences in secretion efficiency from the Golgi apparatus we determined the localization of GFP-Snc1. Consistent with previous observations, GFP-Snc1 localized

primarily to plasma-membranes of the growing daughter cell and at the mother-daughter bud neck in wild-type cells. A similar localization of GFP-Snc1p was observed for all cells grown at the permissive temperature of 25°C. Similar to the invertase secretion defects a shift to the non-permissive temperature of 37°C in the *sec14*^{ts} strain resulted in Golgi-localized GFP-Snc1. Cells of the single bypass *sec14*^{ts} *ckil* had GFP-Snc1 localized to both the plasma membrane as well as internal structures consistent with a delay in the budding of GFP-Snc1 containing vesicles from the Golgi. The *sec14*^{ts} *kes1* and *sec14*^{ts} *ckil kes1* cells displayed GFP-Snc1 localization similar to wild-type cells indicating that transport and recycling of GFP-Snc1 between the Golgi and plasma membrane occured at normal rates in these cells. In addition, these results indicate that the CDP-choline pathway does not influence *kes1*-mediated bypass.

7.1.5. A ROLE FOR PI-4P IN SEC14 DEPENDENT GOLGI VESICULAR TRANSPORT

Initial work involving Sec14, combined with the observation that Sec14 could transfer PI and PC suggested that Sec14 invoked its effects by regulating the metabolism of PI and PC (reviewed by Ile et al., 2006). However, the involvement of lipids in the Sec14 pathway appears to be more complex and includes potential roles for phosphatidic acid, DAG, and PI-4P. Examination of PI-4P levels in cells containing the *sec14*^{ts} *(sec14-1)* allele used in the current study showed a near 50% decrease in the production of PI-4P when cells were shifted to the restrictive temperature, but no alterations in PI-3P (Routt et al., 2005). Consistent with this observation, increased expression of the PI-4-kinase *PIK1* increased the non-permissive temperature of cells carrying the *sec14-1*^{ts} allele (Hama et al., 1999). Examination of the total phosphorylated PI levels in the *sec14 ckil* strain (with a chronic lack of Sec14 function) demonstrates a reduction of nearly 50% in the levels of PI-3P, PI-4P and PI-4,5P_2. Clearly, Sec14 either directly or indirectly has a role in the regulation of the levels of these lipids. This raises the possibility that some of the synthetic lethal interactions we identified using the *sec14 ckil* strain are due to an underlying depletion of one of the phosphorylated PI species.

S. cerevisiae contain three PI-4-kinases, the essential ones Pik1 and Stt4, along with the non-essential Lsb6 (Audhya et al., 2000; Han et al., 2002). Cells containing reduction-of-function alleles of *PIK1* are known to have defects in secretion and also

accumulate the same aberrant Golgi structures as *sec14*^{ts} cells. Recent studies have also demonstrated that Pik1 localizes to the Golgi and that its PI-4 kinase activity is essential for Golgi function. Together, the results clearly demonstrate a role for Pik1 produced PI-4P in the Golgi for proper function. Recently, an SGA analysis was performed using a modest (~20%) reduction-of-function allele of *PIK1 (pik1-139*^{ts}*)* and identified 15 nonessential genes with described functions in membrane trafficking as resulting in a further loss of fitness. Of these 15 genes, seven genes are shared with the *sec14 cki1* cells including, *TRS65/KRE11, TRS33, YPT31, VPS4, VPS1, VPS51* and *ARL1*. This is consistent with the premise that the reduction of PI-4P levels in the *sec14 cki1* cells is the underlying cause of some of the synthetic interactions identified in our screen, with the above proteins being possible PI-4P effectors. Genetic replacement of *KES1* alleviates defects associated with *sec14* cells and also increases the growth rate of cells with severely compromised Pik1 activity. Kes1 itself has been demonstrated to bind PI-4P or PI-4,5P_2 under several different types of experimental conditions, with Pik1 activity being required for the proper localization of Kes1 to the Golgi (Fairn and McMaster, 2005a; Knodler and Mayinger, 2005; Li et al., 2002; Raychaudhuri et al., 2006). Therefore, the combined physical and genetic results suggest that Kes1, possibly through its ability to bind PI-4P, acts as an inhibitor of secretion by binding to available PI-4P and competing with pro-secretory factors that require PI-4P. This situation becomes critical in cases were Golgi-localized PI-4P is limiting as is found in *sec14 cki1* cells and cells with compromised Pik1 function. This raises the possibility that Sec14 controls PI-4P production through substrate presentation or delivery to Pik1. Consistent with this observation, co-over-expression of *FRQ1* and *PIK1* suppressed *sec14*^{ts} growth defects.

7.1.6. SGA ANALYSIS SUGGESTS Kes1 REGULATES THE GOLGI LOCALIZED PI-4P SIGNALING PATHWAY

The modes of Sec14 bypass appear to be distinct and additive as *sec14 cki1* cells shares synthetic lethal partners with *pik1-139*^{ts} cells, while *sec14 kes1* does not, suggesting that Kes1 attenuates PI-4P signaling initiated by Pik1.

The tertiary structure of Kes1 revealed that it binds sterols within the core of the protein with significant conformational changes associated with Kes1 upon sterol-

binding. However, Kes1 was found to bind PI-4P (or PI-4,5P_2) by several methods and *KES1* replacement increased the growth of cells containing various *pik1^{ts}* hypomorphic alleles. Also, point mutations within Kes1 that abolished its binding to phosphorylated PIs *in vitro* also prevent Kes1 from acting as a negative regulator of vesicular transport from the Golgi. Clearly, PI-4P binding by Kes1 is important for its biological function. A few things remain unresolved concerning sterol and phospholipid binding by Kes1. The work by Prinz and co-workers showed that in addition to transferring sterols *in vitro,* Kes1 could also transfer PI-4,5P_2 (Raychaudhuri et al., 2006). Is the PI-4P (or PI-4,5P_2) binding site on the exterior of the protein, or is it internal, or both? Is there more than one PIP binding site? Furthermore, can Kes1 simultaneously bind to both sterols and PIPs? The experiments with the cross-linkable PI-4,5P_2 from the Bankaitis lab, did not distinguish between surface binding and occupancy of the PIP_2 molecule in the binding pocket (Li et al., 2002). While the experiments used mixed micelles as a vehicle for PIP_2 presentation, the samples were simply resolved by SDS-PAGE.

Lipid protein overlay assays by their very nature investigate the interaction between phospholipids and binding surfaces on the exterior of the protein. Lipid protein overlay assays suggest that Kes1 has a least one region on the surface of the protein that can bind to mono-phosphorylated inositides (Fairn and McMaster, 2005a).

Ergosterol is far more abundant in the cell than PIPs, so it is unlikely that PIPs would occupy the interal-binding cavity of Kes1 unless it bound PIPs with a much higher affinity. Conversely, an alternative could be that loading of Kes1 with PIPs could be under the influence of another protein, which would provide specificity for PIPs over sterols.

