REGULATION OF LIPID METABOLISM AND MEMBRANE TRAFFICKING BY THE OXYSTEROL BINDING PROTEIN SUPERFAMILY MEMBER KES1

by

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ABSTRACT

The *Saccharomyces cerevisiae* oxysterol binding protein homologue Kes1/Osh4 is a member of an enigmatic class of proteins found throughout *Eukarya*. This family of proteins is united by a β-barrel structure that binds sterols and oxysterols. An N-terminal lid is thought to both sequester sterols inside the core and promote localization of Kes1 to regions of high membrane curvature *via* a predicted ArfGAP lipid packing sensor motif. Additionally, a phosphoinositide-binding region on a discrete surface of Kes1 has also been identified. In this thesis, structure-function analysis of Kes1 determined that phosphoinositide binding is required for membrane association *in vitro*, and *in vivo* phosphoinositide binding is required for localization to the Golgi. Ergosterol, the major sterol in *S. cerevisiae*, and membrane curvature had minimal effects on membrane association. This study also revealed a role for Kes1 in the regulation of both phosphatidylinositol-4-phosphate and phosphatidylinositol-3-phosphate homeostasis. Phosphoinositide and sterol binding by Kes1 are necessary for it to alter phosphatidylinositol-4-phosphate, but not phosphatidylinositol-3-phosphate homeostasis. Misregulation of phosphatidylinositol-4-phosphate homeostasis by Kes1 manifested itself in an inability of the v-SNARE Snc1 to traffic properly and was consistent with a defect in *trans*-Golgi/endosome trafficking. I went on to demonstrate a role for Kes1 in regulating the conversion of phosphatidylinositol-4-phosphate to phosphatidylinositol for the synthesis of sphingolipids, and I present a model for the role of Kes1 at the Golgi. Kes1 acts as a sterol sensor that regulates phosphatidylinositol-4-phosphate to sphingolipids metabolism, which ultimately regulates the delivery of proteins that assemble into lipid rafts for their transport from the Golgi to the plasma membrane. I also uncovered a previously unknown role for Kes1 in the regulation of the cytoplasm-to-vacuole and autophagy trafficking pathways, which is mediated by the ability of Kes1 to regulate phosphatidylinositol-3-phosphate homeostasis.

LIST OF ABBREVIATIONS USED

ALPS: ARF GAP lipid packing sensor
bp: base pair
CERT: ceramide transfer protein
CPY: carboxypeptidase Y
CVT: cytoplasm to vacuole
DAG: diacylglycerol
DHS: dihydrosphingosine
DIC: differential interference contrast
DMSO: dimethyl sulfoxide
DTT: dithiothreitol
EDTA: tthylenediaminetetraacetic acid
EM: electron microscopy
ENTH (Epsin-N-terminal homology)
ER: endoplasmic reticulum
FERM (4.1, Ezrin, Radixin, Moesin)
FFAT: two phenylalanines in an acidic tract
FYVE: Fab1, YOTB, Vac1, EEA1
GAL: galactose
GEF: guanine-nucleotide exchange factor
GFP: green fluorescence protein
GOLD: Golgi dynamics
GTP: guanosine triphosphate
g: gram
HIS6x: hexahistidine
HPLC: high performance liquid chromatography
IgG: immunoglobulin G
KANMX4: kanamycin cassette
Kbp: kilobase pairs
L: liter
LB: Luria-Bertani
LCB: long chain base
mg: milligram
μg: microgram
mL: milliliter
μl: microliter
min: minute
MIPC: mannose-inositol-phosphoceramide
$M(IP)_2C$: mannose-(inositol-P)$_2$-ceramide
MTOC: microtubule organizing center
MVB: multivesicular body
NATMX4: nourseothricin cassette
ORD: OSBP related domain
ORF: open reading frame

ORP: oxysterol related protein
ORP1L: ORP1 long
ORP1S: ORP1 short
ORP4S: ORP4 short
ORP4L: ORP4 long
ORP9L: ORP9 long
OSBP: oxysterol binding protein
OSH: oxysterol binding protein homologue
PAS: pre-autophagosomal structure
PBS: phosphate buffered saline
PC: phosphatidylcholine
PCA: perchloric acid
PCR: polymerase chain reaction
PE: phosphatidylethanolamine
PEG: polyethylene glycol
PH: pleckstrin homology
PHS: phytosphingosine
PI: phosphatidylinositol
PIP: phosphatidylinositol phosphate
PIP_2: phosphatidylinositol bis-phosphate
PI-3P: phosphatidylinositol-3-phosphate
PI-4P: phosphatidylinositol-4-phosphate
PI-3,4P_2: phosphatidylinositol-3,4-bisphosphate
PI-3,5P_2: phosphatidylinositol-3,5-bisphosphate
PI-4,5P_2: phosphatidylinositol-4,5-bisphosphate
PI-3,4,5P_3: phosphatidylinositol-3,4,5-triphosphate
PITP: PI transfer protein
PLC: phospholipase C
PMSF: phenylmethylsulfonyl fluoride
PS: phosphatidylserine
PTB: phosphotyrosine binding domain
PVDF: polyvinylidene fluoride
PX: phox domain
RAF: raffinose
RILP: Rab7-interacting lysosomal protein
RFP: red fluorescence protein
SC: synthetic complete
S. cerevisiae: Saccharomyces cerevisiae
SD: synthetic defined
SDS: sodium dodecyl sulfate
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec: seconds
SEC: secretion
SGA: synthetic genetic array
SNARE: soluble NSF attachment receptor
StAR: steroidogenic acute regulatory protein

START: StAR related lipid transfer
TAE: tris base, acetic acid, EDTA
TAP: tandem affinity protein
TEV: tobacco Etch Virus
TLC: thin layer chromatography
TOR: target of rapamycin
VAP: VAMP associated protein
VLCFA: very long chain fatty acid
YPD: yeast extract peptone dextrose
YFG: your favourite gene
YNB: yeast nitrogen base

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It is very hard to come to realization that I have reached a point in my life where I can proudly say that I am writing and defending my PhD thesis. When I first started my career in research I never thought I would be able to reach this point. However, attaining this goal in my life would have been impossible without the help and support of many wonderful people. I would like to take a moment to thank all of these people.

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CHAPTER 1 INTRODUCTION

The Golgi apparatus plays a central role in the regulation of vesicular transport pathways and is thus crucial to intracellular trafficking of proteins and membranes. The integrity of many cellular membranes and compartments is dependent on the proper trafficking of molecules to and from the Golgi. An important characteristic of these membranes is that they are comprised of a specific variety of lipids. However, how lipid metabolism and vesicular trafficking are inter-related is still poorly understood.

A role for lipids in the regulation of Golgi-derived secretion was identified in *Saccharomyces cerevisiae* when Sec14 was shown to be essential for vesicular transport from the Golgi apparatus (Bankaitis et al., 1990). Sec14 was classified as a phosphatidylinositol (PI) transfer protein (PITP) as it has the ability to bind phosphatidylcholine (PC) and PI and transfer them between liposomal membranes *in vitro* (Bankaitis et al., 1990). However, while PI and PC binding are certainly components of Sec14 function, it seems that its transfer activity is not relevant *in vivo*. Instead, Sec14 acts as a sensor of membrane composition, which in turn regulates PC and PI metabolic pathways (Ile et al., 2006). Consequently, this provides an appropriate lipid environment at the Golgi that is crucial for vesicular transport (Ile et al., 2006). Nonetheless, inactivation of other lipid metabolic genes can alleviate the essential requirement for Sec14 function. *OSH4*, also known as *KES1*, is one such gene.

Kes1 is a member of the oxysterol binding protein family (OSBP) in *S. cerevisiae* (Cleves et al., 1991). *S. cerevisiae* has seven OSBP homologues collectively known as Oxysterol binding protein homologues (Osh) proteins that are identified by a common structural domain that binds sterols and oxysterols (Beh et al., 2001). The Osh protein

family is thought to have an overlapping essential function related to regulation of sterol metabolism (Beh et al., 2001). Kes1 is unique among the Osh family as it is the only Osh protein involved in Sec14-mediated regulation of vesicular transport at the Golgi (Beh et al., 2001). The studies described in this thesis investigated how Kes1 regulates lipid metabolism and how this affects membrane trafficking.

1.1 OVERVIEW OF OSBPS IN *S. CEREVISIAE*

S. cerevisiae contains seven Osh proteins (Beh et al., 2001). All seven Osh proteins contain a C-terminal OSBP related domain (ORD domain). Part of this ORD domain is the signature motif, which is the most conserved region between all family members (Beh et al., 2001). Four of the Osh proteins (Osh4-Osh7) only contain the ORD domain and are referred to as short Osh proteins. The remaining three Osh proteins (Osh1-Osh3) are known as the long Osh proteins, and contain additional N-terminal domains (Figure 1) (Beh et al., 2001). These include pleckstrin homology (PH) domains, anykrin repeats, Golgi dynamic domains (GOLD), and two phenylalanines in an acidic tract (FFAT) motifs (Beh et al., 2001; Loewen and Levine, 2005). The FFAT motif mediates an interaction with an endoplasmic reticulum (ER)-resident VAMP-associated protein (VAP) homologue, Scs2 (Loewen et al., 2003). Ankyrin repeats and GOLD domains are also involved in protein-protein interactions (Lehto and Olkkonen, 2003; Schmalix and Bandlow, 1994), while PH domains mediate binding to phosphatidylinositol phosphates (PIPs) and proteins. The presence of different protein- and lipid-binding domains in Osh proteins suggests that these proteins may be able to shuttle between organelles depending on cellular conditions (Ridgway et al., 1992). Interestingly, Kes1, a short Osh protein containing only the ORD domain, has also

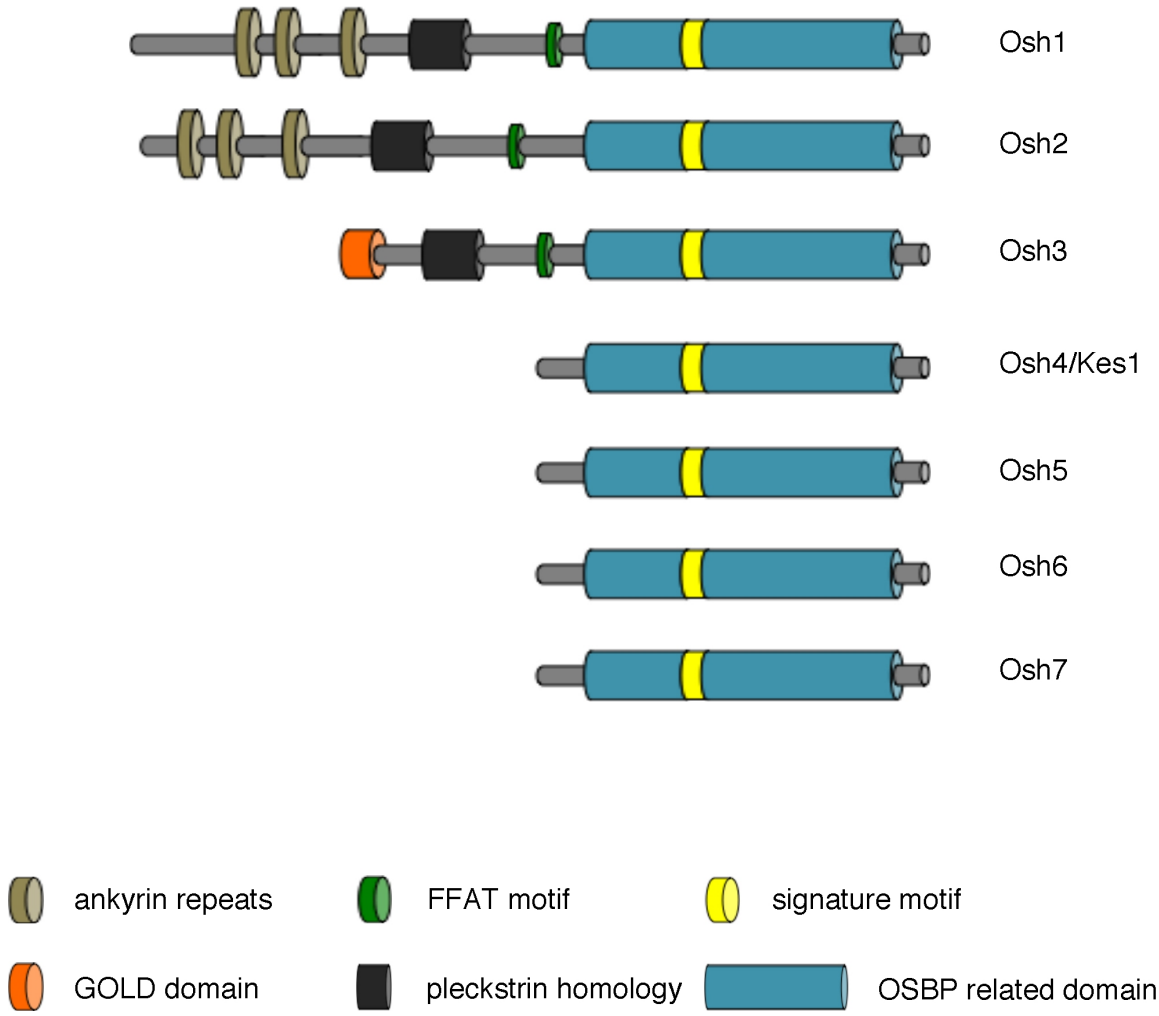


Figure 1 ***S. cerevisiae*** **Osh protein family**. Blue, OSBP related domain (ORD); yellow, signature motif; black, pleckstrin homology (PH) domain; green, two phenylalanines in an acids tract (FFAT) motif ; brown, ankyrin repeats; orange, Golgi dynamics (GOLD) domain.

demonstrated an ability to bind PIPs (Raychaudhuri et al., 2006; Wang et al., 2005a). This affinity of Kes1 for phosphatidylinositol-4-phosphate (PI-4P) is thought to be partially responsible for directing Kes1 to the Golgi (Li et al., 2002a).

The disruption of any six *OSH* genes in combination has minimal to mild affects on cellular growth (Beh et al., 2001). However, inactivation of all seven *OSH* genes results in a substantial decrease in growth, indicating that the Osh proteins most likely share an overlapping essential function (Beh et al., 2001; Beh and Rine, 2004). Additional defects observed upon loss of function of all seven Osh proteins include a 3.5-fold increase in ergosterol level, ergosterol accumulation in intracellular compartments, and decreased endocytosis (Beh and Rine, 2004). Since the ORD is highly conserved between all Osh proteins it is likely that the shared ancestral function of sterol binding underlies the essential function of the Osh family. Whether this function involves the regulation of ergosterol metabolism has yet to be determined. In addition to this shared function, the presence of unique domain combinations within the Osh protein family allows each protein to regulate specific cellular functions. However, these properties are only beginning to be uncovered.

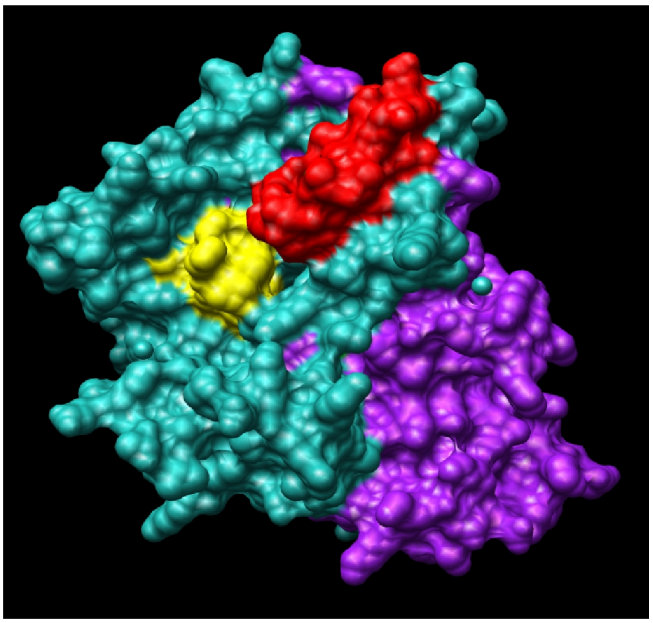
Recent studies of Osh6 and Osh7 suggest that these proteins influence endosomal sorting. This occurs through their regulation of Vps4, an endosomal AAA-ATPase required for efficient transport within the multivesicular body (MVB) sorting pathway (late endosome pathway) (Wang et al., 2005b). Osh6 is a short Osh protein comprised of the ORD domain for sterol binding and a putative coiled-coil motif for protein-protein interaction (Wang et al., 2005a). Both domains are essential for Osh6 function in regulation of vesicular transport. Osh7 and Osh6 directly interact with Vps4, but in the

presence of ergosterol the interaction is abolished (Wang et al., 2005b). Deletion of *VPS4* increases Osh6 and Osh7 interaction with cellular membranes, and decreases sterol esterification. Furthermore, overexpression of *OSH7* in cells that have an inactivated *VPS4* results in no sterol esterification defect. This suggests that Osh6 and Osh7, along with Vps4, are involved in transport of ergosterol out of the endosomal pathway and into an esterified-sterol storage pool (Wang et al., 2005b).

1.1.1 Structure of Kes1 in *S. cerevisiae*

Roles of other Osh proteins remain to be described. Much of our knowledge about the Osh family of proteins, both biochemical and genetic, has come from studies of Kes1. A crystal structure of Kes1, with and without sterols bound to the core of the protein, was previously solved (Figure 2) (Im et al., 2005). The core of Kes1 is a central antiparallel β-sheet of 19 strands (residues 115-293) that forms a near complete anti-parallel β-barrel (Im et al., 2005). Sterols bind to the central tunnel of the β-barrel in a head-down orientation with the 3-hydroxyl buried at the bottom of the tunnel. The side chain touches the inner surface of the N-terminal lid. Unlike other sterol-binding proteins, there are no direct hydrogen bonds between sterol hydroxyl groups and conserved amino acid side-chains. The N-terminal lid, residues 1-29, is flexible and when Kes1 is bound to sterols it protects the ligand, within the central tunnel of the β-barrel, from the solvent. It was suggested that the lid is flexible since no Kes1 crystal structure could be obtained in the absence of sterol unless the first 29 amino acids were removed (Im et al., 2005). As well, the crystal structure showed that Kes1 does not contain a PH domain within the ORD as was previously thought (Li et al., 2002a). On the exterior surface around the lid there are

A



B

Figure 2 **Crystal structure of Kes1 with known domains**. Red, N-terminal lid (amino acid 2-29); purple, phosphoinositide-binding domain; yellow, OSBP related domain (ORD) domain; green, amino acids that interact with ergosterol; (B) pink, ergosterol

six highly conserved basic residues available for phosphate binding when Kes1 is not bound to sterols (Im et al., 2005).

Point mutations of Kes1 were made altering the basic residues of the tunnel entrance, the structural core, the lid, and the sterol-binding domain to correlate structure with biological activity (Im et al., 2005). Residues K109, H143-H144 and K336, near the tunnel entrance, were found to be essential for function *in vivo*, while basic residues farther from the tunnel entrance, K168A and R344, were not. *In vitro*, mutants K109A, K168A and K336A caused a significant decrease in basal cholesterol transport by the mutant Kes1 derivatives, while the double substitution H143-144A only caused a small decrease in transport. However, this major biochemical defect in sterol transport did not always correlate with decreased function within cells (Im et al., 2005). Other substitutions within the tunnel (Y97F) and within the sterol-binding domain (L111D and E117A) significantly decreased sterol binding and transport *in vitro*, and biological activity *in vivo* was abolished (Im et al., 2005). This data indicates that sterol binding within the core of the protein is required for Kes1 function in the cell.

1.1.2 Kes1 Binding to Membranes

Originally, Kes1 was found to localize predominantly to the yeast cytosol (Fang et al., 1996). Later investigation using microscopy and a functional Kes1-green fluorescent protein (GFP) chimera showed that Kes1 is diffused throughout the cytosol and also localized to punctate spots that were determined to be the Golgi apparatus (Fairn et al., 2007; Li et al., 2002a). Studies with deletion and point mutants of Kes1 and its mammalian homologue, ORP1 Short (ORP1S), led to the identification of a PIP-binding site (primarily for PI-4P and phosphatidylinositol-4,5-bisphosphate (PI-4,5P_2)) that is

separate from the sterol-binding site (Fairn and McMaster, 2005; Knodler and Mayinger, 2005; Li et al., 2002a). Binding of PIPs by Kes1 is hypothesized to promote its association with Golgi membranes (Fairn et al., 2007). Inactivation of Pik1, the Golgi PI-4 kinase, also causes Kes1 to localize to the cytoplasm from the Golgi and in fact, when Kes1 cannot bind PIPs it can no longer regulate Sec14 function (Fairn and McMaster, 2005; Knodler and Mayinger, 2005; Li et al., 2002a). This data imply that PI-4P binding is required for both Kes1 function and Golgi localization. Kes1 may indeed be one of the most abundant PI-4P-binding proteins in yeast cells (Knodler and Mayinger, 2005). *S. cerevisiae* contains ~ 32 000 molecules of Kes1 per cell, while all the other Osh proteins combined make up ~ 10 000 molecules per cell (Ghaemmaghami et al., 2003).

Kes1 is also unique among Osh proteins in that it is predicted to possess an ArfGAP1 lipid-packing sensor (ALPS) motif in the N-terminal lid (residues 7-29) (Drin et al., 2007). The ALPS motif is a helix that preferentially binds to model lipid membranes that are highly curved (Drin et al., 2007). The full-length Kes1 protein associates best to curved membranes, and a peptide comprising of residues 1-29 of Kes1 showed increased curvature-dependent binding to these liposomes. Thus, the presence of an ALPS motif within the N-terminus of Kes1 provides a mechanism for targeting Kes1 to specific membranes. This would include areas of low lipid packing such as Golgi-derived vesicles (Drin et al., 2007).

Taken together, these findings suggest that Kes1 can bind membranes *via* its PIP-binding region and ALPS motif. However, how these lipid-and membrane-binding activities of Kes1 are coordinated to regulate vesicular transport at the Golgi is not known.

1.2 PHOSPHOINOSITIDE METABOLISM IN *S. CEREVISIAE*

PI is an essential phospholipid in eukaryotic cells and plays a major role in the composition of biological membranes (Nikawa and Yamashita, 1997). Phospholipids are characterized by a glycerol backbone with two long-chain fatty acids at the first two positions of the glycerol backbone, and a phosphate serving as a linker for various head groups at the third position. PI contains inositol as the head group, while PC, the most abundant phospholipid in most eukaryotic cells, contains choline. PI is unique among head groups in that it can be further modified by the addition of phosphates at specific positions on the inositol ring to produce PIPs (Figure 3). The various PIPs are involved in multiple cellular processes such as vesicular trafficking, glycolipid anchoring of proteins (Shields and Arvan, 1999), signal transduction (Divecha and Irvine, 1995), and mRNA export from the nucleus (Odom et al., 2000).

1.2.1 Inositol Uptake into *S. cerevisiae*

Uptake of inositol from the medium is mediated by the major inositol permeases Itr1 and Itr2 (Figure 4) (Nikawa et al., 1991). The levels of inositol in the medium regulate both the transcription of the *ITR1* gene and the activity of the Itr1 protein. Therefore, removing inositol results in an up-regulation of *ITR1* transcription and an increase in Itr1 activity (Lai and McGraw, 1994; Nikawa et al., 1991). However, *ITR1* mRNA levels drop drastically upon addition of inositol to the medium, and within 2 hours, the uptake of inositol is almost completely down regulated (Lai and McGraw, 1994). In the absence of exogenous inositol, *S. cerevisiae* has the ability to synthesize inositol from glucose.