7.1.7. REPLACEMENT OF *KES1* AMELIORATES DEFECTS ASSOCIATED WITH REDUCTION OF PIK1 FUNCTION

Pik1 is a PI 4-kinase that is responsible for the production of PI-4P in the Golgi and in the nucleus. Loss of Pik1 function results in a block in secretion from the *trans*-Golgi, the accumulation of aberrant Golgi structures, and a block in cytokinesis (Audhya et al., 2000; Garcia-Bustos et al., 1994; Walch-Solimena and Novick, 1999). It was postulated that the PI-4P produced by Pik1 serves as a molecular beacon for proteins required for secretion from

the *trans*-Golgi. I estimated that the Golgi contains ~ 25,000-50,000 molecules of PI-4P, while the cell contains ~32,000 molecules of Kes1 (Ghaemmaghami et al., 2003). Therefore, Kes1 could effectively compete with other PI-4P binding proteins for the available PI-4P in the Golgi.

Most organelles involved in vesicular traffic have distinct phosphoinositide composition, which may serve as "postal codes" for proteins required to target specific organelles. For instance, the Golgi is enriched in PI-4P (Roy and Levine, 2004), the endosomes enriched with PI-3P (Burd and Emr, 1998), the vacuole contains $PI3,5P_2$ (Dove et al., 2004) and the plasma membrane is enriched for $PI-4,5P_2$ (Stefan et al., 2002). One potential effector of PI-4P is the TRAPPII complex, a 10 subunit containing protein complex that appears to act as the gatekeeper for exit from the *trans*-Golgi network (Morozova et al., 2006; Sacher et al., 2001).

Cells containing the *pik1-83*^{ts} allele have defective secretion from the *trans*-Golgi and die as large budded cells at the non-permissive temperature. The trafficking of GFP-Snc1 is altered even at the permissive temperature of 25°C. Due to its highly dynamic trafficking cycle of Golgi to plasma-membrane to endosomes to Golgi, even minor defects in any step create a bottleneck for Snc1 trafficking. In *pik1-83*^{ts} cells the defect is in exit from the *trans*-Golgi, as a result GFP-Snc1 accumulates in the Golgi. Ablation of *kes1* in these cells restored normal trafficking to *pik1-83*^{ts} cells. Hsp150 is a glycoprotein that is upregulated and secreted by yeast under conditions of heat stress (i.e. 37°C). Cells carrying the *pik1-83*^{ts} allele when incubated at 37°C, no longer secrete Hsp150 and accumulates in an intracellular compartment in its fully glycosylated form. Again, genomic replacement of *KES1* restores the secretion of Hsp150 to levels comparable to wild-type cells.

Null mutants of *PIK1* are inviable but some spores carrying a disruption allele germinate only to arrest with a single large bud and fully divided nuclei. Cells carrying the *pik1-12* allele display this same phenotype when shifted to 37°C (Garcia-Bustos et al., 1994). This is not limited to the *pik1-12* allele, with *pik1-83* containing cells also have the same terminal phenotype. Again, this block in cytokinesis is removed upon elimination of the *KES1* gene, demonstrating that *KES1* is a negative regulator of *PIK1*.

Frq1 is a small 190 amino acids calcium binding protein and is the yeast homologue of frequenin (also referred to as neuronal calcium sensor-1) (Hendricks et al., 1999) . In

yeast, the sole essential target of Frq1 is Pik1. Frq1 and Pik1 bind in a 1:1 ratio and exist mainly in a heterodimeric complex as determined by size exclusion chromatography (Huttner et al., 2003). Frq1 appears to both recruit Pik1 to the Golgi and stimulate its kinase activity. Clearly, *FRQ1* is a positive regulator of *PIK1*.

The viability of cells containing simultaneous elimination of *KES1* and *FRQ1* demonstrated that the loss of a positive regulator of Pik1 is tolerable when a negative regulator is also removed as well. The re-introduction of *KES1* into cells lacking *FRQ1* resulted in lethality suggesting that *frq1* is directly bypassed by ablation of *KES1* and not through chronic alterations in metabolism.

7.1.8. Kes1 INHIBITS PI-4P PRODUCTION *in vivo*

Kes1 is abundant and can bind PI-4P, so it is possible that a major role of Kes1 imposed secretion inhibition could be due to its ability to bind PI-4P. An additional explanation is that Kes1 influences PI-4P production. In wild-type cells ~2.5% of the total PI is phosphorylated at the D-4 position on the inositol ring. In cells lacking Kes1, the level approaches 3.3% (a 34% increase). The results in strains with the enfeebled Pik1-83 enzyme were even more impressive. PIP levels were measured in *pik1-83^{ts}* and *pik1-83^{ts} kes1* cells at 26°C and 38°C. Of the strains examined all have similar levels of PI-3P at 26°C, however, PI-4P levels are varied with 2.4%, 1.4% and 3.3% for wild-type, *pik1-83^{ts}* and *pik1-83^{ts} kes1* cells respectively. Replacement of *KES1* in the *pik1-83* cells results in a 2.5-fold increase in PI-4P in the cell at 26°C. While replacement of *KES1* leads to an increase in PI-4P, it does not restore the production of PI-4,5P_2, as both cells of strains AAY104 (*pik1-83^{ts}*) and GFY201 (*pik1-83^{ts} kes1*) have levels of PI-4,5P_2 around 1.1-1.2% whereas wild-type cells have levels around 1.7%. At the non-permissive temperature, wild-type and GFY201 cells have the same levels of PI-4P (~1.4%), while the levels drop in the cells of strain AAY104 is further reduced (~0.7%). PI-4P generated by Pik1 at the Golgi is not efficiently converted to PI-4,5P_2 presumably because the PI-4P 5-kinase, Mss4, localizes to the plasma-membrane and to the nucleus (but not to the Golgi) (Audhya and Emr, 2002). In addition, Sjl2 and Sjl3, PIP 5-phosphatases reside in the Golgi and may prevent inadvertent or premature conversion of PI-4P to PI-4,5P_2 before secretory vesicles reach the PM (Stefan et al., 2002). The results in the *pik1-83*

and *pik1-83 kes1* cells support this observation. While levels of PI-4P in *pik1-83 kes1* cells reach that of wild-type cells when growing at 38°C, the levels of PI-4,5P_2 do not increase. This also suggests that Kes1 only regulates Pik1 at the Golgi and not in the nucleus. The production of PI-4,5P_2 in the nucleus remains low due to the limited production of PI-4P in the nucleus by Pik1.

There is considerable evidence that PI-4,5P_2 is present in the *S. cerevisiae* nucleus and is cleaved by Plc1, to generate IP_3 and the production of other more highly phosphorylated IPxs (Flick and Thorner, 1993; York et al., 1999). Highly phosphorylated IPxs have apparent roles in regulating mRNA export and modulating transcription (Odom et al., 2000; York et al., 1999). If there are regulators of Pik1 activity in the nucleus, it does not appear that Kes1, or for that matter Frq1, is one of them.

To date we have been unable to demonstrate direct inhibition of Pik1 *in vitro* by Kes1. While weak direct physical interactions have been demonstrated, the interaction appears to be difficult to capture. If the inhibition Kes1 imposes upon Pik1 is through a combination of direct protein-protein and protein-lipid interactions, this environment will be hard to reconstitute *in vitro.* Perhaps Kes1 and Pik1 have to be engaged with the Golgi membrane to interact and regulate PI-4P production. The use of mixed-micelles used in the PIk1 assay, have the advantage that all of the PI substrate is available to the enzyme. However, due to the small size of mixed micelles, unnatural state, and the presence of detergent, it might be difficult for Kes1 to bind to a PI-Triton X-100 micelle and to inhibit Pik1. In addition, if Kes1 must be bound to PI-4P to exert its effects, this would further complicate the situation. Assaying Pik1 activity with liposomes is an alternative to using micelles, however purified Pik1 enzyme should be included in these assays (as Vps34 and Stt4 can use liposomes as substrates). As liposomes are larger, do not contain any detergent, can vary in shape, and in composition of phospholipids, the use of liposomes may provide a useful alternative.

7.1.9. Arf AND ArfGAPs HAVE A HEIGHTENED REQUIREMENT AT ELEVATED TEMPERATURES - Gcs1 ACTIVITY IS ALTERED BY DAG

Diacylglycerol has been the focus of much of the theory surrounding the function of Sec14 and Sec14 bypass. Early studies demonstrated a role for Sec14 in secretion from

the *trans*-Golgi (Bankaitis et al., 1989). The discovery that inactivation of the CDP-choline pathway for PC biosynthesis circumvents the requirement for Sec14 lead to the prediction that too much PC or too little DAG was responsible for the essential cellular defects in Sec14-deficient cells (Bankaitis et al., 1990; Cleves et al., 1991). Because Sec14 could transfer PC *in vitro,* perhaps the removal of excess PC from the *trans*-Golgi was the primary function of Sec14 (Bankaitis et al., 1990). Later it was shown that the ability to transfer PC was not sufficient to bypass the requirement for Sec14 (Skinner et al., 1993) and Sec14 function did alter the PC content in the Golgi (McGee et al., 1994). In wild-type yeast cells, PC comprises about 50% of the total phospholipid content, however, the PC content in the Golgi is only around 30%. In *sec14*^{ts} cells shifted to the non-permissive temperature, the cellular PC content was still around 50%, however PC now comprised about 50% of the phospholipid in the Golgi. Replacement of *ckil* in *sec14*^{ts} cells restored the PC content in the Golgi back to 30% (McGee et al., 1994). Subsequent studies lead to the identification of PC bound Sec14 as an inhibitor of Pct1, the rate-limiting step in the CDP-choline pathway (Skinner et al., 1995). The addition of short-chain water-soluble DAG (di8:0) improved secretion of *sec14*^{ts} strains, whereas the expression of the bacterial DAG kinase further impaired secretion (Kearns et al., 1997). The evidence suggests that excess PC synthesis and the resulting consumption of DAG by the CDP-choline pathway was a defect in *sec14*^{ts} cells at the elevated temperature. The cellular levels of DAG do not change in *sec14*^{ts} or *sec14*^{ts} *ckil* cells and it is unknown if the levels of DAG are altered in the Golgi. It is possible that specific pools of DAG are consumed in *sec14* lacking cells.

Do these alterations in DAG pools and PC content impose a block in secretion through alterations in biophysical properties of the membrane, or are there specific molecules that are recruited and/or activated by DAG that promote secretion? In mammalian cells, Arf activates PLD1 leading to the production of PA (and DAG) *in vivo* (Brown et al., 1993). This led to the observation that vesicles containing PA and DAG stimulated ArfGAPs *in vitro* (Antonny et al., 1997). The same held true for the yeast ArfGAPs Gcs1 and Age2 (Yanagisawa et al., 2002). As *GCS1/AGE2* form a synthetic lethal pair and display defects in post-Golgi trafficking, it is possible that they are a target

of the DAG pool maintained by Sec14 (though it should be noted that yeast Arf1/2 does not activate Spo14 activity) (Poon et al., 2001; Rudge et al., 1998).

Cells lacking *AGE2* and containing an enfeebled *gcs1 (gcs1-4)* fail to grow at elevated temperatures, however like *sec14^{ts}* cells they are rescued by the over-expression of Sfh proteins. Rescue is dependent upon a functional Spo14, and that the addition of di8:0 DAG suppressed growth defects (Figure 56, (Wong et al., 2005). Furthermore, in addition to allowing for cell growth at the non-permissive temperature, invertase secretion was increased (Figure 57). However, unlike *sec14^{ts}, age2 gcs1-4^{ts}* cells are not rescued by genetic replacement of *KES1*. This suggests that Sec14 influences two pathways, with Kes1 regulating one of them, and an additional pathway containing DAG-based activation of Gcs1 and Age2.

Previous work demonstrated that Gcs1 and Age2 were downstream of Sec14 and Spo14 and required for the viability of *sec14^{ts} pct1* and *sec14^{ts} kes1* strains grown at the non-permissive temperature for the *sec14^{ts}* allele (Li et al., 2002; Yanagisawa et al., 2002). As ArfGAPs inactivate Arf1/2, it was a bit of a paradox to find that while Sec14-bypass strains required ArfGAP activity, they also had a heightened requirement for Arf1. However, it is naïve to believe that Arf and ArfGAPs have only one function in the cell. Recently, Gcs1 has been described as possessing ArlGAP activity that may control retrograde transport to the Golgi from endosomes (Liu et al., 2005). As mentioned in the earlier, *AGE2* and *GCS1* were not identified in our SGA analysis performed at 30°C. Furthermore, random-spore analysis confirmed that neither *sec14 cki1* or *sec14 kes1* cells required Gcs1 or Age2 at 30°C, however, they were required at 37°C. This suggests that in the absence of *sec14* there is sufficient DAG to stimulate the remaining ArfGAPs normal temperatures. However, when transferred to the elevated temperatures, there is no longer enough DAG to stimulate ArfGAP activity.

Previously, it was suggested that Kes1 may influence the Arf GTP-GDP cycle, and that this is the way in which Kes1 regulates secretion (Li et al., 2002). However, I prefer an alternative model for the function of Kes1 and the Sec14 pathway as a whole. Cells lacking Arf1 (but still containing the less abundant Arf2) are hyper-sensitive to fluoride ions (F^-), have abnormal Golgi structure and have reduced levels of PI-4P (~30%) (Audhya et al., 2000; Stearns et al., 1990) . However, these cells secrete invertase

at a normal rate, although, it is less efficiently glycosylated (Gaynor et al., 1998a; Stearns et al., 1990). Arf1 is required for the generation of COPI vesicles, and for retrograde transport between the Golgi cisternae (Gaynor et al., 1998b). Cells with diminished recycling of the Golgi resident glycosylation enzymes will have a reduction in efficiency of glycosylation. *arf1Δ* cells also show a delay in CPY trafficking to the vacuole, however, CPY and invertase are first transported to the endosomes in clathrin-coated vesicles and subsequently CPY is transported to the vacuole while invertase is secreted to the cell surface (Abazeed et al., 2005). As invertase secretion is normal in *arf1* cells, the defect in CPY transport must be in the endosomes to vacuole step, or the retrieval of Vps10 (the CPY receptor) from endosomes. *ARF1* and *PIK1* form a synthetic lethal interaction (Sciorra et al., 2005). The observed reduction of PI-4P in *arf1* cells suggests that Arf1 could be upstream of Pik1 and that Kes1 may act by inhibiting Arf1. In cells, carrying the *pik1-83* allele and incubated at the non-permissive temperature, Kes1 and PH^{CERT} relocalize to the ER, while PH^{OSBP}, PH^{FAPP} PH^{Osh1} remain associated with the Golgi, because they interact with Arf1 physically (Roy and Levine, 2004). Kes1 has never been shown to interact with Arf1, and the mechanism by which Arf1 influences PI-4P levels is unknown. It is possible that in *arf1* cells Frq1 is not properly localized to the Golgi. It is currently unknown if Frq1 is a resident of COPI vesicles transported to the *cis*- or *medial*-Golgi cisternae. This may be the mechanism of impairment of Pik1 activity.