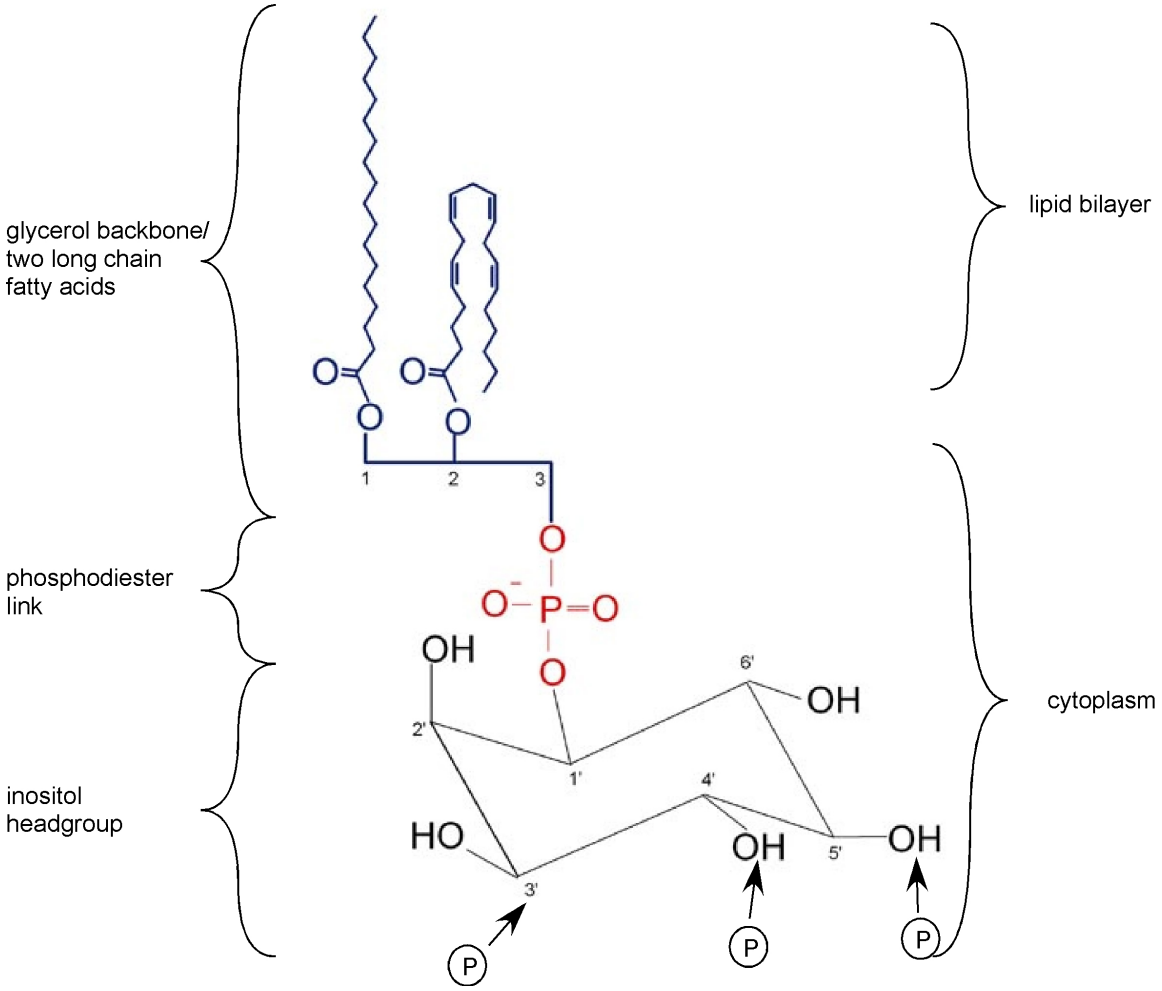


Figure 3 **PI structure**. The inositol headgroup is linked *via* a phosphodiester bond to diacylglycerol- a glycerol backbone with two long-chain fatty acids. The hydroxyls in position 3, 4 and 5 are available for phosphorylation by kinases. The phosphorylated inositol headgroup can be dephosphorylated by phosphatases. The fatty acids are embedded in the lipid bilayer while the headgroups are located in the cytoplasm.

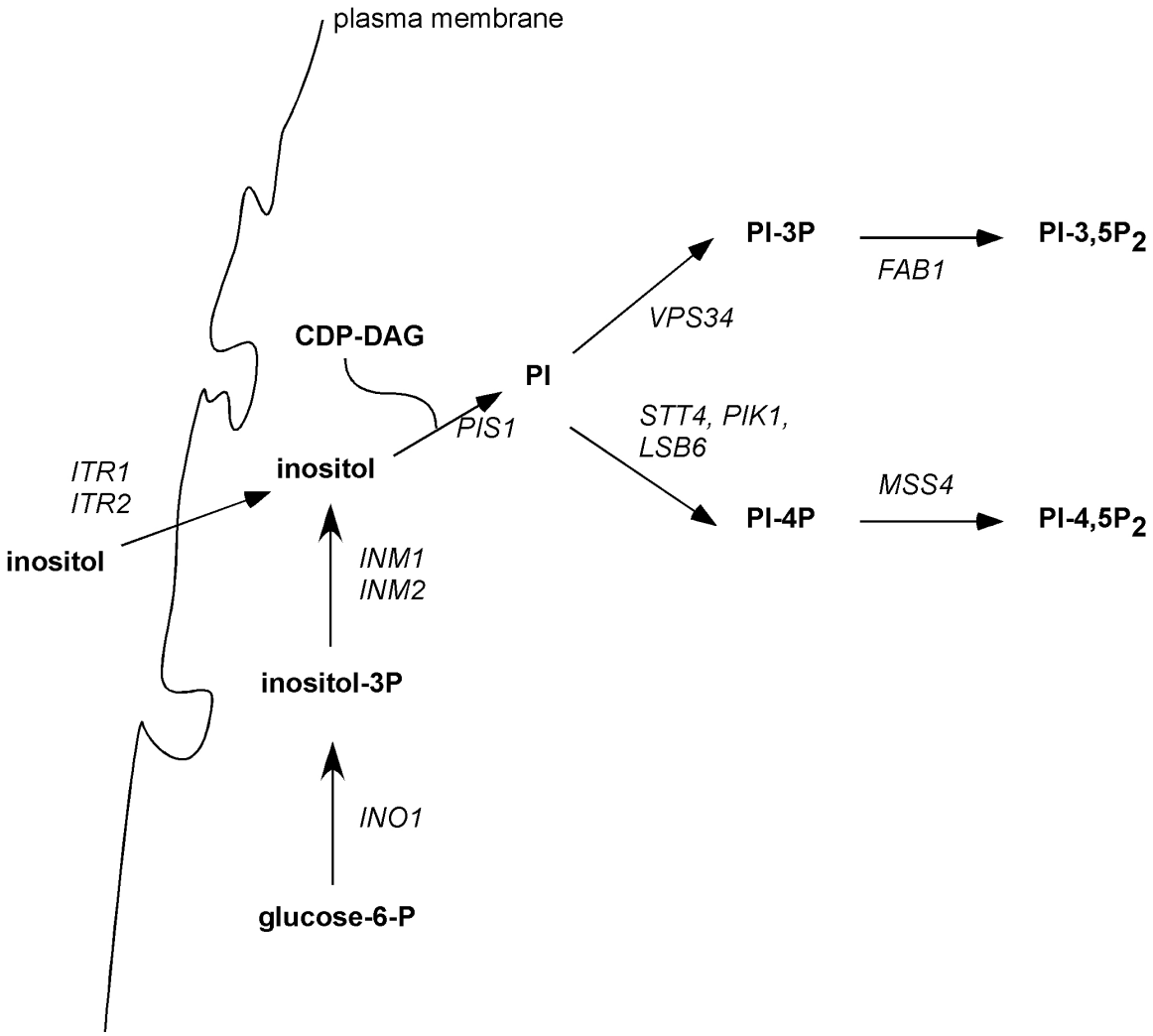


Figure 4 **PI and PIP synthesis in *S. cerevisiae***. The structural genes that encode the enzymes involved in the synthesis of PI and PIPs are indicated in italics. Intermediates are in bolded font.

1.2.2 *De Novo* Synthesis of Inositol and PI

The absence of inositol results in *INO1* being fully expressed (Hirsch and Henry, 1986). *De novo* synthesis of inositol requires *INO1*, a structural gene that encodes inositol-3-phosphate synthase, which converts glucose-6-phosphate to inositol-3-phosphate (Figure 4) (Donahue and Henry, 1981; Klig and Henry, 1984). In turn, inositol-3-phosphate is dephosphorylated to produce inositol (Culbertson et al., 1976; Murray and Greenberg, 2000).

Synthesis of PI (either from inositol uptake or *de novo* synthesis of inositol) is through the CDP-diacylglycerol (DAG) pathway (Figure 4). The PI synthase, Pis1, is localized to the endoplasmic reticulum (ER) and synthesizes PI from CDP-DAG and inositol (Fischl et al., 1986).

In the absence of inositol in the medium, PI comprises 10-12% of the total phospholipid pool. However, upon adding inositol, the PI level increases to 27-30% of the total phospholipid (Gaspar et al., 2006; Kelley et al., 1988). This increase is thought to be due to the fact that the K_m of Pis1 for inositol is 210 μM, almost 10-fold higher than the actual intracellular concentration of inositol (24 μM). This extra inositol supply from the growth medium would therefore affect cellular Pis1 activity (Fischl et al., 1986; Gaspar et al., 2006; Kelley et al., 1988).

1.2.3 PI Phosphorylation and Dephosphorylation

Although PI is a significant component of *S. cerevisiae* phospholipid content, PIPs are present in much lower concentrations, comprising less than 1% of cellular

phospholipid mass. However, small changes in PIP concentration can lead to profound effects on the cell.

The six-membered inositol ring of PI has three hydroxyl groups (positions 3, 4 and 5) available for phosphorylation in a cell by various PI and PIP kinases. (Figure 3) (Balla, 2005). Phosphorylation of PI is a reversible covalent modification as the phosphate groups can be removed by PIP phosphatases. The PI kinases of *S. cerevisiae* are specific for a single substrate, while the PIP phosphatases tend to show more promiscuity in substrate selection (Carpenter and Cantley, 1996; Fruman et al., 1998; Matsuura-Tokita et al., 2006). Therefore, there are many different combinations of phosphorylation/dephosphorylation events that can produce numerous PIPs.

The *S. cerevisiae* genome contains three genes whose products possess PI-4 kinase activity: *LSB6*, *PIK1* and *STT4* (Figure 4). However, only *STT4* and *PIK1* are essential for cell viability, with each responsible for the production of almost half of the cellular PI-4P pool (Audhya et al., 2000; Cutler et al., 1997; Han et al., 2002; Walch-Solimena and Novick, 1999). Pik1 is a 199 kDa soluble enzyme that has a K_m of 100 μM for PI (Flanagan and Thorner, 1992). Pik1 is known to localize at both the Golgi and nucleus (Strahl et al., 2005), and production of PI-4P by Pik1 is vital for secretion from the Golgi (Walch-Solimena and Novick, 1999). Proper localization of Pik1 to the Golgi, *in vivo*, requires its direct protein-protein interaction with Frq1, a small calcium-binding protein (Strahl et al., 2005). Frq1 is also important for stimulating Pik1 activity *in vivo* and *in vitro* (Hendricks et al., 1999) (Strahl et al., 2005). Stt4 is a 215 kDa soluble enzyme that localizes to the plasma membrane (Audhya and Emr, 2002). Stt4 produces a PI-4P pool that is required for both cell wall integrity and maintenance of vacuoles

(Audhya et al., 2000). Lsb6, the non-essential PI-4 kinase, is a soluble 55 kDa enzyme that can localize to the plasma membrane and the vacuole (Han et al., 2002). While overexpression of Lsb6 can restore growth to cells that harbor a temperature-sensitive *STT4* allele, it cannot rescue a *PIK1* temperature-sensitive allele (Han et al., 2002). PI-4P produced by Pik1, Stt4 or Lsb6 can be used by Mss4 (a PI-4P kinase) as a substrate for PI-4,5P_2 synthesis (Figure 4) (Desrivieres et al., 1998). Mss4 is located at the plasma membrane and at the nucleus and is an essential protein involved in actin cytoskeleton organization and cell integrity (Audhya and Emr, 2003).

Other PIPs are also important in various cellular processes. Vps34 utilizes PI as a substrate to produce phosphatidylinositol-3-phosphate (PI-3P) (Figure 4) (Schu et al., 1993). The PI-3P pool produced by Vps34 is enriched in endosomes and contributes to functions in endosome sorting, trafficking of vesicles to the vacuole, and autophagy (Stack and Emr, 1994; Wurmser and Emr, 1998). Subsequent phosphorylation of PI-3P by Fab1 produces phosphatidylinositol-3,4-bisphosphate (PI-3,5P_2) (Yamamoto et al., 1995). PI-3,5P_2 is highly enriched in vacuolar membranes, and appears to be involved in MVB (late endosome) formation and sorting (Rudge et al., 2004). Although two additional PIPs (phosphatidylinositol-3,4-bisphosphate (PI-3,4P_2) and phosphatidylinositol-3,4,5-triphosphate) (PI-3,4,5P_2)) are known to be synthesized in eukaryotic cells, these have yet to be detected in *S. cerevisiae* (Odorizzi et al., 2000).

PIPs are dephosphorylated in the cell by two types of enzymes, phospholipase C (Plc1) and PIP phosphatases. Plc1 yields diacylglycerol (DAG) and inositol-1,4,5P_3 by hydrolyzing PI-4,5P_2 (Flick and Thorner, 1993; York et al., 1999). In mammalian cells, DAG activates protein kinases. However, a DAG-activated protein kinase has yet to be

identified in yeast. In *S. cerevisiae* inositol-1,4,5P_3 is further phosphorylated to inositol-P_4 – inositol-P_6, with these metabolites regulating transcription and mRNA export out of the nucleus (Flick and Thorner, 1993; York et al., 1999).

Seven PIP phosphatases have been characterized in *S. cerevisiae* (Rudge et al., 2004). These phosphatases are categorized into three different subgroups, based on the catalytic activity of their phosphatase domains. The first group is comprised of five enzymes containing a 'Sac1 domain', which include Sac1, Fig4, Inp51/Sjl1, Inp52/Sjl2 and Inp53/Sjl3 (Erdman et al., 1998; Hughes et al., 2000; Novick et al., 1989). The second group consists of four enzymes each containing an inositol polyphosphate 5-phosphatase domain and are responsible for hydrolyzing phosphates at the D5 position (Whisstock et al., 2002). This group includes Inp51/Sjl1, Inp52/Sjl2, Inp53/Sjl3 and Inp54 (Luo and Chang, 1997; Srinivasan et al., 1997; Stolz et al., 1998a; Stolz et al., 1998b). The last group consists only of Ymr1 (Taylor et al., 2000), a protein that shows considerable homology to mammalian myotubularins (Clague and Lorenzo, 2005). Unlike kinases in yeast, these enzymes show promiscuity for their substrates, which results in overlapping physiological functions among the phosphatases. Indeed, three of the phosphatases (Sac1, Sjl2/Inp52 and Sjl3/Inp53) hydrolyze PI-3P, PI-4P and PI-3,5P_2 (Foti et al., 2001); Sjl1 and Inp54 are specific for the phosphate group at position 5 in PI-4,5P_2 (Wiradjaja et al., 2001); Fig4 is specific for PI-3,5P_2 (Rudge et al., 2004), and Ymr1 is specific for PI-3P (Parrish et al., 2004).

1.2.4 Role of PIPs in Vesicular Trafficking

A role for PI and its derivatives in vesicle-mediated membrane trafficking was first shown through studies with *S. cerevisiae*. Sec14, an essential protein required for

Golgi-derived secretion, was determined to be a PI transfer protein (Bankaitis et al., 1990) and Vps34, a PI-3 kinase (Schu et al., 1993), was shown to be necessary for transport of vesicles between the Golgi apparatus and vacuoles (Herman and Emr, 1990). A link between PI-4P generated from Pik1 and vesicular trafficking was also demonstrated when temperature-sensitive *PIK1* mutants were found to be defective in Golgi-derived secretion at the non-permissive temperature (Hama et al., 1999). The role of PIPs in regulating vesicular trafficking is thought to be *via* the ability of these lipids to recruit proteins to intracellular membranes enriched in a particular PIP (Haslam et al., 1993). PIPs can also regulate endocytosis *via* their involvement in alterations in actin dynamics (Di Paolo and De Camilli, 2006).

PIPs can be recognized and bound by several modular protein domains: these include the PH domain; phosphotyrosine-binding (PTB) domain; Zinc Finger in Fab1, YOTB, Vac1 and EEA1 (FYVE) domain; Epsin-N-Terminal homology (ENTH) domain; phox (PX) domain; and 4.1 EZRIN, Radixin and Moesin domain, as well as polybasic regions in proteins (Balla, 2005). However, knowledge of PIP-binding proteins that regulate vesicular transport, and their mechanisms of action, is scarce.

1.2.5 Role of PIPs in Kes1 Function

Kes1 has been demonstrated to bind a variety of PIPs *in vitro*, with residues 171-317 being sufficient for phosphoinositide-binding (indicated in Figure 2 as phosphoinositide binding domain) (Fairn and McMaster, 2005; Li et al., 2002a). Kes1 proteins with substitutions at residues 236, 242 and 243 are compromised for binding to PIPs and *in vivo* studies using this triple mutant have demonstrated that PIP binding is required for Kes1 to be functional (Li et al., 2002).

In addition to the ability of Kes1 to bind PIPs, there is evidence supporting Kes1 regulation of PIP levels (Fairn et al., 2007). In situations in which PI-4P levels are decreased (*pik1*^{ts} or *sec14*^{ts}), inactivating *KES1* restores PI-4P levels to near wild-type values (Fairn et al., 2007). Kes1 is unique in that it is the only Osh protein known to regulate vesicular transport at the Golgi, presumably *via* its regulation of PI-4P function. As previously noted, Kes1 is normally localized to the Golgi and in situations where Golgi PI-4P synthesis is decreased, Kes1 is no longer at the membrane. Together, these findings implicate PI-4P as a major determinant of Kes1 function.

Thus, Kes1 function is clearly modulated by PIP binding, and conversely Kes1 affects cellular PIP levels. However, how Kes1 regulates cellular PIP levels and how PIP binding by Kes1 affects its function are not known.

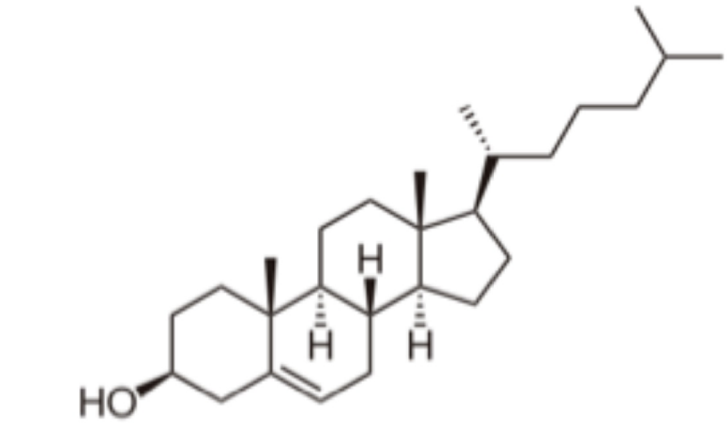
1.3 Sterol Synthesis and Transport in *S. cerevisiae*

Sterols are important components of eukaryotic cell membranes, and as such influence a wide variety of cellular processes (Liscum and Munn, 1999). Variations in sterol concentration within a membrane affects fluidity, and can lead to alterations in signal transduction, membrane trafficking, and the function of integral membrane proteins such as ion channels (Liscum and Munn, 1999; McIntosh and Simon, 2006). Sterols are heterogeneously distributed within the cell, with the highest enrichment of sterols found in the plasma membrane where 60-80 % reside (Liscum and Munn, 1999). The enrichment of sterols in the plasma membrane is maintained through regulation of their rate of synthesis, transport between organelles, and acylation to steryl esters (Holthuis et al., 2003).

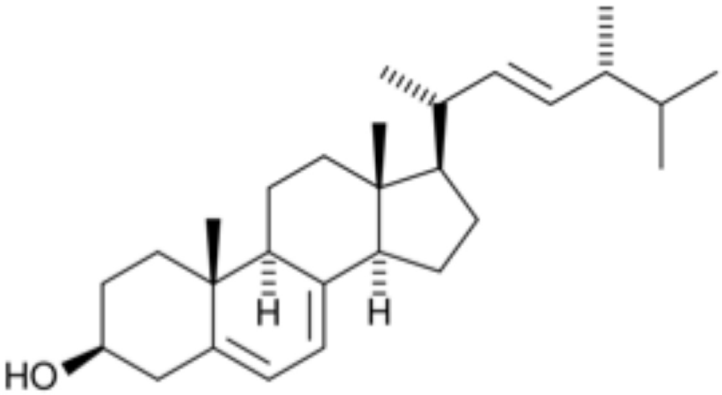
Sterols are synthesized in the endoplasmic reticulum and subsequently transported to the plasma membrane (Holthuis et al., 2003). Sterol transport back into the cell from the plasma membrane is accompanied by constant sorting in and out of transport vesicles (Holthuis et al., 2003). Recently, non-vesicular transport of sterols between donor and acceptor membranes has also been proposed (Maxfield and Menon, 2006). The budding yeast *S. cerevisiae* has been used as a model organism for the study of sterol transport, since vesicular-transport pathways in yeast are similar to those in mammalian cells (Daum et al., 1998). Furthermore, the metabolic pathways for sterol synthesis are also largely conserved between yeast and mammals (Daum et al., 1998).

1.3.1 Ergosterol Biosynthesis and Transport to Plasma Membrane

Ergosterol is the main sterol in *S. cerevisiae*, while cholesterol is the main sterol in mammalian cells (Bagnat et al., 2000). The structure of ergosterol is similar to that of cholesterol but contains two extra double bonds and a methyl group (Figure 5). Ergosterol has similar properties to cholesterol, including an effect on reducing the fluidity of membranes (Bagnat et al., 2000). Ergosterol synthesis begins in the ER by the Erg set of enzymes, and the resulting ergosterol is subsequently transferred to the plasma membrane to provide the high sterol concentration found at this location (Figure 6) (Athenstaedt and Daum, 2006; Daum et al., 1998). Cytoplasmic lipid particles, also known as lipid droplets, are also associated with Erg enzymes (Athenstaedt and Daum, 2006). Lipid droplets are cellular organelles made of a neutral lipid core (steryl esters and triacylglycerol) surrounded by a phospholipid monolayer containing both membrane-anchored and peripheral proteins (Athenstaedt and Daum, 2006; Zinser et al., 1993). Transport to and from these lipid droplets seems to be specific for ergosterol and certain



cholesterol



ergosterol

Figure 5 Structures of cholesterol and ergosterol. Ergosterol has two extra double bonds and an extra methyl group when compared to cholesterol.

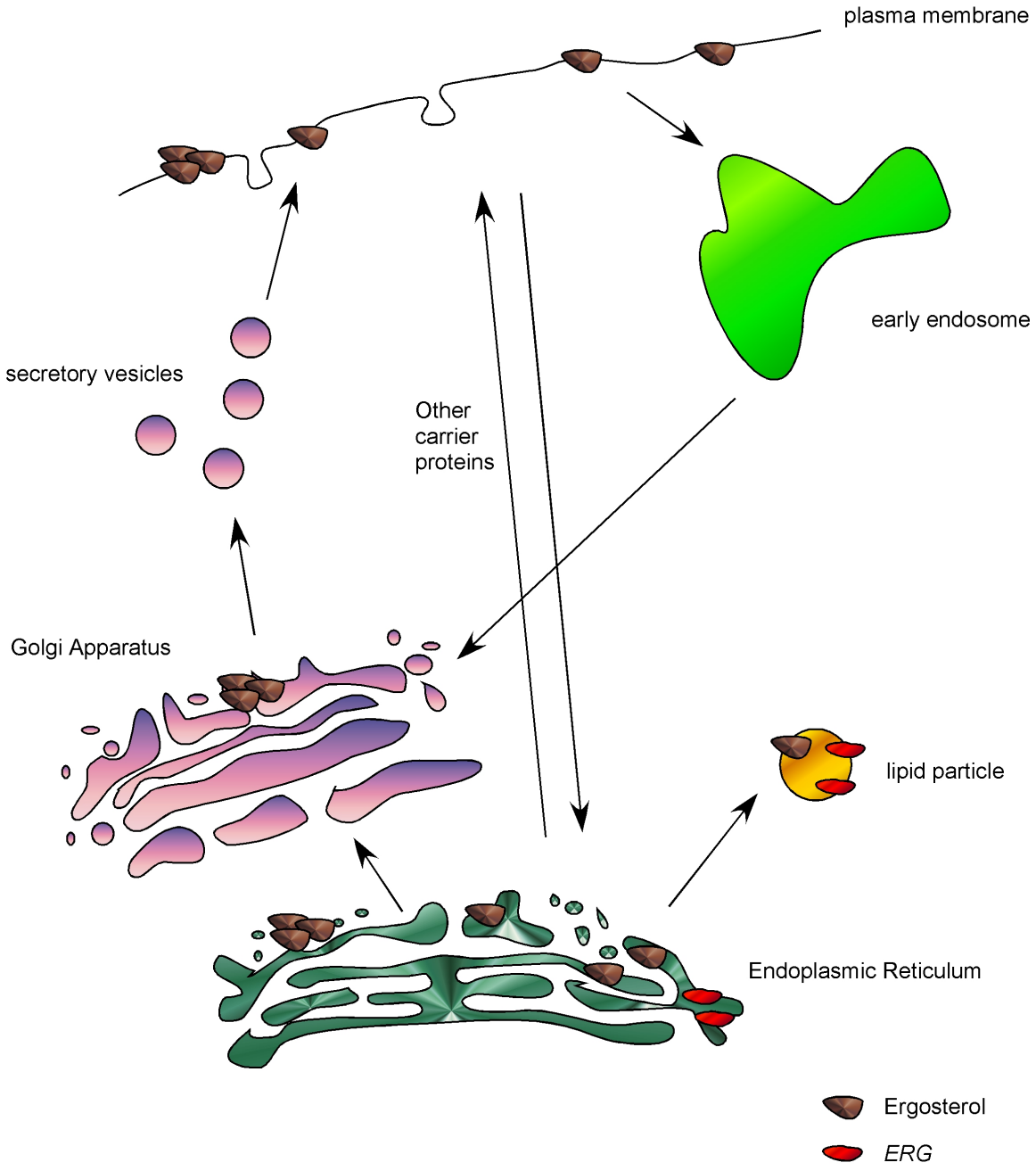


Figure 6 **Sterol transport in *S. cerevisiae***. Known pathways of vesicular transport and proposed non-vesicular sterol transport. Ergosterol synthesis begins in the ER by enzymes of the Erg pathway. Ergosterol may be 1) converted to steryl esters for storage in lipid particles; 2) transported to the cell surface, possibly by carrier proteins such as Osh proteins; or 3) sent to the Golgi for subsequent transport to the plasma membrane in secretory vesicles. Plasma membrane ergosterol can be internalized by endocytosis for transport to the early endosome and then to either the Golgi or vacuole (not shown).

sterol intermediates (Ott et al., 2005); however, precise details concerning the function and regulation of these pathways remain unknown.