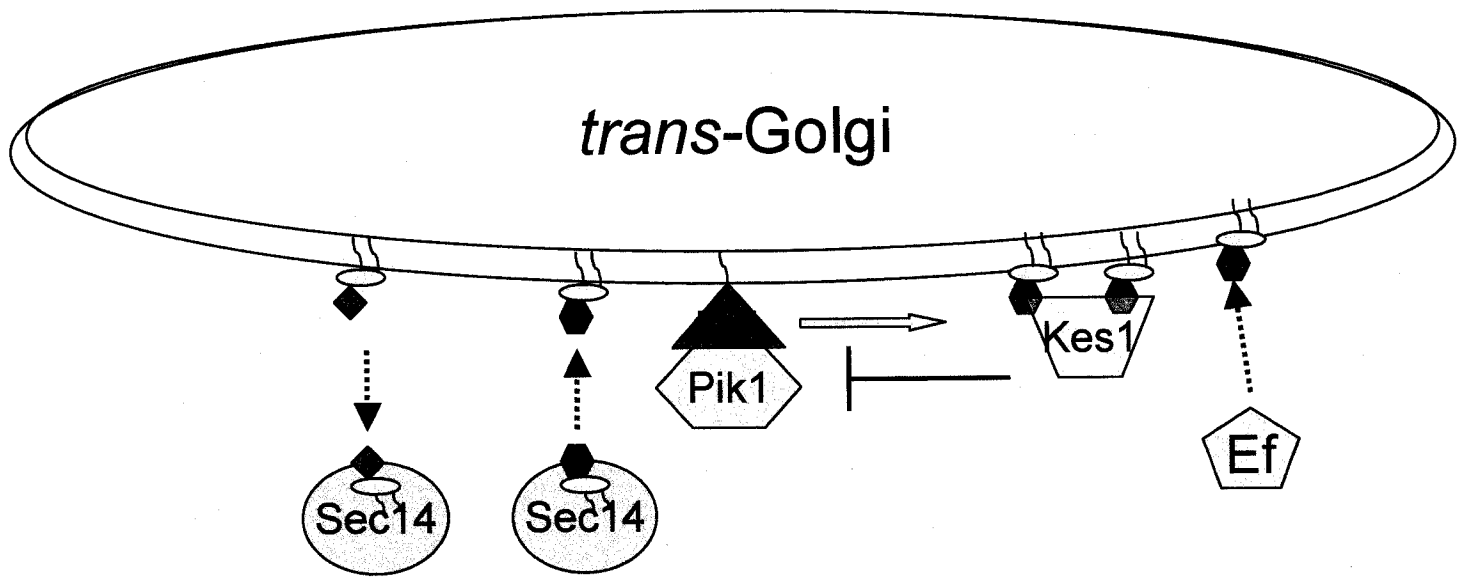
Additional evidence suggests that Kes1 does not regulate Arf1. The *arf1-3 arf2* double-mutant cells are hypersensitive to fluoride (F^-) as are *pik1-101* cells, however, replacement of *KES1* restores resistance to F^- to the *pik1-101* cells, it does not do the same for *arf1-3 arf2* cells (Li et al., 2002). This suggests Kes1 regulates Pik1 and not Arf1/2 or the Arf Cycle. PI-4P and DAG appear to regulate, two distinct and essential pathways. While both pathways are essential, the combination of normally non-lethal defects in both pathways results in lethality, as seen by the synthetic lethal interaction between the normally non-essential *GCS1* and *YPT31* (Robinson et al., 2006).

7.1.9. CO-ORDINATION OF DAG AND PI-4P METABOLISM IN THE GOLGI

DAG is of critical importance in the formation of TGN to plasma membrane transport carriers in mammalian cells. Part of this action is mediated by the recruitment and activation of protein kinase D (Baron and Malhotra, 2002). Protein kinase D activity is important in the fission of transport vesicles from the TGN (Liljedahl et al., 2001; Yeaman et al., 2004). One PKD substrate is the mammalian PI 4-kinase that is further activated upon phosphorylation. In this manner increased Golgi DAG production leads to increased Golgi PI-4P production (Bard and Malhotra, 2006; Hausser et al., 2005). While there is no true homolog of PKD in yeast, the yeast Pik1 has some of the same potential phosphorylation sites as the mammalian PI 4-Kinase. It is currently unclear if Pkc1 in *S. cerevisiae* is responsive to DAG (Bard and Malhotra, 2006; Ogita et al., 1990; Watanabe et al., 1994). The yeast Pkc1 localizes mainly to sites of polarized cell growth and therefore may not be the best candidate for phosphorylating Pik1 (Denis and Cyert, 2005).

7.1.10 THE SEC14 PATHWAY

The Sec14 pathway for Golgi function is illustrated in Figure 73. The principle lipids are shown and underlined. Phosphatidylinositol 4-phosphate (PI-4P) and diacylglycerol (DAG) are both important for secretion from the Golgi and their levels are both influenced by Sec14. In this genetic map, truly essential genes are in green, quasi-essential genes are in blue and essential gene pairs are in purple. Both the pathways illustrated are essential for organization and secretion from the Golgi, but at the molecular level perform different functions. Due to the essential nature of the pathways synthetic lethal interactions can be observed for components within each pathway represented by the single-headed arrows. Furthermore, due to their importance simultaneous impairment of each pathway also results in synthetic lethal interactions as illustarted with the double-headed arrows. Phenotypes associated with the DAG-Arf1/2 axis are more prominent at elevated temperatures, corresponding with an increase in the activity of the CDP-choline pathway that results in a presumed lowering of the Golgi DAG pool and an increase in the PC content. Kes1 is recruited to the Golgi by PI-4P, with Kes1 acting as a negative regulatory of PI-4P production. Genomic replacement of



Lipids

PC

PI

PI-4P

Figure 74. Coordinated production of PI-4P at the Golgi. Pik1 is the PI-4 kinase responsible for the production of PI-4P on Golgi membranes. Pik1 is recruited to the Golgi through direct protein-protein interactions with the Golgi localized and myristolated protein Frq1. Sec14 through its ability to transfer PI, serves to provide substrate for Pik1. PI-4P generated by Pik1 can serve as a molecular beacon to recruit/activate effector proteins (Ef), possibly including TRAPP II components. Kes1 is a highly abundant soluble protein that can bind PI-4P localized within Golgi membranes. In situations in which the PI-4P is reduced (limiting) Kes1 competes with Ef for available PI-4P. Ablation of KES1 also leads to increased production of PI-4P suggesting that Kes1 may act as a sensor and feedback inhibitor of the process. The ability of Sec14 to remove excess PC from the membrane may have the added effect of enriching the PI-4P signal.