Cells harbouring temperature-sensitive mutations in their secretory trafficking pathways are still capable of rapidly transporting ergosterol from the ER to the plasma membrane. Thus it has been suggested that a mechanism for non-vesicular transport of sterols may exist (Baumann et al., 2005). Yet, the need for efficient and regulated transfer of sterols suggests transport proteins would be responsible for mediating non-vesicular transport, instead of simple diffusion (Baumann et al., 2005).

1.3.2 Exogenous Sterol Uptake and Transport

Oxygen is required for endogeneous sterol synthesis in *S. cerevisiae*; however, exogenous uptake of sterols is enhanced dramatically under anaerobic conditions (Ness et al., 1998). Uptake of exogenous sterols can also be enhanced in the presence of oxygen when there is a mutation affecting the transcription factor Upc2, or when the transcription factor Sut2 is over-expressed (Bourot and Karst, 1995; Lewis et al., 1988). Upc2 is involved in regulating the expression of some *ERG* genes, while the *SUT1* gene is expressed in the absence of oxygen (Bourot and Karst, 1995; Lewis et al., 1988). How these proteins facilitate sterol uptake is poorly understood. However, a non-vesicular pathway for internalization of sterols from the plasma membrane to the ER has been proposed, since endocytosis mutants can properly esterify exogenously supplied sterols at the ER (Li and Prinz, 2004).

1.3.3 Sterols and Endocytosis

S. cerevisiae cells with mutations in *ERG* genes display defects in endocytosis. It is not clear if this is due to a lack of ergosterol needed for the function of endocytic mediators, or due to the physical defects of membranes that are associated with the accumulation of sterol intermediates when ergosterol synthesis is inhibited (Heese-Peck et al., 2002; Munn et al., 1999).

1.3.4 Non Vesicular Transport of Sterols by Kes1

Since Kes1 can bind to sterols within its core it was hypothesized that Osh proteins could function as sterol transporters (Im et al., 2005). As previously mentioned, endocytic mutants can transport sterols from the plasma membrane to the ER. In addition, newly formed sterols can be transported from the ER to the plasma membrane even when there are blocks in vesicular transport (Baumann et al., 2005). This observation suggests that a protein-mediated non-vesicular transport pathway could exist. Steroidogenic acute regulatory protein (StAR) related lipid transfer (START) domain proteins are thought to transport sterol this way in mammalian cells (Barros et al., 2005). Since the START-domain proteins and Kes1 demonstrate a similar tertiary structure, it has been suggested that Osh proteins could be sterol transporters (Figure 6) (Tsujishita and Hurley, 2000).

In vitro experiments have shown that Kes1 can bind cholesterol and oxysterols with dissociation constants of 0.3 μM and 0.05 μM, respectively (Im et al., 2005). Kes1 was also shown to be able to extract cholesterol from liposomes and transfer the ligand to acceptor membranes (Raychaudhuri et al., 2006). All but one sterol-binding point mutant examined (H143-144A at the mouth of the tunnel) showed a decreased ability in extracting and transporting cholesterol (Raychaudhuri et al., 2006). An *in vivo* assay that

measured the uptake of radiolabeled sterols from the medium, and subsequent conversion to sterol esters in the ER (Raychaudhuri et al., 2006), was used as a surrogate measure for non-vesicular transport of sterol. Cells that were lacking all Osh proteins showed a decrease in sterol esterification (~50%), with Osh3 and Osh5 being the largest contributors to this process (Raychaudhuri et al., 2006). Deleting the other *OSH* genes individually, including *KES1*, showed little to no affect on sterol transfer (Raychaudhuri et al., 2006).

In vitro, Kes1 can also extract and transfer PI-4,5P_2, but less efficiently than cholesterol, suggesting that Osh proteins may transfer lipids other than sterols. As well, PI-4,5P_2 stimulated sterol transfer by Kes1. This effect might be accomplished by increasing the affinity of Kes1 for PI-4,5P_2-containing membranes where Kes1 can bind to phosphatidylinositol (PIP_2) head groups on the bilayer (Raychaudhuri et al., 2006).

A recent study proposed that yeast Osh proteins can interact with two membranes simultaneously to coordinate sterol transfer (Schulz et al., 2009). Kes1 was used as a model for all Osh proteins, where it was identified to have a 'distal' membrane-binding site away and one at the mouth of the sterol-binding pocket. The distal-binding site was required for wild-type function of Kes1, in addition to *in vitro* sterol transfer and stimulation of sterol transfer by PI-4,5P_2. This information supports previous thoughts that Kes1 and other ORPs can extract sterols from one membrane, diffuse throughout the cytosol and deliver the bound sterol to a second membrane (Im et al., 2005; Raychaudhuri et al., 2006); however, it proposes that closely apposed membranes allow Kes1 binding to two membranes *via* both of its membrane-binding sites, and therefore enhance sterol extraction and delivery (Schulz et al., 2009). Directional transfer has also

been proposed whereupon the presence of PIPs in some membranes but not others (i.e, PI-4,5P_2 at the plasma membrane but not at the ER) can provide a mechanism by which Osh proteins deliver sterols to a specific subset of membranes (Schulz et al., 2009). Indeed, this has been found in mammalian cells, where the oxysterol related protein (ORP) ORP9 Long (ORP9L) and OSBP transport of cholesterol is stimulated by the presence of PI-4P in the acceptor membanes (Ngo and Ridgway, 2009).

It has also been proposed that Osh proteins located at membrane contact sites could act to alter sterol concentration in one organelle in response to a signal, such as PIP levels in another organelle (Schulz et al., 2009). In fact, four of the seven Osh proteins (Osh2, Osh3, Osh6 and Osh7) were enriched at regions where the ER is closely apposed to the plasma membrane, presumably PM-ER contact sites (Levine and Munro, 2001; Loewen et al., 2003; Schulz et al., 2009; Wang et al., 2005b; Wang et al., 2005c). Other yeast Osh and mammalian ORP proteins have also been located at other membrane contact sites, mainly yeast Osh1 at a nuclear-vacuole junction and mammalian ORP1 long (ORP1L) between the ER and endosome (Levine and Munro, 2001; Rocha et al., 2009). This way, Osh proteins would act as sterol sensors responding to lipid composition in two membrane organelles, and therefore help to define highly specialized structures that are characterized by both proteins and lipids (Schulz et al., 2009).

Although Kes1 can transfer sterols *in vitro*, the evidence does not support a role for Kes1 as a major transporter of sterols in cells. Other lines of experimentation predict that Osh proteins, and Kes1, function as lipid sensors rather than transporters (Wang et al., 2005a).

1.4 SPHINGOLIPID SYNTHESIS IN *S. CEREVISIAE*

In addition to sterols and phospholipids, sphingolipids are also particulary abundant in the plasma membrane. Since the initial recognition of sphingolipids being second messengers in signal-transduction pathways, their roles as key lipid mediators that regulate various cellular functions including (but not exclusively) translation, cell cycle, sporulation, endocytosis, stress response and actin cytoskeletion rearrangements (Dickson et al., 2006; Obeid et al., 2002; Sims et al., 2004) have been revealed. *S. cerevisiae* has served as a model organism for the identification of most of the genes that encode sphingolipid metabolizing enzymes, and for understanding sphingolipid metabolism.

1.4.1 Sphingolipid Biosynthesis and Transport to the Plasma Membrane

Sphingolipids are composed of a long-chain base (LCB), a fatty acid, and a polar head group. In *S. cerevisiae*, the LCBs are dihydrosphingosine (DHS) and its 4-hydroxy derivative, phytosphingosine (PHS). While the yeast DHS can contain 16, 18 or 20 carbons, PHS only contains 18 or 20 carbons (Lester and Dickson, 2001). Conversely, the fatty acid is usually saturated and 26 carbons long (Dickson and Lester, 2002).

Sphingolipid biosynthesis begins in the ER, where serine palmitoyltransferase condenses serine with a fatty acyl-CoA to form 3-ketodihydrosphingosine and CO_2 (Figure 7). Next, 3-ketodihydrosphingosine is reduced to DHS by Tsc10 (Beeler et al., 1998). DHS is further processed by either of two ceramide synthases (acyl-CoA:sphingosine N-acyltransferase), Lag1 or Lac1 (Guillas et al., 2001; Schorling et al., 2001). These synthases amide-link DHS to a C_{26} fatty acid, giving rise to ceramide, which is further hydroxylated at Carbon-4 by Sur2/Syr2 to yield phytoceramide.

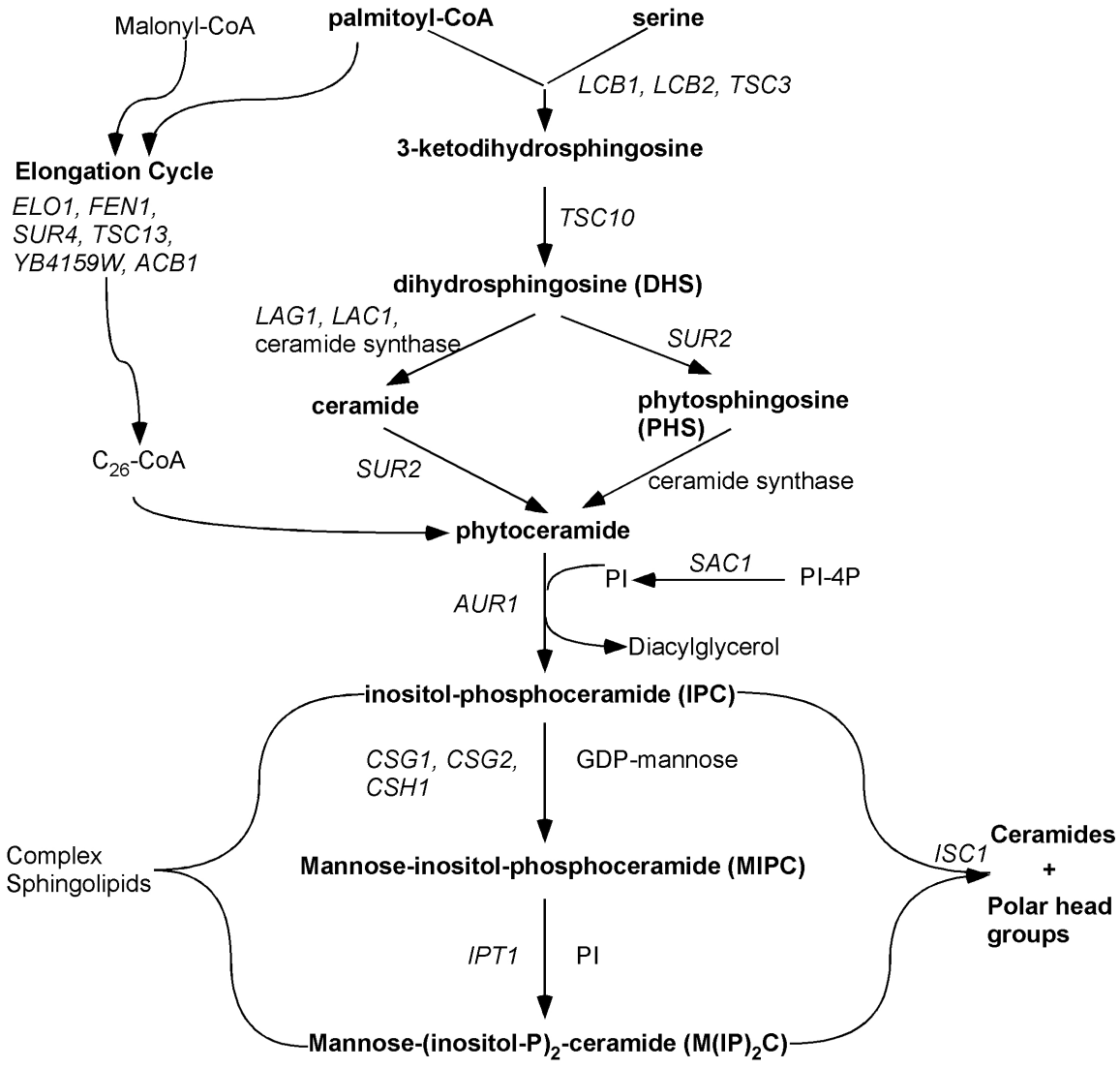


Figure 7 **Sphingolipid synthesis in *S. cerevisiae*.** Phosphatidylinositol plays a central role in the synthesis of sphingolipids. Gene names are shown in italics. Metabolic intermediates and complex sphingolipids are shown in bolded font.

Phytoceramide can also be synthesized by hydroxylating DHS to PHS, which is further amide-linked to a C_{26} fatty acid (Grilley et al., 1998; Haak et al., 1997). The C_{26} fatty acid in phytoceramide can contain no hydroxyl, one hydroxyl at C-2, or two hydroxyls at C-2 and C-3 (Smith and Lester, 1974).

The very-long-chain fatty-acid (VLCFA) synthetase complex is responsible for elongating the C_{14}-C_{18} fatty acids to C_{26} in the ER. The multienzyme complex carries out four reactions, starting with condensation of malonyl-CoA with an acyl-CoA to form a 3-ketoacyl-CoA. The 3-ketoacyl-CoA is reduced to 3-hydroxy acyl-CoA, which is then dehydrated to an enoyl intermediate. The last step involves the enoyl intermediate being reduced to yield an acyl-CoA that is now 2 carbons longer. The family of elongase proteins, including Elo1, Fen1 and Sur4, has specificity in determining chain length (Jakobsson et al., 2006; Oh et al., 1997; Toke and Martin, 1996).

For the polar head group to be added to ceramide, ceramide must be transported from the ER to the Golgi. Both vesicular and non-vesicular transport systems have been proposed to transport ceramide in *S. cerevisiae* (Funato and Riezman, 2001). This is similar to the situation in mammalian cells, where the ceramide-transfer protein (CERT) extracts ceramides from the ER and delivers them to the Golgi membrane *via* a non-vesciular transport mechanism (Hanada et al., 2003; Kumagai et al., 2005). No CERT-like proteins have been found in yeast.

Ceramide incorporates into the Golgi membrane, where the head group is added in the lumen of the Golgi. The first head group added to the ceramide C1-OH is inositol phosphate. This reaction is catalyzed by inositol phosphorylceramide synthase (Aur1) to yield inositol-phosphoceramide (IPC), the first complex sphingolipid (Levine et al., 2000;

Nagiec et al., 1997). The enzyme inositol phosphoceramide mannosyl transferase catalyzes the next reaction, where mannose from GDP-mannose is transferred onto the inositol 2-OH moiety of IPC. This yields the second complex sphingolipid, mannose-inositol-phosphoceramide (MIPC). The enzyme that catalyzes this reaction has two forms: one contains Csg1 and Csg2 proteins and the other has Csh1 and Csg2 proteins. The third and last complex sphingolipid that is made in the Golgi is formed by the transfer of a second inositol phosphate from phosphatidylinositol to MIPC, which yields mannose-(inositol-P)$_2$-ceramide (M(IP)$_2$C). The complex sphingolipids made in the Golgi are subsequently transported to the plasma membrane, primarily by vesicular transport (Hechtberger and Daum, 1995). Complex sphingolipids comprise around 30% of phosphorylated membrane lipids and almost 7% of the total mass of the plasma membrane (Patton and Lester, 1991).

1.4.2 Regulation of Sphingolipid Metabolism

Studies suggest that yeast cells promote ceramide and sphingolipid synthesis when growth conditions are favored, while synthesis is decreased under conditions of stress (Figure 8). The target of rapamycin complex 2 (TORC2) regulates the *de novo* synthesis of ceramide and complex sphingolipids. TORC2 has been implicated in controlling the activity of ceramide synthase (Aronova et al., 2008) by sensing nutrients and stresses, and in turn coordinating metabolism (Wullschleger et al., 2006). The Ypk2 kinase appears to act downstream of TORC2 to directly activate ceramide synthase activity (Kamada et al., 2005). Defects associated with decreased ceramide synthase activity and/or Ypk2 activity includes defects in cell wall integrity and in actin polarization. Another Tor protein complex in yeast, TORC1, was originally thought to be

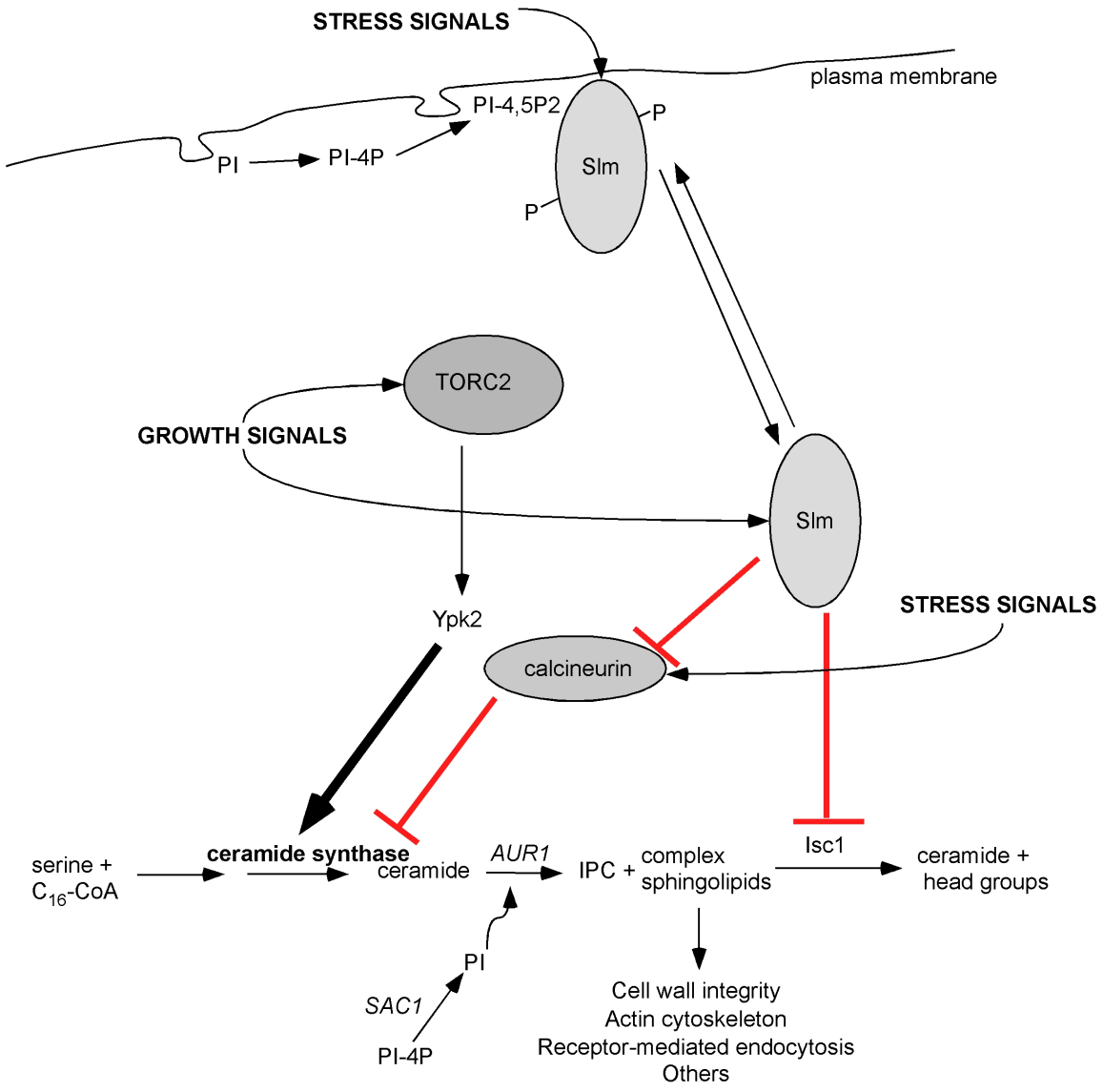


Figure 8 Regulation of sphingolipid synthesis by cellular stress and growth signals Growth signals promote ceramide synthase activity, opposed by calcineurin. Stress signals causes Slm proteins to anchor at the plasma membrane, *via* binding to PI-4,5P_2, no longer inhibiting calcineurin activity. Sac1 promotes sphingolipid synthesis by producing a pool of PI available for the synthesis of complex sphingolipids by Aur1.

independent of TORC2 with its activity being sensitive to rapamycin, a compound that mimics nitrogen starvation in cells (Wullschleger et al., 2006).

The TORC2 complex also binds the Slm proteins at the plasma membrane (Audhya et al., 2004; Fadri et al., 2005). The Slm1 and Slm2 proteins have overlapping functions in the cell, with both being required for cell viability (Kamada et al., 2005). Slm proteins have PH domains that allow them to bind PI-4,5P_2, which can act as an anchor to the plasma membrane in situations of stress (Audhya et al., 2004; Yu et al., 2004). By regulating the breakdown of complex sphingolipids, the Slm proteins act downstream of PIP_2 and TORC2 signaling pathway and control actin cytoskeleton for cell growth, cell wall integrity and receptor-mediated endocytosis. Indeed, the activated Slm proteins (dephosphorylated) can decrease activity of the Isc1 enzyme (Tabuchi et al., 2006). The yeast Isc1 protein shows sequence similarity to the mammalian sphingomyelinases, and has been identified as a phospholipase C enzyme that cleaves polar head groups from sphingolipids (Sawai et al., 2000; Wells et al., 1998). The Slm proteins also regulate the Ca^{2+}/calmodulin-regulated protein phosphatase calcineurin. Calcineurin opposes the TORC2 pathway by downregulating ceramide synthase activity in situations of cellular stress (Tabuchi et al., 2006). In this way, the Slm proteins serve to adapt the regulation of ceramide to complex sphingolipids according to conditions of cellular stress (Tabuchi et al., 2006).

It was recently determined that interactions between the PI-4P phosphatase Sac1 and the PI-4 kinase at the plasma membrane, Stt4, regulate sphingolipid levels *via* Aur1 (Brice et al., 2009). When the gene encoding the lipid phosphatase *SAC1* was inactivated, decreases in the levels of complex sphingolipids were observed. This inhibition was

observed using both steady-state and pulse-labeling experiments, indicating a decrease in both the complex sphingolipids mass and the rate of their synthesis. Total PI levels were also decreased during steady-state and pulse-labeling experiments, indicating that a decreased amount of PI was available for synthesis of complex sphingolipids. The mechanism by which Sac1 regulates the availability of PI for synthesis of complex sphingolipids is thought to be independent of Slm1/2 and PI-4,5P_2. Indeed, inactivation of Slm1/2 does not cause an accumulation of the total sphingoid bases, precursors for the synthesis of complex sphingolipids. However, inhibition of Aur1, or inactivation of *SAC1*, does result in an increase (Brice et al., 2009). Therefore, the mechanism by which Sac1 regulates the synthesis of complex sphingolipids is thought to be independent of Slm1/2 and PI-4,5P_2, and appears to be *via* the regulation of Aur1 (Brice et al., 2009).

Sac1 is a phosphatase that primarily localizes to the ER (Foti et al., 2001), while Stt4 is the PI-4 kinase at the plasma membrane (Audhya and Emr, 2002). Although the relationship between Stt4 and Sac1 is clear, the mechanism by which PI-4P travels to the binding site of Sac1 is unknown. Further research must be conducted to understand how and where the PI pool produced by Sac1 provides a key component for synthesis of complex sphingolipid at the Golgi.

1.4.3 Role of Sphingolipids and Kes1 in the Secretory Pathway

A genome-wide screen revealed a role for both sphingolipids and ergosterol in the regulation of cell-surface delivery in yeast. Sterol mutants, sphingolipids mutants and *KES1* mutants were all found to be defective in the delivery of a cell surface marker protein that travels *via* lipid rafts to the plasma membrane (Proszynski et al., 2005). Another study determined that sterols and sphingolipids are required for the delivery of

lipid rafts to the plasma membrane (Klemm et al., 2009). Lipid rafts are dynamic nanometer-sized membrane domains primarily formed by sterols, sphingolipids, saturated glycerophospholipids, and proteins. They are thought to facilitate the sorting of lipids at the *trans*-Golgi (Klemm et al., 2009) and may also be involved in the generation of the lipid gradients in the secretory pathway (Hancock, 2006; Klemm et al., 2009; Simons and Vaz, 2004). These lipid rafts also contain proteins like Pma1, an essential 10-membrane spanning protein pump, which must be delivered to the plasma membrane (Bagnat et al., 2000; Gong and Chang, 2001; Lee et al., 2002; Wang and Chang, 2002). Cells lacking sphingolipids, or ergosterol, cannot properly deliver Pma1 to the plasma membrane (Proszynski et al., 2005).

A role for Kes1 has yet to be identified in the regulation of sphingolipids or in the formation and delivery of lipid rafts to the plasma membrane. However, this remains an interesting hypothesis as sterols, sphingolipids and Kes1 are all implicated in delivery of cell surface marker proteins, sterols and sphingolipids are important for the formation of lipid rafts at the *trans*-Golgi, and Kes1 is a sterol-binding protein that primarily localizes at the *trans*-Golgi.

1.5 VESICULAR TRAFFICKING IN *S. CEREVISIAE*

Secretion is initiated at the ER, where protein synthesis, folding and modification occur. The process of secretion and vesicular transport involves the initiation, budding, scission, uncoating, tethering, docking and fusion of transport vesicles (Bonifacino and Glick, 2004). During the initiation step, “early” coat proteins, soluble NSF attachment receptor (SNARE) proteins, and cargo are recruited to the donor membrane. The coat proteins are recruited to the donor membrane by binding to GTPases or PIPs. Budding

consists of recruitment of additional coat proteins to the nascent vesicle, concentration of the cargo, and an increase in membrane curvature. During scission, the membrane vesicle is released from the donor membrane with the aid of coat proteins and accessory factors. The inactivation of small GTPases and/or alterations in PIP metabolism is thought to facilitate scission. A vesicle tethers to its target membrane with the aid of GTP-bound Rab (small GTPase) proteins. Docking is the process by which v-SNAREs (on the vesicle) and t-SNAREs (on the target membrane) form a four-helix bundle (Fasshauer et al., 1997; Sutton et al., 1998), and along with tethering factors, promote fusion between vesicles and the acceptor membrane.

1.5.1 ER-to-Golgi (Anterograde) Transport

Proteins destined for locations other than the ER are sorted into vesicles for transport to the Golgi and beyond. Sec12, a transmembrane guanine-nucleotide exchange factor (GEF), initiates vesicular transport from the ER by activating the small GTPase Sar1 (Barlowe and Schekman, 1993). Activated Sar1 then recruits components of the vesicle coat, Sec23 and Sec24, and subsequently, Sec13 and Sec31 (Barlowe et al., 1994). This protein coat, referred to as the COPII protein coat, remains part of the vesicle until the vesicle is released from the ER. Prior to vesicle budding, cargo proteins are concentrated into nascent COPII vesicles (Balch et al., 1994). To facilitate trafficking, transmembrane cargo can bind directly to COPII coat proteins through their ER export signals (Kuehn et al., 1998). Soluble protein cargo cannot bind directly to COPII vesicles and must bind *via* transmembrane export receptors such as Erv14 to facilitate export (Powers and Barlowe, 2002). Sec23, a GTPase-activating protein, then stimulates GTP hydrolysis by Sar1 to initiate vesicle uncoating (Yoshihisa et al., 1993). Recent evidence

has shown that the initial interaction of a vesicle with its target membrane requires the tethering complex TRAPPI (and specifically the Bet3 subunit) to bind to the coat subunit Sec23 (Cai et al., 2007; Sacher et al., 2001). *In vitro* studies have shown that the interaction between Sec23 and Bet3 targets TRAPPI to COPII vesicles. This process mediates vesicle tethering with another COPII vesicle, or the Golgi (Cai et al., 2007). Proteins that participate in anterograde transport are recycled back from the Golgi to the ER for further rounds of transport (Figure 9).

1.5.2 Golgi to ER (Retrograde) Transport

Transport from the Golgi to the ER is similar in mechanism to anterograde transport. Retrograde transport is mediated by COPI vesicles and components including Ret1, Ret2, Ret3, Sec21, Sec26, Sec27 and Sec28 (Letourneur et al., 1994). COPI-vesicle formation depends on the small GTPases Arf1/2 (Duden et al., 1994; Liang and Kornfeld, 1997). Arf1/2 are activated by GEFs (Gea1, Gea2, Sec7 and Syt1), and inactivated by GTPase-activating proteins (Gcs1 and Glo3) (Figure 9) (Jones et al., 1999; Peyroche et al., 1996; Poon et al., 2001).

1.5.3 Golgi Trafficking

The Golgi apparatus is involved in modifying newly synthesized proteins that are destined for secretion, or for transport to the vacuole. In *S. cerevisiae*, cisternal maturation is responsible for transporting proteins from the *cis*-Golgi to the *trans*-Golgi (Glick and Malhotra, 1998; Matsuura-Tokita et al., 2006). Cisternal maturation refers to vesicles from the ER that fuse to form the *cis*-Golgi. The *cis*-Golgi then matures into the *medial*-Golgi, which in turn develops into the *trans*-Golgi. Although the Golgi apparatus

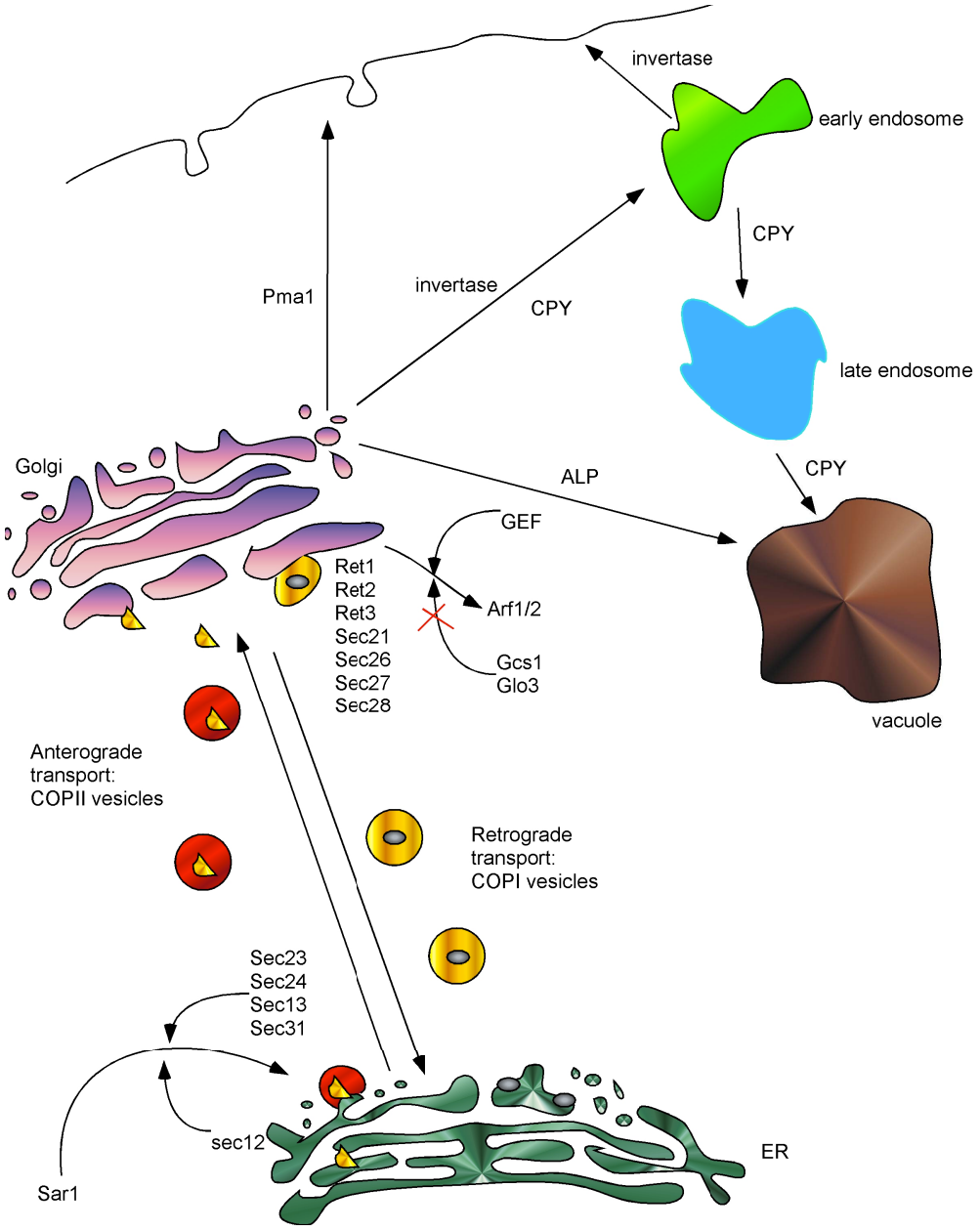


Figure 9 **Vesicular transport in *S. cerevisiae*.** During anterograde transport, Sec12 (a transmembrane GEF) activates Sar1 (small GTPase) to recruit the COPII components Sec23, Sec24, Sec13 and Sec31. Sec23 (a GTPase-activating protein) initiates uncoating by stimulating GTP hydrolysis by Sar1. Vesicle fusion at the receptor membrane (Golgi) requires the Bet3 subunit of TRAPPI. Retrograde transport is mediated by COPI vesicles with Ret1, Ret2, Ret3, Sec21, Sec26, Sec27 and Sec28 as components. Arf1/2 activates vesicle formation and is activated by GEFs (Gea1, Gea2, Sec7, Syt1) and inactivated by GTPase activating proteins (Gcs1, Glo3). The Pma1 pathway is a direct path for transport from the Golgi to the plasma membrane. Invertase is transported to the plasma membrane *via* endosomes. ALP and CPY are transported from the Golgi to the vacuole *via* seperate pathways

in *S. cerevisiae* is less defined than in mammalian cells, the *cis*-, *medial*- and *trans*-Golgi compartments can be identified by the presence of particular marker proteins (Franzusoff et al., 1991; Morin-Ganet et al., 2000). COPI vesicles are responsible for returning proteins to the *cis*-Golgi at each step of cisternal maturation, which ensures that the required proteins are present at the *cis*-Golgi for maturation to occur. The *trans*-Golgi forms transport vesicles that travel to the vacuole or the plasma membrane. However, prior to vesicle formation at the *trans*-Golgi, sorting of protein cargo occurs (Glick and Malhotra, 1998). Another model, referred to as vesicle shuttling, had previously been proposed to explain transport within the Golgi (Glick and Malhotra, 1998). In this model, the *cis-, medial*- and *trans*-Golgi are static, COPI vesicles deliver the cargo between the cisternae, and the vesicles bud from the *trans*-Golgi. However, in *S. cerevisiae*, evidence supports the cisternal maturation model over vesicle shuttling.

The *trans*-Golgi is the site of protein sorting and packaging for trafficking to the vacuole and plasma membrane. There are several transport pathways from the *trans*-Golgi network. For instance, the Pma1 (plasma membrane ATPase) pathway is responsible for direct trafficking between the Golgi and the plasma membrane *via* lipid rafts (Bagnat et al., 2000; Harsay and Schekman, 2002). Additionally, the transport of invertase typifies another pathway to the plasma membrane (Figure 9).

Two routes have also been identified by which proteins traffic from the Golgi to the vacuole. The ALP (alkaline phosphatase) pathway is a direct route from the *trans*-Golgi to the vacuole (Vowels and Payne, 1998). A second route to the vacuole is exemplified by carboxypeptidase Y (CPY) where trafficking to the vacuole is a two-step pathway *via* the early and late endosome (Figure 9) (Jung et al., 1999).

1.5.4 Secretory Mutants

Secretory (*sec*) mutants in *S. cerevisiae* were first identified on the basis that cells defective in protein secretion would become denser due to the accumulation of proteins and membranes. Consequently, these secretory-mutant cells could be separated from wild-type cells by density gradient centrifugation (Novick et al., 1980; Novick and Schekman, 1979). Using this method, mutagenized cells that showed accumulation of proteins and membrane were isolated and identified as *sec* mutant. (Novick et al., 1980; Novick and Schekman, 1979). When a yeast cell is defective in secretion, invertase also accumulates within the cell (Novick et al., 1980). Synthesis and glycosylation of invertase occurs at the ER. Subsequent transport to the Golgi for further glycosylation occur before the protein is transported to the plasma membrane and secreted out of the cell (Esmon et al., 1981). Therefore, the secretion efficiency of these *sec* mutants was measured by looking at the ratio of secreted invertase to intracellular invertase.

A genetic screen identified temperature-sensitive *sec* mutants where upon a shift to the non-permissive temperature, cells stopped growing at all stages of the cell cycle (Novick et al., 1980). One of the mutants, *sec14*, exhibits a temperature-sensitive phenotype associated with the accumulation of Berkeley bodies (a unique organelle thought to form upon distorted vesicular trafficking from the Golgi) and secretory vesicles (Bankaitis et al., 1990; Novick et al., 1980).

Further investigation of the *SEC14* temperature-sensitive mutant ($sec14^{ts}$) included the isolation of spontaneously occurring mutants that allowed the growth of $sec14^{ts}$ cells at the non-permissive temperature (Cleves et al., 1991). Many of these spontaneous mutations that suppress defects associated with the $sec14^{ts}$ phenotype were

shown to be not simply revertants, or secondary mutations within the *sec14*^{ts} allele, but rather second-site mutations affecting other genes. Not only could these new mutants grow normally at the non-permissive temperature for the *sec14*^{ts} allele (37^{o}C), but the secretion deficiencies previously shown for *sec14*^{ts} cells were also significantly suppressed (Cleves et al., 1991). Further analysis identified two dominant complementation groups (encompassing 81 of the 107 spontaneous suppressors), and five recessive complementation groups. These so-called "*SEC14* bypassers" were able to suppress defects associated with loss of function of *SEC14*, but not of the other known 22 *sec* mutants (Cleves et al., 1991).

The genes for the five recessive bypassers of Sec14 deficiency were identified as *SAC1*, *KES1*, *CKI1*, *PCT1* and *CPT1* (Cleves et al., 1991; Fang et al., 1996). The dominant alleviating genes are still unidentified, and are referred to as *BSD1* and *BSD2*. As described above, Sac1 is a PIP phosphatase and Kes1 is a member of the OSBP protein family that binds both sterols and PIPs. Cki1, Pct1 and Cpt1 are the three enzymes of the CDP-choline pathway that carries out PC biosynthesis (Cleves et al., 1991). Inactivation of any of these enzymes alleviates the essential requirement for Sec14 function. As inactivation of genes that code for PC biosynthetic enzymes or genes that affect PIP metabolism alleviate defects associated with inactivation of Sec14, the ability of Sec14 to bind PC and PI appears to be closely related to its function within the cell.

A genome-wide screen performed on Sec14 demonstrated that the modes of bypass of the essential function of Sec14 by altered PC metabolism and mutant Kes1 are distinct (Fairn et al., 2007). It was suggested that Pik1 and Kes1 might act in concordance to regulate PI-4P levels and vesicular transport at the Golgi. Pik1 is the PI-4 kinase that

functions at the Golgi and, when absent, results in vesicular-trafficking defects similar to those seen upon loss of function of Sec14 (Audhya et al., 2000; Sciorra et al., 2005; Strahl et al., 2005; Walch-Solimena and Novick, 1999). By deleting *KES1*, growth and secretory defects of both *pik1*^{ts} and *sec14*^{ts} cells at non-permissive temperatures were relieved. In these two mutant situations, there is a 50% decrease in PI-4P at the Golgi, which can also be returned near normal levels by inactivating *KES1* (Bankaitis et al., 1990; Fairn et al., 2007). However, the mechanism by which the absence of Kes1 activity restores PI-4P levels in either of these situations is not known.

1.5.5 Role of PIPs at the *trans*-Golgi

The role of PIPs, and their effectors, at the *trans*-Golgi is poorly understood. Nonetheless, PI-4P and PI-3P are both required for trafficking pathways within and from the *trans*-Golgi (Audhya et al., 2000; Hama et al., 1999; Li et al., 2002a; Walch-Solimena and Novick, 1999). FAPP1 and FAPP2 are two proteins in mammalian cells that are effectors of PI-4P at the *trans*-Golgi (Godi et al., 2004). By binding both PI-4P and Arf (a small GTPase involved in COPI retrograde transport) *via* their PH domains, these effectors localize to the *trans*-Golgi, where they co-ordinate vesicle budding and fission reactions. FAPP1 and FAPP2 also form carriers targeted for fusion at the plasma membrane (Godi et al., 2004; Isakoff et al., 1998).

PI-4P has also been shown to be involved in the recruitment of trafficking proteins (Behnia and Munro, 2005; Carlton and Cullen, 2005). At the *trans*-Golgi, PI-4P is required for targeting a major clathrin adaptor, AP-1 (Heldwein et al., 2004; Wang et al., 2003), and it is also required for the recruitment of the GGA clathrin-adaptor proteins

(Puertollano et al., 2001; Wang et al., 2007). GGA clathrin adaptors are also dependent on Arf1 for *trans*-Golgi targeting (Puertollano et al., 2001).

Vps34, the PI-3-kinase, is responsible for creating an enriched pool of PI-3P at the *trans*-Golgi and endosome (Odorizzi et al., 2000). Vps34 is activated and recruited to membranes by Vps15, a serine/threonine protein kinase. Subsequently, Vps34 produces PI-3P, which may serve to attract and/or activate effector molecules. These include vesicle coat proteins that are required for proper trafficking (Odorizzi et al., 2000). PI-3P also binds, and possibly activates, proteins that contain FYVE and PX homology domains. FYVE domains are known to bind PI-3P with high specificity (Ago et al., 2001; Vanhaesebroeck et al., 2001). Defects in the lipid-kinase activity of Vps34 result in vacuolar sorting defects, suggesting that the catalytic role of Vps34 is fundamental to endosome-vacuole trafficking (Sotsios and Ward, 2000).

1.5.6 Cytoplasm-to-Vacuole (Cvt) Protein Targeting and Autophagy

Although the major pathway for protein transport to the vacuole is *via* the *trans*-Golgi vesicular trafficking pathway, there are "non-classical" protein trafficking pathways that involve transport from the cytoplasm to the vacuole. Autophagy is a non-specific process that degrades proteins and cytosolic macromolecular components in the vacuole to maintain normal development and differentiation (Levine and Klionsky, 2004). Although autophagy is a dynamic process, it can be divided into different steps. Vesicle formation begins and is followed by selection and packaging of cargo, initiation of vesicle formation (nucleation), vesicle expansion and completion, retrieval of certain autophagy proteins and fusion of the vesicle with the vacuole. The autophagy pathway is completed by the breakdown of the inner membrane of the vesicle and release of the

cargo within the vacuolar lumen (Figure 10) (Klionsky, 2005). Autophagy is a mechanism that is generally used for bulk cargo degradation, although in some instances it can play a role in biosynthesis through the delivery of vacuolar enzymes (Harding et al., 1995). For instance, studies of the trafficking of vacuolar aminopeptidase I (Ape1) first identified a constitutive cytoplasm-to-vacuole targeting (Cvt) pathway involved in this biosynthetic process (Segui-Real et al., 1995). Ape1, as well as the α-mannosidase (Ams1) enzyme, are both synthesized in the cytoplasm as inactive precursors and assemble to form large homo- oligomeric complexes (Hutchins and Klionsky, 2001; Kim et al., 1997). These complexes are sequestered by double-membrane Cvt vesicles and are targeted to the vacuolar lumen (Scott et al., 1997) (Figure 10). Investigation of *cvt* and autophagy (*atg*) mutants determined that both processes use similar machinery (Baba et al., 1994; Harding et al., 1996; Scott et al., 1996). However, although the Cvt vesicles are similar in morphology to autophagosomes, they only include their specific cargo and not other cytoplasmic contents.

Induction of autophagy in yeast is only known to occur during nutritional starvation, although basal-level autophagy is thought to occur under normal conditions. The Tor signaling pathways are the major regulators of autophagy and Cvt (Noda and Ohsumi, 1998). Interaction of Atg13 with the Tor serine/threonine kinase facilitates the switch between the two pathways. Under high nutrient conditions, the Tor kinase is active and Atg13 is phosphorylated (Noda and Ohsumi, 1998). However, in poor nutrient conditions, the Tor kinase is inactive and Atg13 is largely dephosphorylated. This allows Atg13 to interact with Atg1 (Noda and Ohsumi, 1998). The interaction between the dephosphorylated Atg13 and Atg1 is thought to drive the initiation of autophagy over the

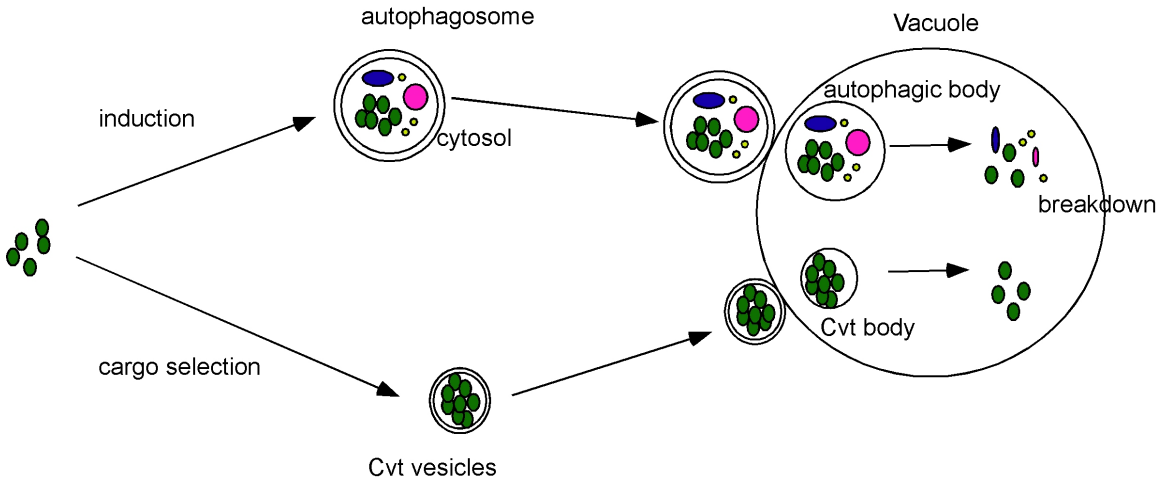


Figure 10 Autophagy and Cvt trafficking pathways in *S. cerevisiae.* The Cvt and autophagy pathways use much of the same machinery for the formation of double-membrane vesicles and for delivery of cargo from the cytosol to the vacuole. Autophagy is non-specific for cargo selection into autophagosomes, whereas the Cvt pathway selects specific cargo into its vesicles. Breakdown of products in the vacuole provides fresh building blocks for the cell in situations of nutrient starvation (autophagy) or acts as a biosynthetic pathway for maturation of enzymes (Cvt).

constitutive Cvt pathway (Kamada et al., 2000; Scott et al., 2000).

Although some molecular machinery is shared between autophagy and the Cvt pathway, differences remain. Atg17, Atg29 and Atg31 are specific for autophagy (Kabeya et al., 2007; Kawamata et al., 2005; Tsukada and Ohsumi, 1993), while Atg11, Atg19 and Atg24 function specifically in the Cvt pathway (Kim et al., 2001; Leber et al., 2001; Nice et al., 2002). The source of the membranes for synthesis of the vesicles carrying the cargo also differs between both pathways. While autophagy requires Sec23/24 and Sec12 for COP II formation (Ishihara et al., 2001), the Cvt pathway utilizes the t-SNARE complex composed of Tlg2 and Vps45 (Abeliovich et al., 1999). Outer membranes of vesicles formed through both pathways fuse with the vacuole membrane, and both pathways require the vacuolar t-SNARE Vam3 to release the vesicles to the vacuole (Darsow et al., 1997; Ishihara et al., 2001).