KES1 alleviates growth and secretion defects associated with loss of Sec14, Frq1 or Pik1 activity (who appear to co-ordinate the production of PI-4P), but Kes1 does not regulate other aspects of the pathways.

7.1.11. SGA ANALYSIS OF SEC14-BYPASS CELLS REVEALS DISTINCT NETWORKS

SEC14 encodes an essential PC/PI transfer protein in yeast. This study extended our understanding of Sec14 regulation by applying SGA to the analysis of two separate strains that contain inactivating 'bypass' mutations in second genes, *ckil* (choline kinase – the first step in the CDP-choline pathway for PC synthesis) or *kes1* (a member of the OSBP family that also binds PI-4P), that allow for life in the absence of the normally essential *SEC14*. In this study, SGA analysis of *sec14 ckil* and *sec14 kes1* strains was employed to identify new positive effectors of the essential protein, Sec14, a phospholipid binding protein required for vesicular transport from the *trans*-Golgi. For lack of a better term, the identified "effectors" could represent any gene that is normally non-essential, but has a heightened requirement in the absence of Sec14. The use of two different bypass suppressors of an essential gene has the added benefit of determining if the two modes of bypass work through similar or discrete modes of action. If the modes of bypass are different, then the set of genes found to be synthetic lethal with each bypass strain will also be different. SGA analyses were performed using *ckil* and *kes1* strains enabling identification of genes that genetically interact with these single mutations (and thus would not be specific to the *sec14 ckil* and *sec14 kes1* bypass cells).

No genes were found to be synthetic lethal with *ckil* or *kes1* indicating that every synthetic lethal genetic interaction observed in the '*sec14* bypass' strains *sec14 ckil* or *sec14 kes1* was specific to the combined loss of function of Sec14 with Ckil or Kes1. We identified 33 genes whose inactivation resulted in reduced fitness in combination with *sec14 ckil* and 16 for *sec14 kes1* (with 12 of these genes common to both screens) (Figure 12). Previous work had identified *SPO14*, coding for the major PC phospholipase D in yeast, as required for both *ckil*- and *kes1*-mediated bypass of *sec14*, and this gene was identified as reducing fitness in both the *sec14 ckil* and *sec14 kes1* strains. Of the 21 genes specifically required for *sec14 ckil* bypass most have described roles in vesicular trafficking from the Golgi. This is consistent with a major function of Sec14 being the

regulation of Golgi-derived vesicular transport. Synthetic lethality unique to *sec14 kes1* bypass was observed for only 4 genes, none of which have known roles in the regulation of Golgi-derived vesicular transport, nor do they appear to possess a common link. Genes determined to be required for growth of both the *sec14 ckil* and *sec14 kes1* cells were enriched in coding for proteins required for cell wall maintenance. Taken together, the results from the SGA analyses confirmed an essential function for Sec14 in Golgi-derived vesicular transport and provided tangible links between Sec14 regulation of phospholipid metabolizing enzymes that affect this process, and protein components of Golgi-derived vesicular transport. A genetic-interaction-map for Sec14 was created using the 37 genes identified in the current screen as synthetically sick/lethal with *sec14 ckil* and *sec14 kes1* using all synthetic genetic interactions contained within the MIPS and GRID databases (Breitkreutz et al., 2003; Mewes et al., 2000). The entire network was comprised of 269 genes displaying 430 interactions. Figure 13 shows genes that possessed at least two synthetic genetic interactions. Clustering of specific genes within this data set revealed that some of the newly uncovered genes that genetically interact with *sec14* regulate cellular processes other than vesicular transport from the Golgi.

7.1.12 VESICLE TETHERING AT THE GOLGI

From the current study, loss of function of genes involved in the tethering of endosomal vesicles to Golgi membranes compromised growth of *sec14 ckil* cells illustrated by the synthetic interaction between Arf-like encoding genes, *arl1* and *arl3*, with the cells of the *sec14 ckil* strain (Behnia et al., 2004; Lu et al., 2001; Setty et al., 2004; Van Valkenburgh et al., 2001). Arl1 and Arl3 are required to target Imh1 (a Golgin protein thought to aid in vesicle tethering to the Golgi) (Setty et al., 2003). As well GTP-bound Arl1 has been found to interact with another tethering complex, the Vps51 tethering complex, which is important for tethering vesicles derived from endosomes to the Golgi apparatus (Conibear et al., 2003; Munro, 2005; Panic et al., 2003). Cells lacking *sec14 ckil* also showed a synthetic lethal phenotype with *vps51*. The clustering analysis also revealed that the deletion of the nonessential conserved oligomeric Golgi complex (COG) genes (*cog 5,6,7,8*) is synthetic lethal with the *arl1* and *arl3*. This suggests that multi-protein complexes involved in tethering retrograde vesicles to the

Golgi and proper function is under the influence of a local lipid environment regulated by Sec14. In this manner, sup-optimal conditions require optimal protein complex function.

7.1.13. SNARE FUNCTION AND VESICLE FUSION

The network demonstrates that defects in '*sec14* bypass' and in the SNARE palmitoyltransferase *swf1* cells both demonstrate a heightened requirement of the phospholipase D Spo14 for viability. Previous studies described an essential role for *SPO14* in *sec14* verified growth and this was identified by our SGA analysis (Xie et al., 1998). However, random-spore analysis and the construction of strains suggest that Spo14 activity only supports bypass, it is required for it in our genetic background. *SPO14* encodes the major PC specific phospholipase D in *S. cerevisiae* and catalyzes the hydrolysis of PC to phosphatidic acid. Recently, Swf1 has been described as catalyzing the transfer of palmitate to SNARE proteins, specifically Snc1, Tlg1, and Syn8. Palmitoylation appears to protect SNARE proteins from ubiquitination and degradation (Valdez-Taubas and Pelham, 2005). Cells lacking *SWF1* have a reduction in Tlg1p protein levels and possibly a reduction in the levels of other SNARE proteins. Previous SGA analysis has demonstrated that *spo14* and *swf1* constitute a synthetic lethal pair, suggesting that phosphatidic acid or its breakdown product DAG is important for cellular function if the global level of SNARE protein(s) is reduced (Tong et al., 2004). Phosphatidic acid produced by Spo14 has been shown experimentally to be also important for Spo20 (a SNARE) mediated vesicle fusion during sporulation (Nakanishi et al., 2006).

Examination of our network shows that two uncharacterized open reading frames *YDL133w* and *YGL007w* are required for the viability of '*sec14* bypass' strains and *swf1* strains. It should be noted that the protein products of these open reading frames are predicted to contain transmembrane domains and may have an unappreciated role in vesicular trafficking or SNARE function. Ydl133w has a predicted size of a partially purified protein with PE/PS phospholipase D (Pld2) activity (Tang et al., 2002). However, cell lysates lacking Ydl133w display the same Pld2 activity as do extracts from wild-type cells.