Only two proteins are present throughout the entire Cvt or autophagy pathway. Atg8 and Atg19 are associated with vesicles, where they remain until they reach the vacuole (Noda et al., 2000). Atg8 is a ubiquitin-like protein that is required for autophagosome formation (Levine and Klionsky, 2004). Atg8 is used as a marker for the induction and progession of autophagy, as this protein is localized on both the pre-autophagosome structure (PAS) (where autophagosomes are formed) and on the autophagosomes themselves (Kabeya et al., 2000; Kirisako et al., 1999; Yoshimoto et al., 2004). Atg8 can be conjugated to the lipid phosphatidylethanolamine (PE), allowing the protein to be anchored to membranes (Ichimura et al., 2000; Kirisako et al., 2000). Further studies suggest that Atg8-PE is involved in the process of autophagosome formation and that the deconjugation of Atg8-PE to Atg8 is important for the recycling of

Atg8 after autophagosome formation (Ichimura et al., 2000; Kirisako et al., 1999; Kirisako et al., 2000).

Another important factor required for both the Cvt and autophagy pathways is the PI-3-kinase Vps34 (Kihara et al., 2001). Vps34 plays a role in vacuolar protein sorting within the endosomal pathway, but it is also complexed with the Cvt-and autophagy-specific factors Atg14 and Atg6 (Kihara et al., 2001). The PI-3-kinase complex is thought to recruit PI-3P-binding proteins such as Atg18 (Guan et al., 2001; Matsuura et al., 1997), which regulates the retrieval of Atg9.

1.5.7 Role of Kes1 in Vesicular Trafficking and Alleviating Sec14 Essential Function

The *KES1* gene was identified when its inactivation alleviated the requirement for the essential function of Sec14 (Cleves et al., 1991). Sec14 regulates secretion from the Golgi and thus a role for Kes1 as an effector of Golgi-derived secretion was hypothesized (Cleves et al., 1991). Only inactivation of *KES1*, and not other *OSH* genes, alleviates defects associated with the essential requirement for Sec14 function (Fang et al., 1996). Thus, Kes1 exhibits functional specificity for its involvement in the negative regulation of Golgi-derived vesicular transport (Fang et al., 1996). Mammalian ORPs and yeast Osh proteins had previously been shown to be involved in the regulation of cholesterol and sphingolipid metabolism. It was suggested that inactivation of *KES1* may result in sterol-metabolism defects, which would alleviate the growth and vesicular-transport defects that occur when *SEC14* is inactivated (Fang et al., 1996). However, in studies where the yeast sterol composition was altered by either 1) inactivating *ERG* genes in the sterol biosynthetic pathway, or 2) treatment with zaragozic acid (a squalene synthase inhibitor)

or lovastatin (inhibitor of HMG-CoA reductase), no differences in secretion or growth by cells containing the temperature sensitive *sec14*^{ts} allele were observed (Fang et al., 1996). This suggested that the absence of Kes1 bypasses the requirement for Sec14 through a mechanism other than membrane-sterol synthesis inhibition.

A genome-wide screen revealed a major function of Kes1 as a regulator of vesicular transport at the Golgi by modulation of PI-4P level and availability (Fairn et al., 2007). As previously noted, decreasing the function of either the Golgi-localized PI-4 kinase Pik1 or Sec14 decreases the level of cellular PI-4P by 50%. Inactivation of *KES1* also results in restored growth and PI-4P to wild-type levels in these cells (Fairn et al., 2007). In cells with impaired Pik1 or Sec14 function, there is improper localization of a fluorescent PI-4P reporter to the Golgi. This mislocalization is no longer observed when *KES1* is inactivated (Fairn et al., 2007). The data reaffirm the notion that the PIP-binding ability of Kes1 affects PI-4P levels and availability at the Golgi. Although a decrease in PI-4P levels by Kes1 directly inhibiting Pik1 activity is an attractive hypothesis, it has yet to be demonstrated. Despite our lack of understanding of the mechanism by which Kes1 regulates PI-4P levels, the role of Kes1 in regulating PI-4P at the Golgi appears to be an important component of Kes1 function (Fairn et al., 2007).

Another role for Kes1 at the Golgi has recently been discovered. Drs2 is a Golgi-resident flippase that has specificity for the phospholipids phosphatidylserine (PS) and PE (Alder-Baerens et al., 2006; Natarajan et al., 2004). Drs2 plays a vital role in vesicle budding at the *trans*-Golgi, independent of coat recruitment. It is thought that the physical placement of phospholipids on a specific membrane leaflet induces curvature that is favourable for vesicle formation and is dependent on AP-1/clathrin (Chen et al.,

1999; Graham, 2004; Liu et al., 2008). Beyond its role in vesicle formation, Drs2 has been proposed to affect the membrane curvature required for establishing lipid raft membrane structures at the Golgi (Muthusamy et al., 2009b). In this model, it is suggested that Kes1 inhibits Drs2 flippase activity to relax membrane curvature (Muthusamy et al., 2009b). Other work has determined that Drs2 flippase activity is dependent on the PI-4 kinase at the *trans*-Golgi, Pik1 (Natarajan et al., 2009). Cross talk between sphingolipids and phospholipid flippase activity has also recently been shown at the plasma membrane, where complex inositol-containing sphingolipids (MIPC) activate Fpk1. Fpk1 in turn inhibits Ypk1, a major regulator of complex-sphingolipid synthesis (Roelants et al., 2010). This balance of flippase activity is important for many membrane functions, including endocytic and exocytic vesicle-mediated transport of proteins (Hua et al., 2002; Muthusamy et al., 2009a), non-vesicular trafficking of sterols (Muthusamy et al., 2009b), and the maintenance of polarized growth through membrane recruitment of Cdc42 (Saito et al., 2007). Indeed, all these cellular processes have been linked to Kes1 functions.

A role for Kes1 and Cdc42 was identified when a study demonstrated that over-expression of several *OSH* genes, including *KES1*, relieves the growth and cell polarity defects observed in cells with impaired function of the small GTPases Cdc42 and Rho1 (Kozminski et al., 2006). This genetic link implicates Osh proteins in the regulation of vesicular transport to the site of polarized cell growth. Localization of Rho1 and Sec4 (a Rab GTPase that regulates exocytosis) is also defective in cells lacking Osh proteins (Kozminski et al., 2006). Since Kes1, and other Osh proteins, can relieve the growth defect associated with *CDC42*-mutant temperature-sensitive cells, it is thought that Osh

proteins have overlapping functions in localizing certain proteins that are transported to sites of polarized growth (Kozminski et al., 2006). This is consistent with a role for all Osh proteins in regulation of vesicular transport.

1.6 MAMMALIAN OSBPS

OSBP, the founding member of the oxysterol binding protein superfamily, was initially identified as a high-affinity cytosolic receptor for oxysterols. Oxysterols are 27-carbon oxygenated derivatives of cholesterol produced by both enzymatic and non-enzymatic mechanisms (Russell, 2000). They were originally thought to act as ligands for nuclear receptors of the liver X receptor family (Cummins and Mangelsdorf, 2006; Olkkonen and Lehto, 2004), but not all of their effects were associated with their ability to regulate gene expression (Schroepfer, 2000). Oxysterols are far less abundant than cholesterol but are more soluble (Schroepfer, 2000). The effects of these potent oxysterol molecules within cells initiated a search for other protein mediators of oxysterol action, and led to the identification of OSBP (Kandutsch et al., 1984; Levanon et al., 1990).

1.6.1 OSBP Family in Mammalian Cells

In humans there are at least 16 predicted oxysterol-related proteins (ORPs) encoded by 12 genes, which undergo pre-mRNA splicing (Lehto et al., 2001). They can be subdivided into six subfamilies based on sequence homology (Lehto et al., 2001). The family members form a protein family joined by the presence of the C-terminal ORD domain (Lehto et al., 2001; Olkkonen and Levine, 2004). Like the OSBP family in yeast, some mammalian OSBP/ORPs contain domains at their N terminus known to mediate various functions (Levine and Munro, 1998; Levine and Munro, 2002). The various

domains include PH domains, FFAT motifs, ankyrin repeats and GOLD domains, like in *S. cerevisiae*. The PH domain of various OSBP/ORP family members can bind PIPs (Johansson et al., 2005; Lehto et al., 2005), while ankyrin repeats and GOLD domains are important in mediating protein-protein interactions. The PH domains and ankyrin repeats help direct localization of OSBP/ORP proteins. The FFAT motif also affects localization of OSBP/ORP, and other lipid-binding proteins such as the ceramide transport protein (CERT) (Loewen and Levine, 2005; Loewen et al., 2003; Wyles et al., 2002; Wyles and Ridgway, 2004). Interaction of the FFAT motif with the ER-resident protein VAP facilitates OSBP and CERT co-regulation of sphingolipid metabolism (Loewen and Levine, 2005; Loewen et al., 2003; Wyles et al., 2002; Wyles and Ridgway, 2004). OSBP appears to stimulate CERT-mediated ceramide transport from the ER to the Golgi (Perry and Ridgway, 2006). The ability of OSBP to localize to the Golgi depends on its PH domain binding to PI-4P, and its interaction with the small G protein Arf1 (Levine and Munro, 2002). OSBP therefore requires its FFAT motif to interact with VAP proteins for localization to the ER, and its PH domain and interaction with Arf1 for localization to the Golgi (Perry and Ridgway, 2006).

Perry *et al* initially studied co-regulation of sphingolipid metabolism by OSBP and CERT. They showed by RNA interference that OSBP is required for CERT-mediated transfer of ceramide from the ER to the Golgi (Perry and Ridgway, 2006). OSBP regulates sphingolipid synthesis by recruiting CERT to the Golgi and increasing the rate of CERT-mediated transport of ceramide from the ER to the Golgi (Perry and Ridgway, 2006). Sterol synthesis and sphingolipid synthesis are closely linked, and OSBP may act as the bridge that regulates the synthesis of these two classes of lipid. By acting as a

sterol sensor, OSBP may in turn coordinate sphingomyelin synthesis through its regulation of CERT-mediated ceramide transport (Perry and Ridgway, 2006).

OSBP has also been identified as a sterol-responsive regulatory scaffold that can interact with two protein phosphatases: the serine/threonine phosphatase PP2A and the tyrosine phosphatase PTPPBS. Both phosphatases are involved in attenuating the activity of the extracellular signal-related kinases1 and 2 (ERK1/2) (Wang et al., 2005c). Sterol binding by OSBP is thought to act as a conformational switch for assembly and disassembly of phosphatase containing protein complexes, which directly regulate the ERK signaling pathway (Wang et al., 2005c). Other ORP proteins have specific roles within the cell, although the role of sterol binding has yet to be determined in many situations.

Two splice variants of ORP1 have been identified. One of the ORP splice variants, ORP1S, is 437 amino acids in length and is comprised of only the OSBP domain (Johansson et al., 2003). ORP1S is the only mammalian ORP that can phenocopy all of the defects associated with the loss of function of Kes1 in yeast (Xu et al., 2001). ORP1L contains the same C-terminal OSBP domain as ORP1S, but also contains three ankyrin repeats and a PH domain (Johansson et al., 2003). ORP1L localizes to the late endosomal compartment and regulates endosomal membrane trafficking through interaction with Rab7 (Johansson et al., 2005). ORP1L physically interacts with Rab7, a central regulator of late-endosomal membrane trafficking, and stabilizes its GTP-bound active state (Johansson et al., 2007). ORP1L also facilitates recruitment of dynein-dynactin complexes to microtubules (Johansson et al., 2007). The cytoplasmic dynein is part of a 1.2-MD multisubunit protein complex that is the major motor for centripetal

transport of membrane cargo along microtubules (Johansson et al., 2007). Dynactin is critical for dynein activity (Johansson et al., 2007). Rab7 recruits the dynein-dynactin motor to late endosomes, resulting in minus-end-driven vesicular transport of the microtubule-organizing center (MTOC). GTP-bound Rab7 can bind to both Rab7-interacting lysosomal protein (RILP) and ORP1L at different sites (Johansson et al., 2007). The RILP-Rab7 complex is responsible for recruiting the $p150^{GLUED}$ subunit of the dynein-dynactin motor complex. In this tripartite complex, RILP interacts with the C-terminal domain of $p150^{GLUED}$; the C-terminal domain of RILP interacts with the active GTP-Rab7; and the N-terminal domain of RILP interacts with the C-terminal fragment of the $p150^{GLUED}$ subunit. Once the tripartite complex is formed, Rab7-RILP- $p150^{GLUED}$ is recruited by ORP1L to βII spectrin, the late-endosome membrane-associated receptor for the dynein-dynactin motor complex (Johansson et al., 2007). Thus, the movement of late endosomes to the minus end of mictorubules requires the activity of Rab7, RILP and ORP1L to facilitate dynactin-dynein interaction with βII spectrin (Johansson et al., 2007). The sterol-binding role of ORP1L in the regulation of late-endosomal trafficking has yet to be identified.

Both splice variants of ORP4 (ORP4S and ORP4L) have been observed to co-localize with the intermediate filament vimentin (Wang et al., 2002; Wyles et al., 2007). ORP4L is the closest homologue of OSBP (Wang et al., 2002). When ORP4S is over-expressed, intermediate filaments aggregate and collapse. It has therefore been implied that ORP4 plays a role in microtubule function (Wang et al., 2002). Binding of sterols to ORP4, however, does not seem to influence its binding to intermediate filaments *in vitro* (Wang et al., 2002).

1.7 MAIN AIMS

Although all members of the oxysterol binding protein superfamily contain the ORD domain, these proteins have been implicated in a variety of cellular processes. In this thesis, I analyzed the OSBP family member Kes1/Osh4 of *S. cerevisiae* to gain insight into the function of this family of proteins. The primary goals of my research were to 1) characterize the lipid-binding properties of Kes1 by structure-function analysis; 2) characterize the role of Kes1 in regulation of lipid metabolism and membrane trafficking and; 3) clarify the roles of Kes1 and Sec14 at the *trans*-Golgi, since biochemical and genetic interactions implicate each in co-regulation of vesicular trafficking.

CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents

All molecular biology reagents used in this study were purchased from New England Biolabs (NEB), Invitrogen, Q-Biogene, Pierce, Stratagene or Qiagen. Custom oligonucleotides were purchased from Integrated DNA Technology (IDT). Materials used for preparation of bacteria and yeast media were purchased from Difco or Q-Biogene. Antifungal compounds G418, L-canavanine and hygromycin B were purchased from Sigma, while nourseothricin (NAT) was purchased from Werner Bioagents. Affinity resins were purchased from Pierce (Cobalt). Protease inhibitors (complete and EDTA-free tablets) were purchased from Roche, and phenylmethylsulfonyl fluoride (PMSF) was from Sigma. [^{3}H]myo-inositol was purchased from American Radiolabelled Chemicals. Phospholipids were purchased from Avanti Polar Lipids, except for phosphorylated phosphatidylinositols PI-3P and PI-3,5P_2, which were purchased from Echelon. Ergosterol was purchased from Sigma.

2.1.2 Media and Growth Conditions

Rich medium included yeast extract peptone dextrose (YPD) broth (2% bacto-peptone, 1% bacto-yeast extract, 2% dextrose) or synthetic complete (SC). SC was made of 0.67% bacto-yeast nitrogen base, 2% dextrose (2% galactose (SGal) and 1% raffinose (SRaf) were also used as alternative sugar sources), 21 mg/L of Adenine, 173.4 mg/L of L-leucine, 8.6 mg/L of para-aminobenzoic acid and 85.6 mg/L of the following: L-alanine, L-arginine HCl, L-asparagine, L-aspartic acid, L-cyteine HCl, glutamine, L-

glutamic acid, glycine, L-histidine HCl, myo-inositol, L-isoleucine, L-lysine HCl, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, uracil and L-valine.

Minimal medium was synthetic defined (SD) with 2% glucose (2% galactose and 1% raffinose were also used as alternative sugar sources), 0.67% bacto-yeast nitrogen base without amino acids and, to satisfy the auxtotrophies occurring most often, amino acids and purine/pyrimidine bases were added: 20 mg/L L-histidine HCl, 100 mg/L L-leucine, 30 mg/L L-lysine, 20 mg/L L-methionine, 20 mg/L L-tryptophan, 20 mg/L uracil and 20 mg/L adenine sulfate. The appropriate nucleoside base or amino acid was omitted to select against auxotrophies and to select for the presence of a yeast plasmid. SD-inositol medium included the appropriate carbon source (2% glucose, 2% galactose or 1% raffinose), yeast nitrogen base (YNB) without amino acids and without inositol, and appropriate supplements added for plasmid maintenance. In cases where selection for a drug-resistance marker was required, the appropriate drug was added to the medium: hygromycin (350 mg/L), G418 (200 mg/L) and NAT (100 mg/L). For media containing G418, yeast nitrogen base with no ammonium sulphate and amino acids (0.17%) was used, and 1% monopotassium glutamate was added as the nitrogen source. Solid medium was made by adding 2% bacto-agar.

E. coli cells were cultured in Luria-Bertani (LB) medium: peptone from casein (10 g/L), yeast extract (5 g/L) and sodium chloride (10 g/L). Ampicillin was added to the LB medium at a final concentration of 100 mg/L for the selection of *E. coli* cells containing a plasmid carrying an ampicillin-resistance gene.

Liquid cultures of *S. cerevisiae* and *E. coli* were cultured in a shaking incubator at 220 rpm. Cultures on solid medium were kept in incubators. Yeast cells were normally cultured at 25ºC, the permissive temperature for the strains used in this study. To assess temperature sensitivity, yeast cells were cultured at 37ºC. *E. coli* cells were also grown at 37ºC. Yeast and *E. coli* cultures were stored long-term in 15% glycerol at -70°C. Yeast and *E. coli* strains that were used frequently were stored on solid medium at 4°C for 2-3 weeks.

2.1.3 Construction of Yeast Strains and Plasmids

The *S. cerevisiae* strains used in my research are listed in Table 1. Described here are the creations of yeast strains where selected genes have been replaced by nutritional markers or drug-resistance markers.

Construction of strain CMY306

The *kes1*::*HIS3* gene was amplified by polymerase chain reaction (PCR) from cells of strain CMY136 using primers hybridizing 500 base pairs (bp) upstream and downstream of the open reading frame (ORF). Primers used were 5' primer 5' AAG CTT ATT CCG TTC GCC TTT TAC TAG and 3' primer 5' GGT ACC ATC GAT TAT GTG GTT CTA. The linear PCR product was transformed into SEY6210 dsPma1-RFP cells and His+ transformants were screened by PCR of genomic DNA using primers hybridizing 600 bp upstream and downstream of the *KES1* ORF for confirmation of *kes1*::*HIS3*.

Table 1. Yeast strains used in this study.

Yeast Strain	Genotype	Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf; (Brachmann et al., 1998)
SEY6210	*MATα leu2-3,112 ura3-52 his3-100 trp1-901 lys2-801 suc2-179*	Emr lab; (Robinson et al., 1988)
Y2454	*MAT***a** *mfa1Δ::MFA1pr-HIS3 can1Δ0 his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	(Tong et al., 2001)
Deletion collection	BY4741 *xxx*::*KanMX4* (*xxx*=non-essential gene)	Euroscarf
KES1-GFP	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 KES1-GFP-HIS3MX6*	Invitrogen
CMY210	Y2454; *kes1*::*NatMX4*	McMaster lab collection; (Fairn et al., 2007)
CMY102	*MATα sec14^{ts} ura3 his3 trp1 leu2* GAL+	McMaster lab collection; (Xu et al., 2001)
CMY136	*MATα sec14^{ts} ura3 his3 trp1 leu2 GAL+ kes1::HIS3*	McMaster lab collection; (Fairn et al., 2007)
CMY505	BY4741 *sec14^{ts}*::*NatMX4*	McMaster lab collection; (Curwin et al., 2009)
CBY1	*MATα leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1Δ901 suc2Δ9*	Beh lab; (Beh and Rine, 2004)
CBY924	*MATα leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1Δ901 suc2Δ9 osh1Δ::KanMX4 osh2Δ::KanMX4 osh3Δ::LYS2 kes1Δ::HIS3 osh5::LEU2 osh6Δ::LEU2 osh7Δ::HIS3 [*pRS414-*KES1]*	Beh lab; (Beh and Rine, 2004)
CBY926	*MATα leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1Δ901 suc2Δ9 osh1Δ::KanMX4 osh2Δ::KanMX4 osh3Δ::LYS2 kes1Δ::HIS3 osh5::LEU2 osh6Δ::LEU2 osh7Δ::HIS3 [*pRS414-*kes1^{ts}]*	Beh lab; (Beh and Rine, 2004)
AAY102	SEY6210 *STT4*::*HIS3* [pRS415-*stt4^{ts}*]	Emr lab; (Audhya et al., 2000)
AAY202	SEY6210 *MSS4*::*HIS3* [Ycplac111-*mss4^{ts}*]	Emr lab; (Audhya et al., 2000)
AAY104	SEY6210 *PIK1*::*HIS3* [pRS314-*pik1^{ts}*]	Emr lab; (Audhya et al., 2000)
vps34^{ts}	SEY6210 *VPS34*::*TRP1* [pRS316-*vps34^{ts}*]	Emr lab; (Audhya et al., 2000)

Yeast Strain	**Genotype**	**Source**
CMY564	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-ERG6*	UCSF; (Curwin et al., 2009)
CMY566	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-SNF7*	UCSF; (Curwin et al., 2009)
CMY557	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-CHC1*	UCSF; (Curwin et al., 2009)
CMY559	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-ANP1*	UCSF; (Curwin et al., 2009)
CMY563	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-SEC13*	UCSF; (Curwin et al., 2009)
CMY562	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-COP1*	UCSF; (Curwin et al., 2009)
SEY6210 *PMA1*-dsRFP	*MAT***a** *ura3-52 leu2-3,112 his3-100 trp1-901 lys2-901 suc2-9 PMA1::tdimer2::KanMX4*	(Bagnat et al., 2000)
CMY306	*SEY6210 PMA1-dsRFP kes1::HIS*	This study
CMY307	*SEY6210 PMA1-dsRFP sec14^{ts}*	This study

Construction of strain CMY307

The CMY102 yeast strain (*sec14^{ts}*:*NatMX4*) was mated to the SEY6210 dsPma1-RFP yeast strain to produce a diploid strain that was heterozygous for both *sec14^{ts}* and the dsPma1-RFP construct. Haploid meiotic segregates were selected for by resistance to both NAT (NatMX4) and G418 (KanMX4). Confirmation of the selected haploid strain derivative was done by assessing growth at 37°C and by fluorescence microscopy to detect red-fluorescent protein (RFP).

Yeast and bacterial expression plasmids

Plasmids that were used are listed in Table 2, while the plasmids that were constructed specifically for this work are listed in Table 3.

Yeast genes were amplified by PCR using HiFi Platinum Taq DNA polymerase (Invitrogen). Genomic DNA from wild-type cells or a plasmid containing the gene of interest was used as the template. Samples were denatured at 95ºC for 5 min, then put through 30 cycles of denaturation, annealing and elongation at 95ºC for 30 sec, at 50ºC for 30 sec, and 68ºC for 1 min/Kbp. Afterwards, a final 7-min elongation at 68ºC was performed. Primers often included restriction enzyme sites for sub-cloning into the pCR2.1-TOPO vector (using the TOPO-TA cloning kit from Invitrogen). Unless the cloned gene was under the control of an exogenous promoter and only the ORF was cloned, the cloned gene contained genomic DNA 500 bp upstream and 500 bp downstream of the coding region. The DNA fragments were subsequently sub-cloned from the pCR2.1-TOPO-vector into the appropriate yeast or bacterial vector using restriction sites located within the yeast or bacterial vector and the pCR2.1-TOPO vector.

Table 2. Plasmids obtained for this study.

Plasmid	**Description**	**Source**
pRS416, pRS426	Yeast shuttle vectors *URA3*; 416 (CEN), 426 (2μ)	Hieter lab; (Sikorski and Hieter, 1989)
pRS415, pRS425	Yeast shuttle vectors *LEU2*; 415 (CEN), 425 (2μ)	Hieter lab; (Sikorski and Hieter, 1989)
pRS423	Yeast shuttle vector *HIS*3; (2μ)	Hieter lab; (Sikorski and Hieter, 1989)
pGFP-Snc1	Promoter of TPI expressing GFP-Snc1	Pelham lab; (Lewis et al., 2000)
Osh2-PH-GFP	PH domain of Osh2 fused to GFP	Levine lab; (Roy and Levine, 2004)
FYVE-GFP	FYVE domain fused to GFP	Emr lab; (Burd and Emr, 1998)
pET23b	Bacterial expression vector	Novagen; (Studier et al., 1990)
pESCURA, pESCLEU	Galactose-inducible yeast expression vectors (P_{GAL1})	Stratagene
pRS41H	Yeast shuttle vector; hphNT1 (hygromycin B) marker	(Studier et al., 1990)
pCB255	*kes1*^{ts} *TRP*1 (CEN)	Beh lab; (Beh and Rine, 2004)
KES1-pRS415	Yeast shuttle vector *LEU*; 415 (CEN)	Personal communication: Dr. Greg Fairn
KES1-pRS314	2.4-kb fragment containing *KES1*; SacI, ClaI cloning sites	Personal communication: Dr. Chris Stefan
kes1^{3E}-pRS314	2.4-kb fragment containing *KES1*; SacI, ClaI cloning sites removes unique EcoRI site 3E- R236E K242E K243E	Personal communication: Dr. Chris Stefan
SEC14-pRS416	SpeI, NotI cloning sites	Personal communication: Dr. Amy Curwin
sec14^{ts}-pRS415	SpeI, NotI cloning sites	Personal communication: Dr. Amy Curwin
sec14^{ts}-pRS425	SpeI, NotI cloning sites	Personal communication: Dr. Amy Curwin

Table 3. Plasmids constructed for this study.

Plasmid	**Gene**	**Construction**	**Restriction Sites Used**	**Backbone**
KES1-HIS6x-pET23b	*KES1*	Amplified from *KES1* open reading frame, TA-cloned, and then subcloned into pET23b using primers 5' Kes1-HIS6x (pET23b) primer 5' CTC CAT ATG ATG TCT CAA TAC GCA AGC TCA TCC TCA TGG 3' Kes1-HIS6x (pET23b) primer 5' GTG CTC GAG CAA AAC AAT TTC CTT TTC TTC GTC CCA CAA C	NdeI XhoI	pET23b
P_{GAL1}-*KES1*-HIS6x-pESCURA	*KES1*	PCR amplified *KES1*-HIS6x from *KES1*-HIS6x-pET23b plasmid, TA-cloned and subcloned into pESCURA using primers 5' Kes1-HIS6x primer 5'GGA TCC ATG TCT CAA TAC GCA AGC 3' Kes1-HIS6x primer 5'AAG CTT CAG CAG CCA ACT CAG CTT CCT TTC	HindIII, BamHI	pESC URA
P_{GAL1}-*KES1*-HIS6x-pESCLEU	*KES1*	Subcloned Kes1-HIS6x cassette from P_{GAL1}-*KES1*-HIS6x-pESCURA	HindIII, BamHI	pESC LEU
P_{GAL1}-*kes1*^{K109A}-HIS6x-pESCURA	*KES1*	Site-directed mutagenesis from PGAL1-KES1-HIS6x-pESCURA using primers 5' Kes1 K109A primer 5' CGT AAT GAA TCC TTA GGT TCT GAG AAA **GCC** CCT TTG AAC CCA TTT CTA GGT GAG TTG 3' Kes1 K109A primer 5' CAA CTC ACC TAG AAA TGG GTT CAA AGG **GGC** TTT CTC AGA ACC TAA GGA TTC ATT ACG	HindIII, BamHI	pESC URA

Plasmid	Gene	Primers	Restriction Sites Used	Backbone
P_{GAL1}-*kes1*^{3E}-HIS6x-pESCURA	*KES1*	Site-directed mutagenesis from P_{GAL1}-*KES1*-HIS6x-pESCURA using primers 5' Kes1 R236E KK242/243EE primer 5' CTT TGT GTT ATT GAA TTT TCA GGT **GAG** GGC TAC TTT TCT GGT **GAG GAG** AAT TCA TTC AAG GCA AGA ATT TAC 3' Kes1 R236E KK242/243EE primer 5' GTA AAT TCT TGC CTT GAA TGA ATT **CTC CTC** ACC AGA AAA GTA GCC **CTC** ACC TGA AAA TTC AAT AAC ACA AAG	HindIII, BamHI	pESC URA
P_{GAL1}-*kes1*$^{2\text{-}29\Delta}$-HIS6x-pESCURA	*KES1*	PCR amplify from *KES1*-HIS6x-pET23b using primers 5' Kes1 2-29Δ primer 5' GGA TCC **ATG CCT** CCA TTC ATT TTA TCT CC 3' Kes1 2-29Δ primer 5' AAG CTT CAG CAG CCA ACT CAG CTT CCT TTC	HindIII, BamHI	pESC URA
41H-*kes1*^{ts}-pRS41H	*Kes1*^{ts}	PCR amplified from pCB255, TA-cloned and subcloned into pRS41H using primers 5' Kes1 (+600 bp) primer 5' TCT AGA GAG CTC CTA AAC GAA CTA 3' Kes1 (-500 bp) primer 5' GGT ACC ATC GAT TAT GTG GTT CTA	XbaI KpnI	pRS41H

Plasmid	Gene	Primers	Restriction Sites Used	Backbone
KES1-GFP-pRS415	*KES1*	PCR amplified from KES1-GFP strain, TA-clone and subclone into pRS415 using primers 5' Kes1 (+500 bp) primer 5' AAG CTT ATT CCG TTC GCC TTT TAC TAG 3' Kes1 GFP (between GFP and HIS ORF) primer 5' CTC GAG GCA GGT CTG CAG CGA GGA GCC	HindIII, XhoI	pRS415
KES1-GFP-pRS416	*KES1*	Subcloned from *KES1*-GFP-pRS415	HindIII, XhoI	pRS416
kes1^{K109A}-GFP-pRS415	*KES1*	Site-directed mutagenesis from *KES1*-GFP-pRS415 using primers 5' Kes1 K109A primer 5' CGT AAT GAA TCC TTA GGT TCT GAG AAA **GCC** CCT TTG AAC CCA TTT CTA GGT GAG TTG 3' Kes1 K109A primer 5' CAA CTC ACC TAG AAA TGG GTT CAA AGG **GGC** TTT CTC AGA ACC TAA GGA TTC ATT ACG	HindIII, XhoI	pRS415
kes1^{3E}-GFP-pRS415	*KES1*	Site-directed mutagenesis from *KES1*-GFP-pRS415 using primers 5' Kes1 R236E KK242/243EE primer 5' CTT TGT GTT ATT GAA TTT TCA GGT **GAG** GGC TAC TTT TCT GGT **GAG GAG** AAT TCA TTC AAG GCA AGA ATT TAC 3' Kes1 R236E KK242/243EE primer 5' GTA AAT TCT TGC CTT GAA TGA ATT **CTC CTC** ACC AGA AAA GTA GCC **CTC** ACC TGA AAA TTC AAT AAC ACA AAG	HindIII, XhoI	pRS415

Plasmid	Gene	Primers	Restriction Sites Used	Backbone
KES1-pRS416	*KES1*	Subcloned from Kes1-pRS314 into pRS416	SacI, ClaI	pRS416
KES1-pRS415	*KES1*	Subcloned from Kes1-pRS416 into pRS415	SacI, XhoI	pRS415
kes1^{3E}-pRS416	*KES1*	Subcloned from Kes1^{3E}-pRS314 into pRS416	SacI, ClaI	pRS416
kes1^{3E}-pRS415	*KES1*	Subcloned from Kes1^{3E}-pRS416 into pRS415	SacI, XhoI	pRS415
kes1^{K109A}-pRS416	*KES1*	Site-directed mutagenesis from Kes1-pRS416 using primers 5' Kes1 K109A primer 5' CGT AAT GAA TCC TTA GGT TCT GAG AAA **GCC** CCT TTG AAC CCA TTT CTA GGT GAG TTG 3' Kes1 K109A primer 5' CAA CTC ACC TAG AAA TGG GTT CAA AGG **GGC** TTT CTC AGA ACC TAA GGA TTC ATT ACG	SacI, ClaI	pRS416
kes1^{K109A}-pRS415	*KES1*	Subcloned from *kes1*^{K109A}-pRS416	SacI, XhoI	pRS415

* bolded letters represent the substitutions made for site-directed mutagenesis mutants

Generated plasmids were sequenced by the DalGEN sequencing facility (Halifax, NS), Macrogen Sequencing (USA), or the Analytical Genetics Technology Centre (Toronto).

Construction of tagged Kes1 proteins

C-terminal hexahistidine (HIS6x) tagged Kes1-HIS6x constructs were made using wild-type (BY4741) genomic DNA as a template for PCR amplification of the gene, followed by TA-cloning and sub-cloning into the pET23b bacterial expression vector. The resulting pET23b-based plasmid contained the entire *KES1* ORF with a HIS6x epitope tag inserted in frame at the 3' end. Using HindIII and BamHI restriction sites, the *KES1*-HIS6x cloned fragment was TA-cloned and sub-cloned into the pESCURA vector and subsequently into the pESCLEU yeast expression vector under the control of the *GAL1* inducible promoter (P_{GAL1}).

C-terminally tagged Kes1-GFP constructs were made using Kes1-GFP (Invitrogen) genomic DNA as a template for PCR amplification of the gene. Subsequent TA-cloning and sub-cloning into the pRS415/pRS416 plasmids, using the HindIII and XhoI restriction enzymes, allowed expression of the gene under its own promoter.

Construction of site-directed mutagenesis plasmids

Kes1 mutant proteins ($Kes1^{K109A}$) and ($Kes1^{3E}$; R236E K242E K243E) were made by site-directed mutagenesis using the Quick Change II XL Site-Directed Mutagenesis Kit (Stratagene). Description of constructed plasmids and primers used can be found in Table 3.

2.2 METHODS

2.2.1 Recombinant DNA Manipulations and Transformations

DNA techniques

Plasmid DNA was isolated from an overnight culture of DH10B, DH5α or TOP10 *E. coli* cells, grown up in LB and ampicillin (100 μg/ml) at 37°C, using QIAprep Spin Miniprep Kit (QIAGEN). DNA fragments were resolved by agarose gel electrophoresis, excised in Glass Milk and then washed with NaI supplied from a Gene CleanII kit (Bio101). Agarose gel electrophoresis was performed as described by Sambrook *et al.* using 1% agarose gels containing 1X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) and ethidium bromide (1 μg/ml). Plasmids or genomic DNA from yeast was isolated from a 5 ml overnight culture grown at 25°C using the Y-DER (yeast DNA extraction reagent) kit from Pierce.

Yeast transformation

Plasmid DNA was transformed into yeast cells using the lithium acetate/polyethylene glycol (PEG) method (Gietz et al., 1995). Yeast cells were grown to early logarithmic phase in 10 ml of YPD medium (per transformation) and collected by centrifugation for 5 min at 3,500 rpm in a bench-top centrifuge. Cells were washed with sterile water and re-suspended in 50 μl of TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA)/lithium acetate (LiAc) (100 mM) (TE/LiAc). Then, 50 μg of heat-denatured herring sperm DNA and 1-1.5 μg of plasmid DNA were added to the re-suspended cells, and 300 μl of PEG (40%) in TE/LiAc was added. The cells were incubated for 45 min at 25°C, collected by centrifugation for 30 sec at 14,000 rpm, re-suspended in 200 μl of TE and spread onto selective solid medium for incubation at 25°C.

For targeted disruption of genes, linear DNA was transformed using the same method described for yeast transformations with some minor adjustments. Cells were grown to very early logarithmic phase in 50 ml of YPD medium and re-suspended in 100 μl LiAc/TE. PCR-amplified disruption cassettes were confirmed by agarose gel electrophoresis and directly added to the transformation mixture. The herring sperm DNA and PEG solution was double from that used for a normal yeast transformation (100 μg and 600 μl, respectively). When the transformation involved a drug-resistance gene (i.e *NatMX4*), cells were grown overnight in YPD liquid medium before being spread onto solid medium containing the appropriate drug.

***E. coli* transformations**

Chemically competent DH5α *E. coli* cells (25 μl) were incubated with 0.1-0.2 μg of plasmid DNA for 30 min on ice. The cells were then heat-shocked at 42°C for 30 sec and put on ice for 2 min, followed by the addition of 200 μl of SOC media (0.5% Yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose). Cells were incubated with shaking at 37°C for 1 hour and were spread on LB solid medium containing ampicillin (100 μg/ml)

For transformations of newly ligated plasmid DNA, DH10B cells (10 μl) (Invitrogen) were thawed on ice and electroporated as per the manufacturer's instructions, with 1 μl of ligation reaction and 60 μl of water. Electroporation was done using a 0.1 cm electroporation cuvette in a Bio-Rad Genepulser II set at 1.56 kV. Cells were incubated with shaking at 37°C for 1 hour and were spread on LB solid medium containing ampicillin (100 μg/ml).

2.2.2 Yeast Genetic Techniques

High-copy suppressor screen

To identify possible high-copy suppressors of the lethality of yeast cells lacking all Osh proteins, a high-copy suppressor screen was performed using the CBY926 strain. A yeast transformation was first done to insert a hygromycin B (41H-*kes1*^{ts}) plasmid containing a *kes1* temperature-sensitive mutant. Cells were grown in medium containing tryptophan to ensure plasmid loss of the previous resident plasmid containing the *kes1* temperature-sensitive mutant *(*pRS314-*kes1*^{ts}*)*. A 100 ml culture was then grown to mid-logarithmic phase at 25°C. Cells were harvested at 3,500 rpm for 5 min, and the pellet was washed in 50 ml of distilled water. Afterwards, the pellet was re-suspended in 800 μl of freshly prepared 1X TE/LiAc, and the competent cells were added to 10 μg of a *S. cerevisiae* high-copy library (Cedarlane Laboratories Limited; ATCC) and 2 mg of denatured herring sperm carrier DNA. 6 ml of PEG (40 %) in LiAc was added, and the cells were incubated for 2 hours at 25°C in a shaking incubator. The cells were chilled on ice for 5 min and harvested by centrifugation for 5 min at 1,000 *g* at room temperature. Cells were re-suspended in 10 ml 1X TE and spread (200 μl per plate) on SC-TRP solid medium containing 350 mg/L hygromycin B at 37°C. Plasmids were isolated from single colonies for sequencing.

Synthetic Genetic Array Analysis

Synthetic genetic array (SGA) refers to a specific type of genetic screen that uses robotics to create yeast mutant strains for subsequent analysis (Tong et al., 2001). This method was developed after the construction of the yeast single-gene deletion collection revealed that approximately 80% of yeast genes are not individually essential for life

(Winzeler et al., 1999). Using the yeast deletion collection of all non-essential genes, along with automated high-throughput robotics, we gain access to thorough yeast genetic screens without the need for library screens for gene identification (Tong et al., 2001). To facilitate high-throughput genetic analysis, an ordered array of a total of 4672 viable yeast deletions mutants, out of the approximate 6200 genes, was constructed (Winzeler et al., 1999). Every open reading frame was disrupted using a KanMX4 cassette providing resistance to the antibiotic G418 (Geneticin). All individual deletion-mutant strains are derivatives of the strains BY4741 (MAT**a** *ura3*Δ*0 leu2*Δ*0 his3*Δ*1 met15*Δ*0*) or BY4742 (MATα *ura3*Δ*0 leu2*Δ*0 his3*Δ*1 lys2*Δ*0*). Traditionally, to perform genetic screens with Your Favorite Gene 1 (*YFG1*), a specific strain (Y2454; MATα *mfa1*Δ*::MFA1pr-HIS3 can1*Δ*0 ura3*Δ*0 leu2*Δ*0 his3*Δ*1 lys2*Δ*0*) is used for replacement of the *YFG1* ORF with a NatMX4 cassette providing resistance to the drug nourseothricin (*yfg1*Δ::*NatMX4*) (Tong et al., 2001). The resulting Y2454 query strain containing *yfg1*Δ::*NatMX4* is mated to each of the 4672 yeast single strains from the single-gene deletion collection in the BY4741 strain background (where the ORF of each non-essential gene has been individually replaced with the KanMX4 drug-resistance cassette). Growth defects can be searched for in the double mutant haploid strain that is not seen in the single mutant strains. For the purpose of this study, we used the query strain CMY210, in which *KES1* is replaced with *NatMX4*. The P_{GAL1}-*KES1*-HIS6x plasmid was transformed in the query strain. When grown under 2% galactose conditions, to increase expression of Kes1, these cells stop growing.

Diploids were selected based on resistance to both NAT (NatMX4) and G418 (KanMX4). These diploids were forced to undergo meiosis to form haploids by limiting

nutrients in the medium. Normal segregation of genes during meiosis resulted in a collection of 4672 strains, where one of every four haploid meiotic progeny on average contains *kes1* inactivated with *NatMX4*, as well as a non-essential gene inactivated with *KanMX4*. To be certain that only haploids grow, the integration of the *HIS3* open reading frame downstream of the mating-type-specific *MFA1* promoter in the Y2454 strain ensures that the *HIS3* gene will only be transcribed in haploid yeast derivatives of the **a** mating type (and not in haploids of the α mating type or in diploids). The deletion collection is *MAT***a** and *his3*Δ, and all diploids will be heterozygous for *his3*Δ (cannot grow on medium lacking histidine). The query strain (*yfg1*Δ::*NatMX4*) is *MAT*α, meaning that the *HIS3* construct described above cannot be transcribed in this strain (Tong et al., 2001). Subsequent to sporulation (meiosis), cells were subjected to growth on solid medium lacking histidine to ensure that the resultant haploid derivatives were indeed the product of a mated diploid, and not a diploid or a non-mated original parental strain.

The query strain also has an additional marker to facilitate counter-selection, to make sure that no diploids grow in the final step. Can1 is an arginine permease which not only transports exogenous arginine into the cell but is also the only transporter for the arginine analogue canavanine (Whelan et al., 1979). Cells consequently incorporate canavanine, instead of arginine, into proteins, causing cell death. Inactivating *CAN1* (*can1*Δ) does not cause this cell defect and lethality, even in the presence of canavanine. Therefore, including canavanine in the final selection medium ensures that *CAN1/can1*Δ heterozygous diploids are non-viable and only *can1*Δ haploids can grow.

Yeast cells were transferred using a 768 floating-pin replicator where the deletion collection is arrayed over 20 plates (384 spots per plate, each corresponding to one specific yeast strain containing a gene disrupted with the *KanMX4* cassette) (Tong et al., 2001). The selection for *MAT***a** haploid double mutant segregates was performed by a series of pinnings. Diploids were first transferred to a synthetic minimal medium (lacking uracil to ensure plasmid maintenance) containing cavanine but lacking histidine and arginine. After meisosis, the grown haploids were transferred to a fresh new medium (the same as above but now containing G418 (200 μg/ml)) and, subsequently, to a medium containing NAT (100 μg/ml). The final product resulted in *MAT***a** double-mutant progeny that harbours the P_{GAL1}-*KES1*-HIS6x-pESCURA plasmid. The final two pinnings were on medium selecting for all markers and containing galactose (2%) to allow Kes1 protein expression and to monitor for the alleviation of growth defects. Monitoring of colony size as a measure of improved growth was done by visual inspection.

Serial dilution to assess yeast growth

Serial dilution was used to assess yeast growth under varying conditions. Yeast cultures were grown to logarithmic phase at 25°C in selective minimal medium or YPD and diluted to an OD_{600} of 0.1 in the appropriate medium. The cells were then serially diluted 1 in 10 and 5 μl of each dilution (x3) was spotted onto the appropriate solid medium and incubated at 25°C and 37°C for 48-96 hours. Images of the resulting cell growth were acquired using a VersaDoc (Bio-Rad).

2.2.3 Biochemical Assays

Purification of Kes1-HIS6x tagged proteins

HIS6x-tagged proteins were purified by over-expression of the protein using a pESCURA vector in yeast (galactose induction). Cells were grown overnight in 10 ml of SRaf-URA medium (1%) and diluted to OD_{600} of 0.25 in 1 L cultures. When cells were in logarithmic phase, 2% galactose was added and the cultures were incubated for 4 hours for protein expression. Cells were harvested by centrifugation at 3,500 rpm for 5 min at 4°C and re-suspended in 20 ml of 20 mM Tris-HCl (pH 7.5) and 10% glycerol. EDTA-free complete protease inhibitor (Roche) was added (1/50 v/v) and the cells were lysed by passage through a French press three times at 8,000 psi. Lysates were centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant was collected. Cobalt resin (Pierce) was re-suspended and 500 µl of the slurry was transferred to a Bio-Rad disposable column. The resin was washed twice using 1 ml of wash buffer (10 mM imidazole, 50 mM sodium phosphate pH 7.0, 300 mM NaCl) and by gravity flow. The cell extract was transferred to the column containing the washed resin twice for optimum binding, and bound Kes1-HIS6x was eluted with 2 ml of elution buffer (150 mM imidazole, 50 mM sodium phosphate pH 7.0, 300 mM NaCl) and by gravity flow. $Kes1^{K109A}$-HIS6x, $Kes1^{3E}$-HIS6x and $Kes1^{2\text{-}29_{\Delta}}$-HIS6x mutant proteins were purified using the same method. Kes1-HIS6x, Kes1-K^{109A}-HIS6x, $Kes1^{3E}$-HIS6x or $Kes1^{2\text{-}29_{\Delta}}$-HIS6x was dialyzed overnight against 1 L of 20 mM Tris-HCl (pH 7.5) and 10% glycerol.

Micro BCA protein assay

Protein concentration of Kes1-HIS6x was measured compared to albumin standards of 1 µg, 2 µg, 5 µg and 10 µg. Micro BCA reagents A, B and C (Pierce) were

used at a ratio of 50:48:2. Micro BCA reagent mix (500 µl) was added to 1-20 µl of purified protein and to the albumin standards in small glass tubes. The reagent mix (500 µl) was used as a blank. The tubes were incubated at 60°C in a water bath for 60 min. After incubation, the tubes were cooled to room temperature fairly quickly by placing them in cold water. Absorbance at 562 nm was measured using a DU-640 spectrophotometer and protein concentration was determined.

SDS PAGE and western blot analysis

Proteins were separated by SDS-PAGE along with pre-stained benchmark markers (Invitrogen) in 10% acrylamide gels. Gels were stained by silver nitrate or Gel Code (Pierce).

For western blot analysis, proteins were transferred to PVDF membranes (GE Healthcare) in 25 mM Tris-HCl, 200 mM glycine, 1% SDS, 20% methanol for 1 hour at 80 Volts. Membranes were washed with distilled water and blocked by drying overnight at room temperature. Membranes were incubated for 1 hour in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic and a pH of 7.4) containing 0.1% Tween 20 and 5% milk powder containing anti HIS6x primary antibody (1:1,000), anti-GFP (1:5,000) or anti-Pgk (1:5,000). Membranes were rinsed three times for 5 min with PBS containing Tween 20. They were then incubated for 30 min in PBS containing Tween 20, 1% skim milk powder and 1:5,000 dilution of the horseradish peroxidase-coupled secondary antibody (Cell Signaling). The membranes were washed again with PBS containing Tween 20. The proteins were detected using Super signal West Pico Chemiluminescent Substrate (Pierce) and membranes were exposed to X-Omat Blue film (Kodak).

Gel staining

Proteins resolved by SDS-PAGE electrophoresis were either silver-stained (Shevchenko et al., 1996) or stained with Gel Code (Pierce). Gels for silver staining were fixed for 20 min with 50% methanol and 5% acetic acid followed by 50% methanol for 10 min and washed with water at 4°C overnight. Gels were sensitized with 0.02% $Na_2S_2O_3$ for 1 min, washed with water and then incubated with cold 0.1% $AgNO_3$ for 20 min at 4°C. After washing the gel with water, it was developed using 0.04% formalin and 2% Na_2CO_3 by changing the developer repeatedly until proteins bands were visible.

Gels that were stained with Gel Code (Pierce) were incubated in Gel Code for 1 hour at room temperature, followed by 3 washes in water or until protein bands were visible.