7.1.14. POLARIZED GROWTH AND CELL WALL MAINTENANCE

Chitin is one of the structural components of the yeast cell wall. Defects in '*sec14* bypass' and chitin synthesis share synthetic interactions with *gas1*. The glycophospholipid-anchored surface protein Gas1p is required for cell wall assembly and is described as possessing β-1,3-glucanosyltransferase activity (Tomishige et al., 2003). Our SGA screen revealed that *gas1* is synthetic lethal with both *sec14 cki1* and *sec14 kes1* cells possibly due to the reduction of PI-4,5P_2. Recently, SGA analysis using a partial-loss of function *MSS4* (the PI-4P 5-kinase in yeast) allele identified *GAS1* as forming synthetic lethal pair with *MSS4*.

Cells lacking *gas1* have increased levels of chitin (possibly to compensate for the reduction of 1,3 β-glucan) and are also synthetic lethal with the major chitin synthase gene *chs3*, as well as *chs5*, which encodes a protein that regulates Chs3p localization (Lesage et al., 2004). Chs3p undergoes constitutive recycling between the cell surface and internal pools with localization to the bud neck during polarized growth. In addition, genes required for polarized cell growth during the cell cycle, including *cla4*, *ste20*, *bem2*, and *bem4*, were each found to be required for growth of Sec14 bypass cells. Thus, Sec14 may regulate aspects of polarized cell growth. This is likely downstream from its known function of regulation vesicle transport out of the Golgi as the Golgi provides new membranes and proteins for the daughter cell during cell division. Thus, the current study of *SEC14* our use of bypass strains unmasked the impact of Sec14 beyond vesicular transport from the Golgi including vesicle tethering to the Golgi, SNARE function, and polarized cell growth.

7.2.1 OSH PROTEINS, STEROL TRANSPORT, SPHINGOLIPID METABOLISM AND CELL POLARITY

Sterols are essential components of eukaryotic membranes and are trafficked through the cell both by vesicular and non-vesicular mechanisms. Recent crystallographic, *in vivo* and *in vitro* transfer assays have suggested that oxysterol binding protein (OSBP) homologues, Oshs (yeast) and ORPs (mammalian) are *bona fide* sterol transports (Im et al., 2005; Raychaudhuri et al., 2006). Kes1 (Osh4) was co-crystallized in the presence of ergosterol, cholesterol and a few oxysterols. The structure

demonstrated that Kes1 bound sterols within the core of the protein. Considering that Kes1 is little more than the OSBP related domain (ORD), it is likely that all of the Osh and ORP proteins contain a similar modular protein domain within their 3-D structure. Comparing the structure of sterol bound Kes1 with an unliganded version demonstrated that the N-terminal region of the protein (or lid region) is likely quite flexible in the absence of ligand, however, when occupied by sterol the lid closes over the binding cavity. The structure of Kes1 occupied with sterol also contains several water molecules within the core. While Kes1 can accommodate sterols within the core, there is room to accommodate larger molecules. To date the structural data and *in vitro* data have not addressed the mechanism of sterol binding and release.

In vivo experiments were designed using the observation that yeast containing an *upc2-1* allele will take up sterols from the growth medium. Yeast will convert excess sterols into sterol esters in the ER, and this is used as an indirect measure of sterol transport. The results demonstrated that cells lacking all Osh proteins had a large (50%) reduction in sterol transport, but not a complete loss of sterol transport (Raychaudhuri et al., 2006). This suggests that an additional protein(s) could also transfer sterols *in vivo*. An alternative hypothesis is that Osh proteins are not sterol transporters *per se,* but influence the process of non-vesicular sterol transport. Of the single yeast deletion mutants tested, cells lacking *osh3* and *osh5* showed the largest decrease in sterol ester formation (Raychaudhuri et al., 2006). While cells lacking *KES1* had no decrease in sterol transport. *Osh3* and *osh5* cells are hypersensitive to the actin monomer sequestering drug latrunculin B. In addition, *osh3* and/or *osh5* cells have reduced rates of sterol transport from the plasma membrane to the ER. An alternative to being direct sterol transporters perhaps, the Osh proteins affect sterol transport from the plasma membrane to the ER, by altering cell polarization, that might be required for non-vesicular transport of sterols.

Sterols are not the only lipid molecules thought to be transported within the cell by non-vesicular mechanisms. Ceramide is produced in the ER and is transported *via* a non-vesicular mechanism to the Golgi for conversion to sphingolipids (Funakoshi et al., 2000; Funato and Riezman, 2001). Recently, the mammalian ceramide transfer protein was identified and is referred to as CERT (for ceramide transport) (Hanada et al., 2003).

However, the tertiary structure of CERT is not yet available but from primary sequence comparison the START domain of CERT likely transports ceramide. The yeast genome has only one START domain-containing protein based upon sequence homology. However, the tertiary structure of the START domain and the structure of Kes1 are similar, in that they have cavities the appropriate size to accommodate a single lipid molecule, and a lid to protect the lipids from the aqueous environment. CERT also contains a PH domain and an FFAT motif that appear to be critical for its proper targeting to the Golgi and ER, respectively. To assess the potential of Osh proteins as ceramide transporters, single *osh* deletion mutants (*osh1-osh6*) cells were labeled with [^{3}H]serine to follow the conversion of ceramide to inositolphosphoceramide and complex sphingolipids. While there was a slight variation in the labeling profile when compared to wild-type cells, ceramide appeared to be readily converted to IPC. However, cells lacking *OSH3, OSH4/KES1, OSH5,* or *OSH6* appear to have alterations in the conversion of IPC to the more complex sphingolipds. This is largely an initial observation, and the work would need to be repeated this time including *osh7* and the *osh1Δ-osh7Δ osh4-1^{ts}* strains in addition to the others at permissive and non-permissive temperatures to further the investigation. This suggests that the yeast genome encodes an additional protein(s) to transfer ceramide from the ER to the Golgi.

Previously, it was reported that yeast deficient in Osh proteins had a defect in endocytosis (Beh and Rine, 2004). As well, Osh-deficient cells have defects in polarized cell growth and exocytosis (Kozminski et al., 2006b). A recent screen identified Kes1 as a dosage suppressor of polarity defects associated with temperature sensitive *CDC42* allele. Cells deficient in Osh proteins also mislocalized the small GTPases Cdc42 and Rho1. Furthermore, factors required for bud emergence, septin rings, and cortical actin patches, were depolarized (Kozminski et al., 2006b).