In vivo analysis of phosphoinositides

Cells were grown overnight to an early logarithmic phase at 25°C in 15 ml of SC medium (2% glucose or 1% raffinose) lacking the appropriate supplements. When needed, 2% galactose was added to allow protein induction and cultures were then incubated for 4 hours. Cells were harvested at 3,500 rpm for 5 min, washed twice in inositol-free medium and resuspended in 1 ml of inositol-free medium. For cells carrying a temperature-sensitive mutant protein, they were incubated at 37°C for 1 hour before changing to an inositol-free medium for another 1 hour incubation at 37°C. 5 OD_{600} (5 ml of OD_{600}=1) units were re-suspended in 450 µl of an inositol-free medium and 50 µCi of myo-[2-^{3}H]inositol (American Radiolabeled Chemicals) was added followed by a 1 hour incubation. After that time, cells (500 µl total) were added to 500 µl of cold 9% perchloric acid (PCA) and 500 µl of glass beads to allow cell lysis by vortexing for 5

min. Extracts were collected by centrifugation at maximum speed for 10 min at 4°C and vigorously re-suspended in 1 ml of ice-cold 100 mM EDTA. Extracts were collected again by centrifugation at maximum speed for 10 min at 4°C and re-suspended by sonication in 50 μl of ice-cold 100 mM EDTA. Phospholipids were deacylated by using methylamine as described (Hawkins et al., 1986). To the 50-μl sample, 1 ml of deacylation reagent (methanol, methylamine-40%, ddH_20, 1-butanol, 4.6:2.6:1.6:1.1, v/v) was added and the mixture was incubated at room temperature for 30 min followed by 50 min incubation in a 53°C water bath. Un-reacted methylamine was removed by drying the lipids in a Speed-vac overnight and the dried pellet was re-suspended in 500 μl sterile water. After another round of drying in the Speed-vac, re-suspension in 500 μl sterile water and drying in the Speed-vac, the pellet was re-suspended in 300 μl sterile water by sonication to allow the deacylated lipids to be completely dissolved. An equal volume of the extraction reagent was then added (1-butanol, ethyl ether, formic acid ethyl ether/ethyl formate, 4:0.8:0.2). The samples were mixed by vortexing for 30 sec and collected by centrifugation at 13,000 rpm for 3 min. The aqueous phases containing the [^{3}H]glycerophosphoinositides were transferred to new tubes and re-extracted with the extraction reagent. The aqueous phases were collected and dried in a Speed-vac overnight. Samples were re-suspended in 22 μl sterile water. Quantities of [^{3}H] glycerophosphoinositides were analysed by Dr. Christopher Stefan in the lab of Dr. Scott Emr (Cornell University, Ithaca, NY) using an anion-exchange Partisphere SAX column (Whatman Inc.) coupled to a gold high-performance liquid chromatography (HPLC) system (Shimadzu) and an online radiometric detector (Packard Instrument Company) (Stefan et al., 2002).

In vivo measurements of sphingolipids

Measurements of sphingolipids were done in collaboration with Ashley Cowart's laboratory in the Department of Biochemistry and Molecular Biology at the Medical University of South Carolina. Lipid extraction and measurements was done as previously described (Brice et al., 2009). For steady-state labeling, cells were grown overnight to saturation and inoculated into a medium containing 1 μCi/ml [^{3}H]inositol. The cells were grown for 21 hours to mid-logarithmic phase, 10^7 cells were harvested (pipetted into five volumes of ice-cold 10% trichloroacetic acid solution and washed twice) and lipids were extracted. The dried extract was dissolved in 16:16:5 chloroform:MeOH:water and resolved by thin-layer chromatography (TLC) in a solvent system of chloroform:methanol:NH_4OH (9:7:2) as described (Mao et al., 1997).

Liposome preparation

Liposomes were prepared as described (Bigay et al., 2005). Lipids in chloroform, methanol and/or ethanol were purchased from Avanti Polar Lipids (PC, PI, PS, PI-4P and PI-4,5P_2), Echelon (PI-3P and PI-3,5P_2) and Sigma (Ergosterol). A dried film was prepared through the evaporation of a mixture of the indicated lipids, and then re-suspended in 50 mM Hepes pH 7.2 and 120 mM potassium-acetate (KAc) for 1 hour at 25°C in a shaking incubator. The liposome suspension was extruded sequentially through polycarbonate filters of 200-nm or 50-nm pore size.

Liposome flotation experiments

Kes1-HIS6x binding to liposomes was analyzed using liposome flotation as described (Bigay et al., 2005). Kes1-HIS6x (0.5 μM) and liposomes (1 mM) were incubated in HKM buffer (50 mM Hepes pH 7.2, 120 mM KAc, 1 mM $MgCl_2$, 1 mM

DTT) for 5 min at room temperature in a total volume of 150 μl. 100 μl of 75% (w/v) sucrose solution (in HKM buffer) was added and mixed with the liposome-protein mix. 200 μl of HKM buffer containing 25% w/v sucrose was gently overlaid on top of the high-sucrose suspension, followed by the addition of 50 μl of HKM buffer containing no sucrose. The samples were centrifuged at 55,000 rpm for 1 hour in a Beckman swinging rotor (TLS 55). The top (100 μl) and bottom (250 μl) layers were manually collected and analyzed by SDS-gel and western blot for the presence of Kes1-HIS6x.

Co-purification of TAP tag and protein binding partners

The Tandem Affinity Protein (TAP) tag protocol was described by Puig et al., (Puig et al., 2001). Yeast cells expressing *KES1*-TAP (Invitrogen) were grown to mid-logarithmic phase in 4 L of YPD medium at room temperature. Cells were harvested by centrifuging at 3,000 rpm for 10 min at 4°C. Pellets were resuspended in 10 mM K-HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, EDTA-free protease tablet (2 tablets diluted in 4 ml of water), and pepstatin A. Cells were lysed by passing through a French press 3 times at 8,000 psi.

A $1/9^{th}$ volume of 2 M KCl was added to the cell lysate, and the cells were centrifuged at 10,000 rpm for 5 min at 4°C. The supernatent was added to the IgG agarose beads that were previously suspended in IPP150 buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.1% NP-40) and incubated for 2 hours at 4°C. The suspension was added to a 10 ml disposable column and drained by gravity flow. Beads were washed using 30 ml of IPP150 buffer followed by 10 ml TEV cleavage buffer (10% glycerol, 10 mM Tris-Cl pH 8.0, 100 mM NaCl, 0.1% NP-40/Triton X-100, 0.5 mM EDTA, 1 mM DTT). Beads were incubated with 1 ml TEV cleavage buffer and 10 units of acTEV

recombinant protease (Invitrogen) for 2 hours at 16°C. The eluate was recovered by gravity flow.

Calmodulin beads were prepared by washing with a calmodulin-binding buffer (10% glycerol, 10 mM β-mercaptoethanol, 10 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% NP40/Triton X). The washed beads were added to the IgG eluate with 3 ml of calmodulin-binding buffer and 3 μl of 1 M $CaCl_2$. After incubation at 4°C for 1 hour, the suspension was added to a column and washed with 30 ml of calmodulin-elution buffer (10% glycerol, 10 mM β-mercaptoethanol, 10 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 5 mM EGTA, 0.1% NP40/Triton X). The samples were concentrated using ReadyPrep Cleanup Kit (Biorad). Proteins were separated by SDS-PAGE electerophoresis, silver stained and excised for analysis by mass spectrometry. Peptides were sequenced by ms/ms at the Proteomics Facility at Dalhousie University.

Identification of* Kes1-HIS6x *interaction partners

Purified Kes1-HIS6x was re-bound to the Cobalt resin, and yeast cell protein extract was added to Kes1-HIS6x-bound resin or empty resin for determination of Kes1-HIS6x interacting proteins. Proteins were separated by SDS-PAGE electrophoresis, silver-stained, and excised for analysis by mass spectrometry. Peptides were sequenced by ms/ms at the Proteomics Facility at the Faculty of Medicine at Dalhousie University.

2.2.4 Microscopy

Live-cell imaging

A Zeiss Axiovert 200M microscope fitted with a plan-neofluor 100x oil immersion objective lens was used for all microscopy experiments. Cells were visualized using differential interference contrast (DIC), GFP, RFP or UV filters. Images were captured using a Zeiss Axio Cam HR and Zeiss Axiovision 4.5 software.

For immobilization and maintenance of cells in a single plane of focus, the cells were mounted on microscope slides containing agarose pads (Fisher). Concentrated cells (2-3 μl) were added to the agarose pad and a cover slip was placed on top. A seal was formed by adding clear nail polish around the cover slip, and the cells were visualized immediately.

Cells were grown in SC (2% glucose) medium unless otherwise noted. Cells expressing plasmids with the P_{GAL1} promoter were grown in SRaf (1%) medium and transferred to SGal (2%) medium for a further 16-hour incubation to allow protein expression.

Osh2-PH-GFP is a PI-4P reporter that contains the PH domain of Osh2 fused to GFP (Roy and Levine, 2004). The reporter is known to bind PI-4P both at the plasma membrane and at the *trans*-Golgi (Roy and Levine, 2004). The FYVE domain fused to GFP is a PI-3P reporter that has been shown to bind PI-3P at the vacuole and endosome (Burd and Emr, 1998). Snc1 is a v-SNARE that traffics between the *trans*-Golgi, the plasma membrane and the endosome to facilitate numerous rounds of membrane fusion. Snc1 fused to GFP (Snc1-GFP) follows the recycling pathway between these organelles

(Lewis et al., 2000). Cells expressing these chimeras were visualized using DIC and fluorescence microscopy (GFP).

Yeast organelle marker strains express the protein of interest fused to RFP. CMY306 and CMY307 express a genomic ds*PMA1*-RFP. Live cells were imaged using DIC and fluorescence microscopy (RFP).

FM4-64 trafficking to the endosome and vacuole

The lipophilic dye FM4-64 displays a long-wavelength red fluorescence when bound to a lipid (Vida and Emr, 1995). In a time-dependent manner, FM4-64 assesses trafficking from the plasma membrane to the vacuole *via* the endosome. Cultures (15 ml) were harvested by centrifugation at 3,500 *g* for 5 min and re-suspended in the appropriate fresh medium. The cell resuspension was divided for 5, 15, 30 and 45 min time points. Cells were labeled with 40 μM FM4-64 in DMSO for 5 min, washed in fresh medium and incubated further at 25°C for the 4 different time points. Live cells were imaged at the time points indicated above using fluorescence microscopy (RFP).

Lipid droplet staining with BODIPY 493/503

Cells grown to mid-logarithmic phase were stained with 50 μM Bodipy 493/503 for 30 min in a shaking incubator. Cells were washed in PBS, and live cells were visualized using fluorescence microscopy (RFP).

Electron microscopy

Cultures (5 ml) were grown to mid-logarithmic phase, and cells were harvested and washed twice in sterile water. Cells were then incubated in 1 ml of 1.5% $KMnO_4$ for 20 min, harvested at 3,500 rpm for 5 min and washed twice in sterile water. They were then incubated in 1 ml of 1% sodium periodate for 15 min at 25°C, harvested and washed

once with sterile water. Cells were incubated in 1 ml of 1% NH_4Cl for 10 min, harvested and washed once with sterile water, then re-suspended in 500 μl of PBS. Embedding, image capture and processing was performed by the Electron Microscopy Facility of the Faculty of Medicine at Dalhousie University.

CHAPTER 3 RESULTS

3.1 *IN VITRO* CHARACTERIZATION OF LIPID BINDING BY KES1

3.1.1 Kes1-HIS6x Purification

Kes1 is a soluble protein that binds sterols in its core and phospholipids on its surface. In addition to lipid binding, Kes1 can also bind membranes *via* its 29 residue N-terminal lid forming an α-helix that is predicted to contain an ALPS motif (Fairn and McMaster, 2005; Im et al., 2005; Knodler and Mayinger, 2005; Li et al., 2002a; Xu et al., 2001). This motif has been identified to preferentially bind membranes of high curvature (Drin et al., 2007). These same N-terminal 29 amino acids also form a lid that sequesters bound sterols within the core of Kes1. To address the role of sterol, phospholipid, and curved membranes in Kes1 membrane association, a HIS6x tag was appended to the carboxy terminus of Kes1. Kes1-HIS6x protein was expressed in *S. cerevisiae* cells (Figure 11A) and purified to apparent homogeneity using Co^{2+} affinity resin (Figure 11B). Mutant versions of Kes1-HIS6x, either unable to bind PIPs ($Kes1^{3E}$) (Li et al., 2002a), with decreased sterol-binding capacity ($Kes1^{K109A}$) (Im et al., 2005; Raychaudhuri et al., 2006), or with the N-terminal lid deleted ($Kes1^{2\text{-}29\Delta}$ $Kes1^{2\text{-}29_{\Delta}}$), were also expressed in *S. cerevisiae* and purified.

3.1.2 Kes1 Requires a Free Phosphate Group to Associate with Liposomes

Since Kes1 is a PIP- and sterol-binding protein, and can also associate with membranes, I wanted to assess the ability of Kes1-HIS6x to bind to liposomes of different curvature and composition. Liposomes of 50 nm and 200 nm diameter,

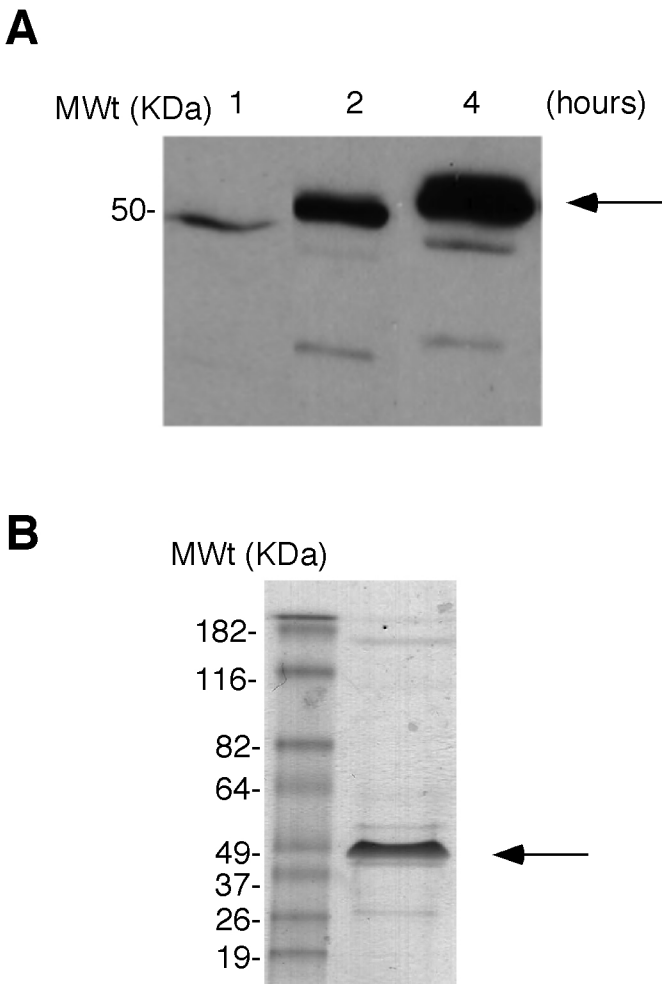


Figure 11 Kes1-HIS6x purification. (A) BY4741 [P_{GAL1}-*KES1*-HIS6x-pESCURA] cells were grown to mid-logarithmic phase in SRaf medium at 25°C and shifted to galactose containing medium for 1, 2 or 4 hours. Protein expression by these cells was analyzed by SDS-PAGE followed by western blot with anti-HIS6x antibodies. (B) BY4741 [P_{GAL1}-*KES1*-HIS6x-pESCURA] cells were grown to mid-logarithmic phase in SRaf medium at 25°C and shifted to galactose containing medium for a 4 hour incubation. Protein purification was performed using cobalt resin. The eluted protein sample was separated by SDS-PAGE and the gel was stained with gel code.

representing vesicles (the average diameter of a transport vesicle in *S. cerevisiae* is 35-50 nm) and planar membranes, were prepared. The liposomes contained various phospholipids in the absence or presence of the major fungal sterol ergosterol. Liposomes were prepared with the major phospholipid PC along with 10% (mol/mol) of another phospholipid and/or 10% (mol/mol) ergosterol. Kes1-HIS6x bound liposomes containing any PIP tested, including PI-4P, PI-3P, PI-4,5P_2 and PI-3,5-P_2 (although less so than the other PIPs), as well as liposomes containing phosphatidic acid (PA) (Figure 12). However, Kes1-HIS6x was unable to bind membranes containing other phospholipids, including PC alone or PC plus 10% (mol/mol) of PI, PE, PS or DAG.

In the presence of PIPs or PA, Kes1-HIS6x bound liposomes of 50 nm only slightly better than 200 nm liposomes. Kes1-HIS6x was unable to bind membranes containing PC and ergosterol, while the addition of ergosterol to PIP- or PA-containing liposomes resulted in only a small increase in Kes1-HIS6x membrane association (Figure 12).

The results indicate that pure Kes1-HIS6x requires a phospholipid with a free phosphate group to associate with membranes. Ergosterol alone is unable to promote Kes1-HIS6x membrane association and the presence of ergosterol in PIP-containing liposomes, or altering membrane curvature, had limited effects on Kes1-HIS6x membrane association.

3.1.3 The Role of PIP and Sterol Binding by Kes1 in Membrane Association

To determine if the PIP-binding domain on the surface of Kes1 is responsible for allowing membrane association, the purified Kes1^{3E}-HIS6x mutant was assessed for its

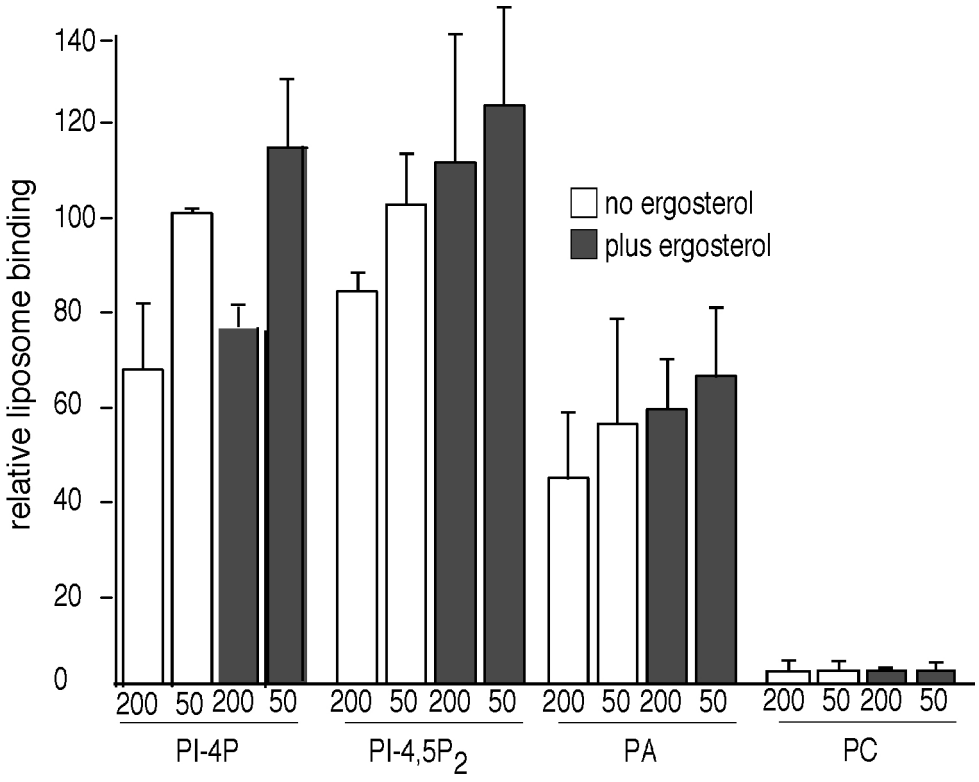


Figure 12 Kes1 association with liposomes requires the presence of membrane phospholipids with a free phosphate group. Liposomes were made of PC + 10% (mol/mol) of another phospholipid and/or 10% (mol/mol) ergosterol. Kes1-HIS6x bound to liposomes was isolated by flotation. Assays contained 0.25 μM Kes1-HIS6x and 1 mM liposomes extruded at 200 nm and 50 nm. After sucrose-gradient centrifugation, the top and bottom fractions were collected and analyzed by SDS-PAGE and western blot. The results were quantified using densitometry. The data is presented as relative liposome association compared to Kes1-HIS6x binding to 50 nm liposomes composed of PC + 10% (mol/mol) PI-4P (100% binding). Kes1-HIS6x binding to liposomes containing PC + 10% (mol/mol) PI, PS, PE or DAG (+/-ergosterol) was similar to the results observed for Kes1-HIS6x binding to PC only liposomes (+/- ergosterol). Error bars show variation (± SE) observed for a minimum of three different experiments using either different set of extruded liposomes and/or different protein preparations.

ability to associate with membranes. Liposomes were composed of PC + 10 % (mol/mol) PI-4P with or without 10% ergosterol (mol/mol). Kes1^{3E}-HIS6x was unable to bind PIP-containing liposomes whether ergosterol was present or not (Figures 13A and 13B). This data is consistent with the requirement of a free phosphate group in the membrane to allow Kes1-HIS6x membrane association.

To examine the role of sterol binding, and the function of the lid as both a sterol sequesterer within the core and a method to target Kes1 to areas of high membrane curvature, Kes1^{K109A}-HIS6x and Kes1$^{2\text{-}29_{\Delta}}$-HIS6x were assessed for binding to liposomes composed of PC + 10% (mol/mol) PI-4P with or without 10% (mol/mol) ergosterol. The Kes1^{K109A}-HIS6x and Kes1$^{2\text{-}29_{\Delta}}$-HIS6x proteins showed similar liposome binding compared to wild-type Kes1, regardless of lipid composition (Figures 13A and 13B). The one exception was the Kes1^{K109A} mutant, which bound 50 nm vesicles in the absence of ergosterol much better than wild-type. One reason may be that Kes1^{K109A} cannot undergo a conformational change, which normally occurs within the sterol-binding pocket when Kes1 binds sterols. This conformational change could presumably allow the release of Kes1-HIS6x from liposomes. When ergosterol is present at a high enough concentration in the membrane, Kes1^{K109A} can bind ergosterol, abeit at a decreased level of wild-type Kes1 (Im et al., 2005; Raychaudhuri et al., 2006), and undergo the conformational change needed to be released from the membrane. The Kes1$^{2\text{-}29_{\Delta}}$-His6x protein did exhibit a slight increase in membrane association with the 50 nm liposomes compared to the other proteins, although this difference was minimal.

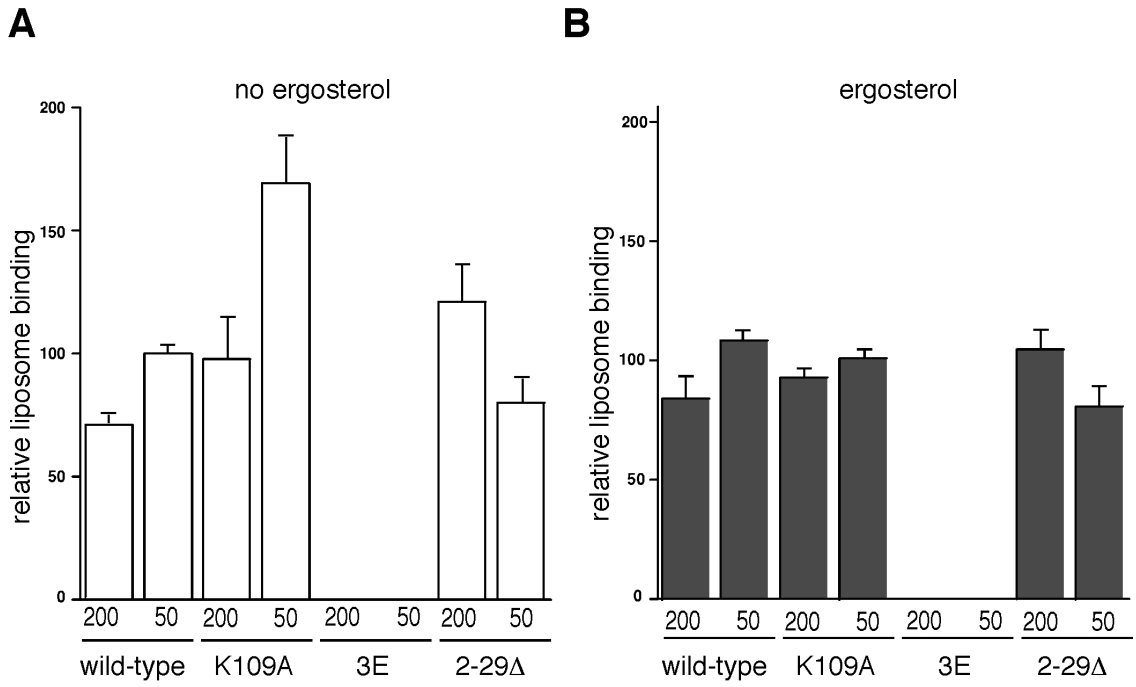


Figure 13 Kes1 binding to PIPs is required for liposome association. (A) Liposomes were made of PC + 10% (mol/mol) of PI-4P and (B) 10% (mol/mol) ergosterol. Kes1-HIS6x, Kes1^{K109A}-HIS6x, Kes1^{3E}-HIS6x or Kes1$^{2\text{-}29_{\Delta}}$ bound to liposomes was isolated by flotation. Assays contained 0.25 μM Kes1-HIS6x (wild-type or mutants) and 1 mM liposomes extruded at 200 nm and 50 nm. After sucrose gradient centrifugation the top and bottom fractions were collected and analyzed by SDS-PAGE and western blot. The results were quantified using densitometry. The data is presented as relative liposome association compared to Kes1-HIS6x binding to 50 nm liposomes composed of PC + 10% (mol/mol) PI-4P. Error bars show variation (± SE) observed for a minimum of three different experiments using either different sets of extruded liposomes and/or different protein preparations.

In summary, the major determinant with respect to the ability of pure Kes1 to associate with membranes is a phospholipid containing a free phosphate group, while ergosterol and membrane curvature have minimal roles.