More than 50 proteins in eukaryotic cells are known to be post-transcriptionally modified by the addition of isoprenoids, via 15-carbon farnesylation and the 20-carbon geranylgeranylation. *CDC42* and *RHO1* encode essential proteins that require geranylgeranylation for proper function (Ohya et al., 1993). As a result the two subunits of the geranylgeranyltransferase I (GGTase-1) *CDC43* and *RAM2* are also essential genes. The essential nature of *CDC43* and *RAM2* can be bypassed by the over-expression

of *CDC42* and *RHO1*. Suggesting that these are the only two essential targets of GGTase-1. Expression of alternative forms of Cdc42 and Rho1 containing farnesylation sites (instead of geranylgeranylation sites) also bypasses the essential nature of GGTase-1. The results demonstrate that proper function of Cdc42 and Rho1 depend upon the proper localization and insertion of isoprenoid modifications into the membrane. Due to the observation that Osh proteins effect both Rho1 and Cdc42, perhaps Osh proteins exert their effects by altering membrane dynamics which in turn alters Rho1/Cdc42 function and activity. There was an apparent decrease in $M(IP)_2C$ production in *kes1* cells, perhaps over-expression of *KES1* might increase $M(IP)_2C$ levels, that in turn could alter Rho1 and Cdc42 functions Crystallographic studies using human Cdc42 and its GEF Dbs, demonstrate that the GEF exchange is dependant upon contact with the membrane (Rossman et al., 2002). Perhaps Osh function affects prenylation itself, insertion of Cdc42 and Rho1 into the membrane, or interaction with the appropriate GEF.

7.2.2. CONSERVATION OF FUNCTION IN THE OSBP FAMILY

OSBP and ORPs (OSBP-related proteins) constitute an enigmatic protein family that is united by a lipid-binding domain that binds sterols and possibly other lipids. In humans, there are 12 genes that, through differential pre-mRNA splicing, code for at least 16 predicted OSBPs/ORPs. The transcript of the human ORP1 gene is differentially spliced, resulting in the production of two mRNAs encoding ORP1L (long) and ORP1S (short). ORP1L and ORP1S transcripts were found to be expressed ubiquitously, with ORP1L predominating in lung and macrophages, and ORP1S in heart and muscle. The ORP1L transcript encodes a 950-amino-acid protein whose N-terminus contains ankyrin repeats, a PH domain, and a FFAT motif for endoplasmic reticulum targeting, while the C-terminus is composed of the signature ORP lipid binding domain. The ORP1S transcript encodes a protein comprised of the C-terminal 437 amino acids of ORP1L and contains only the diagnostic ORP family lipid-binding domain (Johansson et al., 2003). Previously, it was demonstrated that human ORP1S, but not the very similar human ORP2, was able to phenocopy Kes1 function with respect to acting as a negative regulator of Sec14-derived vesicular transport (Fairn and McMaster, 2005a; Xu et al., 2001). Purified ORP1S protein was observed to bind phosphatidic acid and

phosphoinositides, and it was suggested that ORP1S/Kes1 might regulate vesicle transport through their ability to bind one or more of these lipids. Subsequent studies on Kes1 have determined that purified Kes1 bound several acidic phospholipids and phosphoinositides bound with the highest affinity (Fairn and McMaster, 2005a; Knodler and Mayinger, 2005; Li et al., 2002). Subsequent genetic and cell biological analyses implied that PI-4P was the primary candidate to be the Kes1p ligand *in vivo* (Li et al., 2002).

As described in the results, we have shown that human ORP9S, but not human ORP10S is also able to substitute for the function of endogenous Kes1 with regards to inhibiting vesicular transport and cell growth in cells lacking Sec14. With the emerging evidence of Kes1 being an inhibitor of Pik1 activity, we expressed human ORPs in the *pik1-83 kes1* cells. Consistent with the previous results involving Sec14-bypass, human ORP1S and ORP9S were able lower the non-permissive temperature of the *pik1-83 kes1* cells. The results also suggest that ORP1S was better at complementing Kes1 function than ORP9S, although it appears ORP1S is expressed at higher levels than ORP9S. Because the expression of PI-4P binding PH domains also inhibits growth and secretion of cells with reduced levels of Golgi PI-4P, it is possible that ORP1S and ORP9S bind Golgi PI-4P. Currently, the ability of ORP1S to regulate PI-4P production is being assessed *in vivo*. These results will illuminate the mode by which ORP1S can substitution for Kes1 function.

While inhibition of secretion is one phenotype associated with Kes1, the other major phenotype is that Kes1 can support life in yeast cells lacking the other six Osh proteins (Beh et al., 2001; Beh and Rine, 2004). To further investigate the ability of Kes1 homologs to substitute for Kes1 function, human ORP1S, ORP2, ORP9S and ORP10S were each expressed in cells of a strain lacking the genomic copies of the *OSH* genes (*osh1Δ-osh7Δ*) and kept alive with a plasmid-borne copy of a temperature sensitive *KES1 (osh4-1)* (Beh and Rine, 2004). Cells grown in the presence of galactose and carrying the human ORPs were shifted to the non-permissive temperature for *osh4-1* and only cells containing human ORP1S continued to grow. Previously, a separation of function allele of Kes1 was discussed, a Kes1$^{(R344A)}$ mutant that could rescue Osh-deficient cells but not inhibit the growth of *sec14^{ts} kes1* cells. Here, through the investigation of human ORPs

we have identified an ORP protein, ORP9S that can regulate secretion but not provide the overlapping essential function of the Osh proteins. Future investigations may reveal the basis of these observations.

Human ORP10S binds specifically to PI-3P, a lipid specifically found in endosomal compartments. Like ORP10S, Osh6 and Osh7 cannot regulate the Sec14 pathway and they too appear to bind several phosphoinositides including PI-3P (Beh et al., 2001; Beh and Rine, 2004). Recently, it was identified that yeast Osh6 and Osh7 localized to endosomes and interacted with Vps4 (Wang et al., 2005a). The pre-loading of Osh6/7 with ergosterol inhibited the interaction between Osh6 and Osh7. The C-terminal region (amino acids 366-437) appeared to mediate the interaction with Vps4 as demonstrated by two-hybrid and pull-down assays. In addition, over-expression of the C-terminal 71 amino acids of Osh7 lead to a multivesicular-body sorting defect similar to *vps4* cells. Vps4 also appeared to regulate the association of Osh6/7 with the endosomal membranes as cells lacking Vps4 had a higher percentage of their Osh6/7 associated with endosomal membranes. Perhaps Osh6 and 7 recycle ergosterol out of the endosomes and that Vps4 aids in the loading of the ergosterol into the binding pocket of Osh6 and Osh7. Upon ergosterol binding the interaction with Vps4 is weakened and Osh6 and Osh7 is released into the cytosol. The over-expression of the C-terminal 71 amino acids of Osf7 appears to titrate away Vps4 and results in endosomal sorting defects. Perhaps ORP10S performs the same role in human cells though this awaits direct experimentation.

7.3.1. CELLS LACKING THE CDP-CHOLINE PATHWAY REQUIRE OPTIMAL VACUOLE FUNCTION

This study subjected yeast strains deficient in the CDP-choline pathway for PC biosynthesis to SGA analysis. No genes were found to be synthetic lethal with *cki1*, previous work demonstrated that even the simultaneous elimination of the quasi-redundant *cki1* and *eki1* did not result in lethality (Choi et al., 2004). Cells lacking choline and ethanolamine kinase activity simply used the CDP-DAG pathway and subsequent decarboxylation and methylation to produce their PE and PC. As a result, *CKI1* appears dispensable.

Cells lacking *PCT1 or CPT1* formed several synthetic genetic interactions. The majority of the interactions were found with genes involved in transport of proteins to the vacuole. Cells lacking *PCT1* displayed additional interactions with components of the vacuolar ATPase, suggesting that cells with a complete block in the CDP-choline pathway have a heightened requirement for a fully functional and acidified vacuole. Yeast vacuoles are constantly undergoing fission and fusion reactions, with typical wild-type cells displaying 1-3 large vacuoles. Defects in vacuole fusion lead to the fragmentation of the vacuole. Vacuole fusion requires a proper vacuole membrane potential, generated by an active vacuolar ATPase, as well as components of the HOPS (homotypic and protein sorting) complex identified in my the screen (Seeley et al., 2002).

The results suggest that in cells lacking a functional CDP-choline pathway, that trafficking to the vacuole and/or fusion to the vacuolar membrane might be mildly impaired. When combined with the more severe mutants (as the ones identified in the screen) the result is synthetic sickness or lethality. Mechanistically, the CDP-choline could be important to produce PC for the subsequent consumption by Spo14 in the endosomes to aid in sorting, or possibly to aid in vesicle fusion after docking. This is not an unprecedented role for Spo14, as during sporulation Spo14 activity is required for the fusion of Spo20 (SNARE) containing vesicles with the prospore membrane after docking (Coluccio et al., 2004; Nakanishi et al., 2006). An alternative role for the CDP-choline pathway would be to consume DAG that may interfere with sorting in the endosomes. This work is not the focus of this thesis, however it does provide clues into additional functions of the CDP-choline pathway in yeast that should be investigated further.

7.4.1. FUTURE DIRECTIONS

Identification and characterization of genes required for Sec14-bypass has suggested that Sec14 may influence both exit and entrance at the *trans*-Golgi, as well as influence vesicle fusion and cell polarity. Sec14 regulates the production of phosphoinositides and the consumption of DAG to produce PC by the CDP-choline pathway. The previously described DAG responsive genes, *GCS1* and *AGE2,* were only required for Sec14-independent life at elevated temperatures. This suggests that Sec14-

bypass mediated by an elevation in DAG maybe artificial or indirect at normal temperatures.

Replacement of *KES1* ameliorates all of the cellular defects examined in *sec14*^{ts} and *pik1-83*^{ts} strains, as well as bypasses the essential nature of Frq1. Ergo Kes1 appears to regulate secretion by inhibiting the availability and/or production of PI-4P in the Golgi. I propose that PI-4P effector proteins are now not efficiently recruited/activated at the Golgi apparatus. Indeed, using a fluorescent probe of PI-4P cells with enfeebled Pik1 activity displayed a large reduction in PI-4P levels and a reduction in the localization of the fluorescent probe. Replacement of *KES1* restored the production of PI-4P and resulted in the return of the fluorescent probe to the Golgi. Over-expression of PH domains capable of binding PI-4P were able to inhibit growth of cells with reduced Golgi PI-4P production. The ability of these PH domains to compete with natural PI-4P effectors suggested that under limiting conditions PI-4P sequestering caused further detriments to the cell. I have estimated the Golgi pool of PI-4P at 25,000-50,000 molecules/cell. Kes1 is expressed in the cells at about 32,000 molecules/cell, and due to its ability to bind PI-4P may also influence secretion in this manner as well. The identification of Kes1 mutants that cannot bind PI-4P, or alter PI-4P production, will help distinguish between the effects of both properties on secretion.

From a biochemical standpoint, much needs to be examined with regards to the Kes1-Pik1 interaction and possible inhibition of kinase activity. The use of liposomes may allow for reconstitution of Pik1 activity the inhibition *in vitro*. The generation of Kes1 fusion proteins other than GST-tagged versions may help better capture the interaction between Kes1 and Pik1. While ergosterol appears to mildly enhance the Pik1-Kes1 interaction, there may be a role for additional lipids in regulating this interaction.

The results suggest that Golgi PI-4P is under the control of several proteins in yeast, including Pik1, Frq1, Sec14 and Kes1. For each of these yeast genes there exists homologues in metazoans that can phenotypically copy their yeast homologues. This suggests that in metazoans, PI 4-kinase, frequenin/neuronal calcium sensor 1 (NCS-1), PITPα/β, and select oxysterol binding protein related proteins may function in a similar regulatory circuit. NCS-1 is found primarily in excitable cells with over expression leading to increased neurotransmitter release and exocytosis (Hendricks et al., 1999;

Zheng et al., 2005). NCS-1 stimulates phosphatidylinositol 4-kinase and enhances neuronal secretion by stimulating vesicular trafficking in a phosphoinositide dependent manner. More recently, over-expression of NCS-1 has been shown to protect neurons from death by several stimuli, with the proposed mechanism involving an increased production of PIP_3, that in turn leads to the activation of protein kinase B (Nakamura et al., 2006). In addition to its role in neurons, NCS-1 has been shown to positively regulate insulin secretion in pancreatic cells and exocytosis in mast cells (Gromada et al., 2005; Kapp-Barnea et al., 2003). It would appear that NCS-1 is important in "professional secretory" cells. Due to the tissue variability of human ORPs it is unclear if any ORPs can influence the processes just mentioned. The observation that ORP1S can phenocopy *KES1* and that NCS-1/frequenin can phenocopy *FRQ1* (remembering *KES1* and *FRQ1* have opposing functions), raises the possibility that ORP1S (or other ORPs) expression in professional secretory cells has a significant role in regulating secretion.

The results show that there has been some conservation of function among the oxysterol binding protein homologs. ORP1S and ORP9S can both regulate secretion while only ORP1S can provide life to Osh-deficient yeast. In contrast, human ORP2 and ORP10S appear unable to regulate either. Due to the relative ease of experimentation with *S. cerevisiae*, the ability of human ORPs to complement yeast Osh functions (as they emerge) should help elucidate their specific functions.

The ability of "PC-only" Sec14 to rescue *sec14 cki1 ypt31* cells is still puzzling. Previously, it was suggested that "PC-only" Sec14 may retain some of its PI binding ability *in vivo* (Rudge et al., 2004b). That could be the case, though the characterization of the protein suggested that this was completely deficient in PI binding. It is possible that unliganded Sec14 still has function(s) that remain uncharacterized. The development of a Sec14 molecule unable to bind any phospholipids may provide insight. Another possibility is that the ability of "PC-only" Sec14 to remove and transfer PC from the Golgi may influence the cell. Removal of PC (a bulk phospholipid) from the Golgi would have the effect of increasing the localized concentration of PI-4P and DAG (pro-secretory lipids). Perhaps by engineering a permanent Golgi-membrane-bound Sec14 molecule would illuminate this situation. This could also allow researchers to determine if the ability of Sec14 to diffuse throughout the cell has an important role. Furthermore, if a

Golgi restricted Sec14 was successfully generated it could be useful in determining the reason why *sec14* cells accumulate vesicles at the plasma membrane.

The continued identification of precise roles of the lipid and protein machinery involved in TGN to plasma membrane transport in yeast will help further address some of the observations and theories presented in this thesis.

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