3.2 *IN VIVO* CHARACTERIZATION OF LIPID BINDING BY KES1

3.2.1 Kes1-GFP Localization

A functional GFP-tagged version of Kes1 (Fairn et al., 2007), as well as the Kes1^{K109A} and Kes1^{3E} mutants, were expressed under control of the *KES1* promoter on a low-copy (1-3 copies per cell) plasmid, and transformed into *kes1*Δ cells. A western blot to detect the GFP tag determined that these proteins were expressed at similar levels *in vivo*, indicating that the mutations did not affect Kes1 abundance. The position of the bands on the western blot corresponded to the mass of the Kes1-GFP fusion proteins (Figure 14). This is indicative that the fusion proteins are intact and verifies their usefulness as probes for their localization in cells. Kes1-GFP localization was similar to that observed previously, with Kes1 present in the cytoplasm and in punctate spots (Figure 15). These punctate spots have been demonstrated previously to co-localize with Golgi marker proteins (Fairn et al., 2007). The Kes1^{K109A}-GFP mutant was also present in punctate regions but this was clearly not the case for Kes1^{3E}-GFP. This observation supports the *in vitro* data indicating that Kes1 must be able to bind PIPs to associate with membranes.

3.2.2 *In Vivo* Phosphoinositide Levels

To determine if there is a functional link between the ability of Kes1 to bind membranes containing PIPs *in vitro* and PIP metabolism in cells, I assessed PIP levels

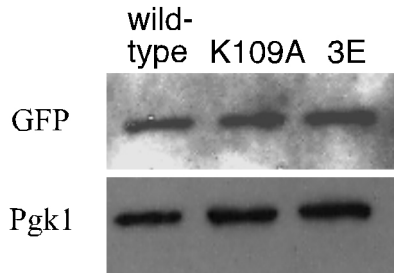


Figure 14 Kes1^{K109A} and Kes1^{3E} mutations do not alter expression levels of Kes1 protein. (A) *kes1*Δ [*KES1*-GFP-pRS415], *kes1*Δ [*kes1*^{K109A}-GFP-pRS415] and *kes1*Δ [*kes1*^{3E}-GFP-pRS415] cells were grown to mid-logarithmic phase in SC medium at 25°C. Cells lysates were analyzed by SDS-PAGE followed by western blot with anti-GFP antibodies and anti-Pgk1 antibodies.

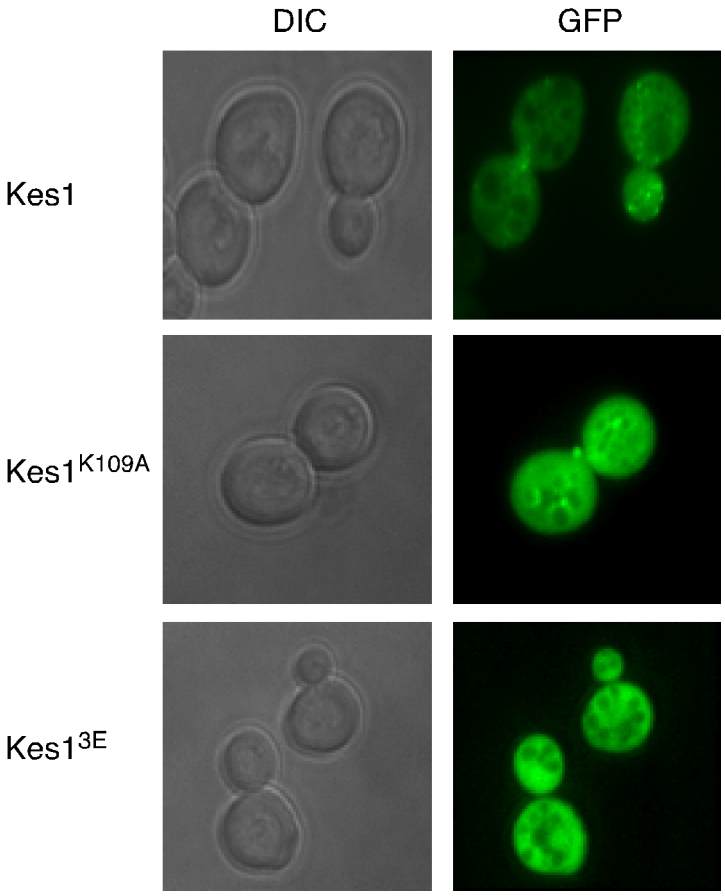


Figure 15 Kes1 binding to PIPs is required for association with Golgi membranes. *kes1*Δ [*KES1*-GFP-pRS415], *kes1*Δ [*kes1*^{K109A}-GFP-pRS415] and *kes1*Δ [*kes1*^{3E}-GFP-pRS415] cells were grown to mid-logarithmic phase in SC medium at 25°C. Live cells were visualized using fluorescence (GFP) and DIC filters.

when the *KES1* gene was inactivated and when *KES1* expression was increased. Inactivating *KES1* resulted in 40% increases in cellular PI-4P and PI-3P levels and smaller increases in PI-3,5P_2 and PI-4,5P_2 levels (Figure 16). After a 4-hour induction using the P_{GAL1} promoter to drive transcription of *KES1*, the levels of PI-3P and PI-4P were decreased by ~50%. The levels of PI-4,5P_2 also decreased, although this could be due to a decrease in the precursor PI-4P, respectively (Figure 17).

3.2.3 PIP Distribution and Availability

PIPs are spatially localized in cells, as they act as cues to define organelle identity and affect protein recruitment to, and function at, specific intracellular membranes. After I observed that altering expression of *KES1* caused changes in both PI-4P and PI-3P levels, I went on to assess PIP distribution using well-characterized fluorescent reporters for PI-4P (Osh2-PH-domain-GFP) and PI-3P (FYVE-domain-GFP).

Consistent with previous observations (Burd and Emr, 1998; Roy and Levine, 2004), in wild-type cells, distribution of the PI-4P reporter partitioned between the plasma membrane and punctate regions in the cytoplasm (known to be Golgi), while the PI-3P reporter primarily decorated the vacuolar membrane and was present in small punctate spots (known to be endosomes). Although the PIP reporters are primarly seen at these organelles, re-distribution can be due to a change in the ratio of the PIP at different organelles, or simply due to a decrease in the PIP available for binding.

Inactivation of *KES1* resulted in redistribution of the PI-4P reporter toward more punctate staining (Figure 18), indicative of more PI-4P being available for the reporter to bind at the Golgi. Both an increased level of PI-4P in these cells and the absence of Kes1,

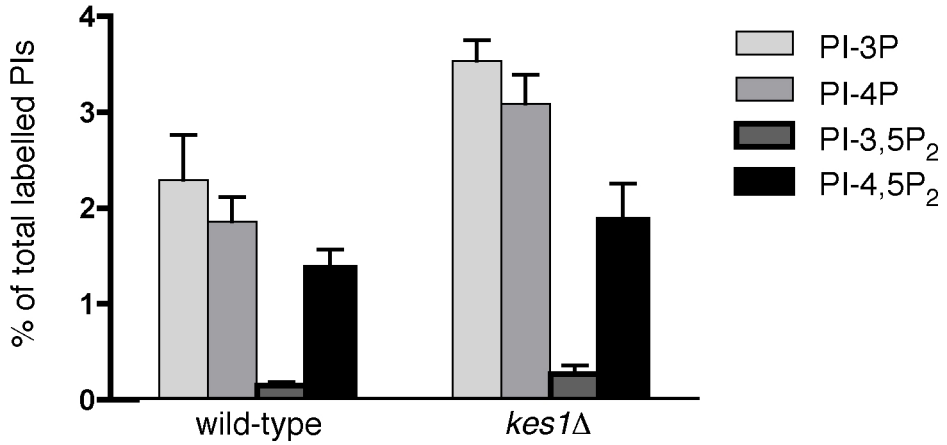


Figure 16 Inactivation of *KES1* increases PI-3P and PI-4P levels. BY4741 (wild-type) and *kes1*Δ cells were grown to mid-logarithmic phase in SC medium at 25°C. Cells were washed in inositol-free medium and labeled with *myo*-[^{3}H]inositol for 1 hour. Phosphoinositides were extracted, deacylated, separated by high-performance liquid chromatography and quantified using an online radiometric detector. Data is presented as percentage of the total number of counts for inositol-containing phospholipids and are expressed as mean ± SE of a minimum of three separate experiments.

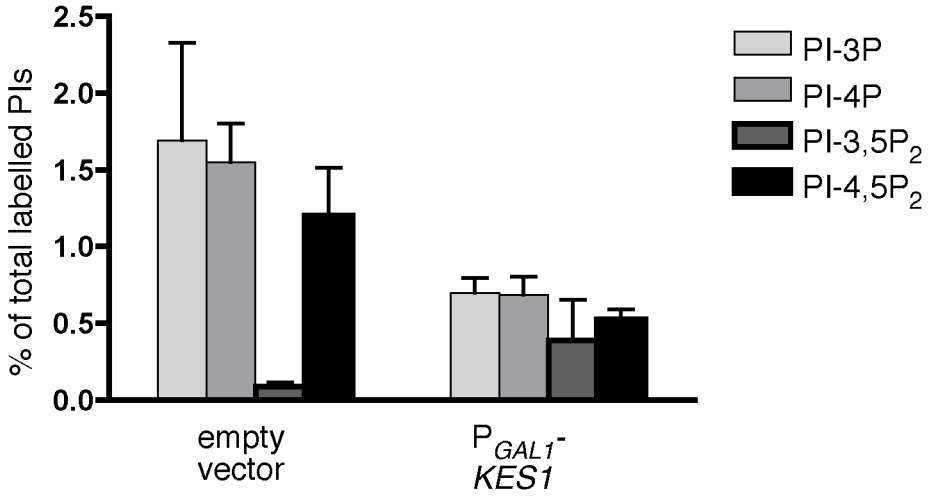


Figure 17 Kes1 negatively regulates PI-3P and PI-4P levels. BY4741 [vector, pESCURA] (wild-type) and *kes1*Δ [P_{GAL1}-*KES1*-HIS6x-pESCURA] cells were grown to mid-logarithmic phase in SRaf medium at 25°C. For over-expression of *KES1*, using the galactose inducible P_{GAL1} promoter, galactose was added to the medium and the cells were incubated for an additional 4 hours. Cells were washed in inositol-free medium and labeled with *myo*-[^{3}H] inositol for 1 hour. Phosphoinositides were extracted, deacylated, separated by high-performance liquid chromatography and quantified using an online radiometric detector. Data is presented as percentage of the total number of counts for inositol-containing phospholipids and are expressed as mean ± SE of a minimum of three separate experiments.

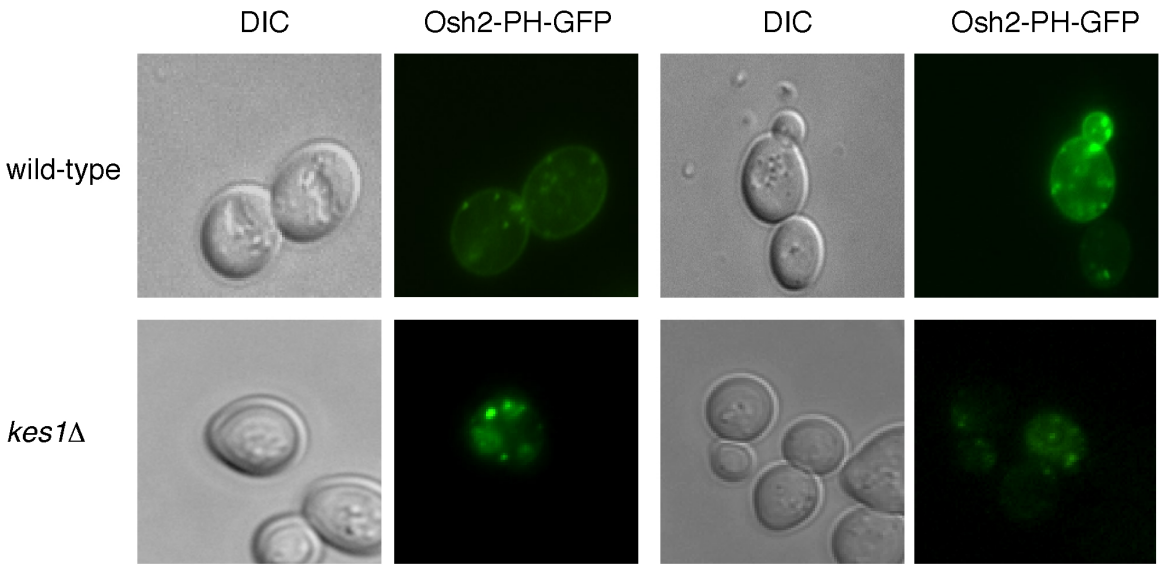


Figure 18 Inactivation of *KES1* increases PI-4P availability at the Golgi. BY4741 (wild-type) and *kes1*Δ cells were transformed with a low-copy plasmid expressing a PI-4P reporter that contains the PH domain of Osh2 fused to GFP. Cells were grown to early-logarithmic phase in SC medium at 25°C and visualized by using fluorescence (GFP) and DIC filters.

a PI-4P-binding protein, most likely contribute to the increased PI-4P available at the Golgi. The distribution of the PI-3P reporter did not appear to be altered in cells with an inactivated *KES1* gene (Figure 19).

Increased expression of *KES1* resulted in a more diffuse localization of the PI-4P probe (Figure 20), indicative of less available PI-4P in membranes for binding by the PI-4P probe. Since these cells exhibit a decrease in PI-4P level and a large increase in the PI-4P binding protein Kes1, both conditions could explain the re-distribution of PI-4P to a more diffuse localization. Localization of the PI-3P probe remained similar to that observed in wild-type cells, suggesting that increased expression of *KES1* does not affect PI-3P availability (Figure 21). Combined, the data imply that the major intracellular PIP that Kes1 binds is the Golgi pool of PI-4P.

3.2.4 The Role of Lipid Binding in Kes1 Regulation of PIP Metabolism and Availability

To assess whether the ability of Kes1 to bind to sterols or PIPs is important in regulating PIP levels, I measured *in vivo* PIP levels in cells in which the Kes1 mutant deficient in sterol binding ($Kes1^{K109A}$), or unable to bind PIPs ($Kes1^{3E}$), was expressed. For cells with an inactivated *KES1* gene, I observed that when expression of either Kes1 mutant was increased, the level of PI-4P was in between that of cells expressing an empty vector and that of cells in which wild-type Kes1 was over-expressed. Increased expression of $Kes1^{K109A}$ or $Kes1^{3E}$ decreased PI-3P levels to those observed for over-expression of wild-type Kes1 (Figure 22). This suggests a bi-functional role for Kes1 in the regulation of PIPs. On the one hand, binding PIPs and sterols enhances the ability of Kes1 to regulate PI-4P metabolism. However, Kes1 can still modulate the level of PI-3P even if PIPs or sterols cannot be bound.

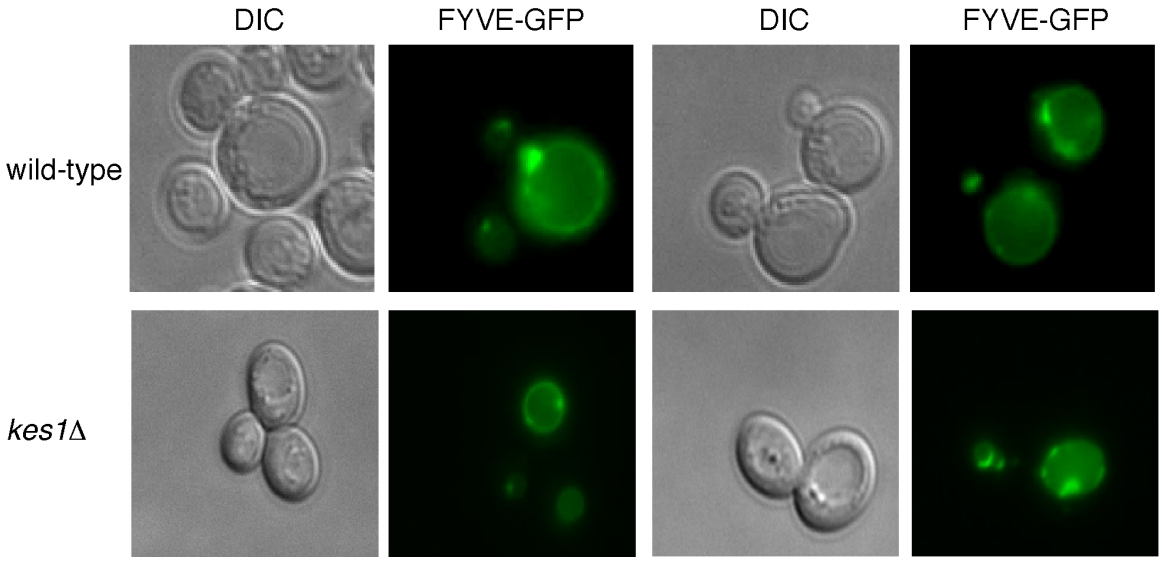


Figure 19 Inactivation of *KES1* does not change PI-3P availability. BY4741 (wild-type) and *kes1*Δ cells were transformed with a low-copy plasmid expressing a PI-3P reporter that contains the FYVE domain fused to GFP. Cells were grown to mid-logarithmic phase in SC medium at 25°C and visualized by using fluorescence (GFP) and DIC filters.

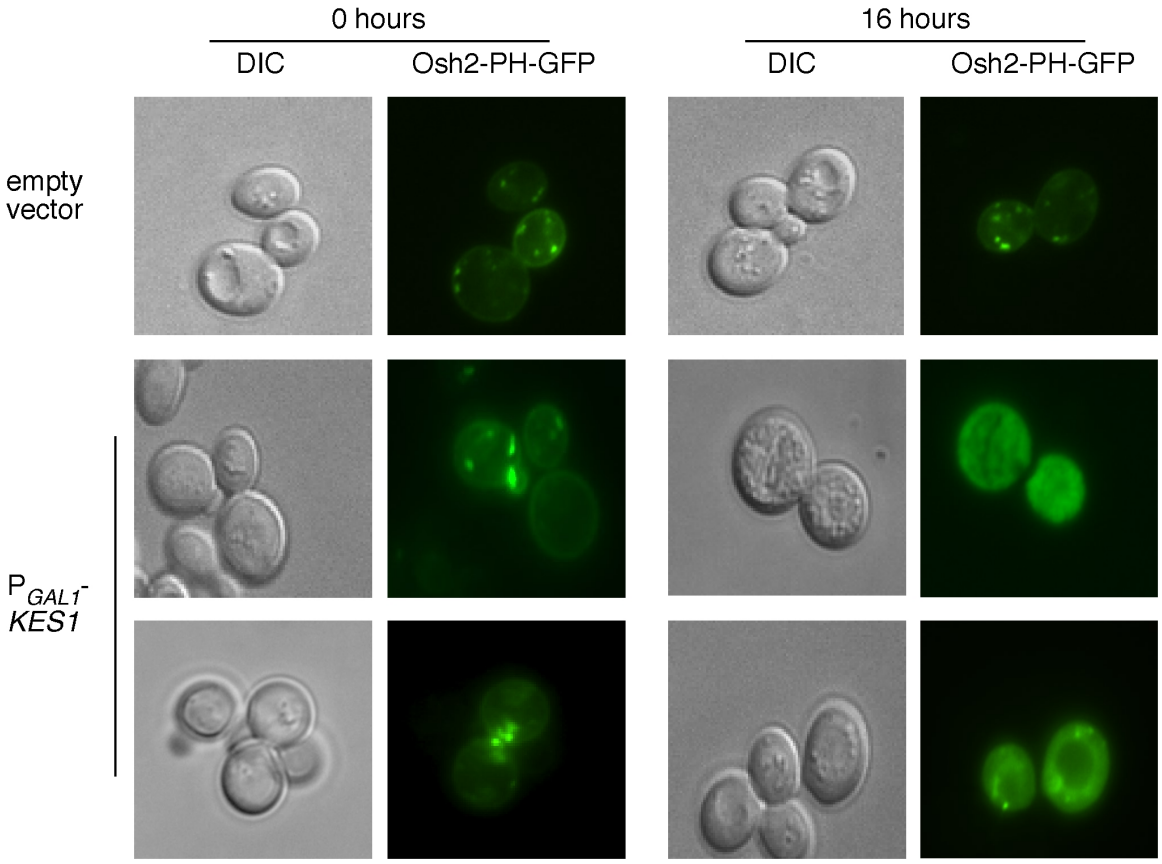


Figure 20 Increased expression of *KES1* decreases PI-4P availability at the Golgi. BY4741 [vector, pESCLEU] (wild-type) and *kes1*Δ [P_{GAL1}-*KES1*-HIS6x-pESCLEU] cells were transformed with a low-copy plasmid expressing a PI-4P reporter that contains the PH domain of Osh2 fused to GFP. Cells were grown to mid-logarithmic phase in SRaf at 25°C and transferred to SGal for a 16-hour incubation to induce Kes1-HIS6x protein expression. Cells were visualized by using fluorescence (GFP) and DIC filters.

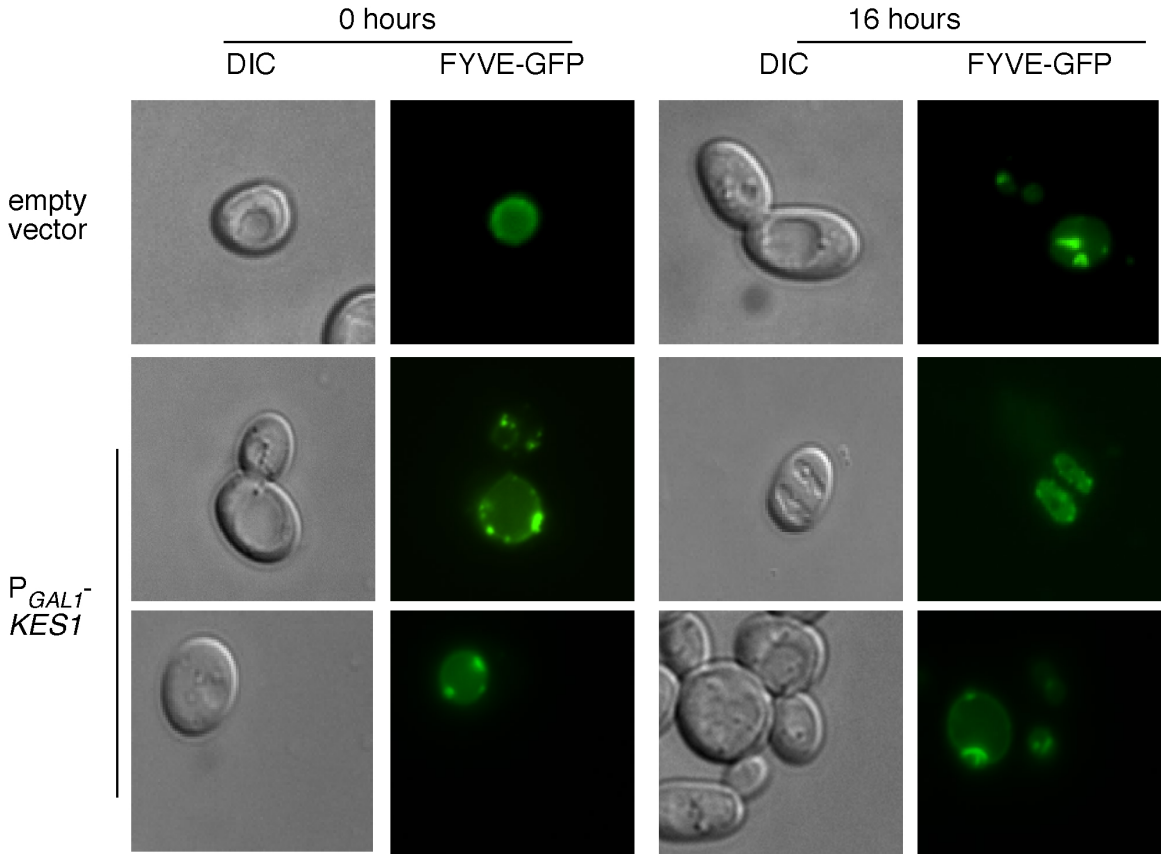


Figure 21 Increased expression of *KES1* does not change PI-3P availability. BY4741 [vector, pESCLEU] (wild-type) and *kes1*Δ [P_{GAL1}-*KES1*-HIS6x-pESCLEU] cells were transformed with a low-copy plasmid expressing a PI-3P reporter that contains the FYVE domain fused to GFP. Cells were grown to mid-logarithmic phase in SRaf medium at 25°C and transferred to SGal for protein expression for 16 hours. Cells were visualized using fluorescence (GFP) and DIC filters.

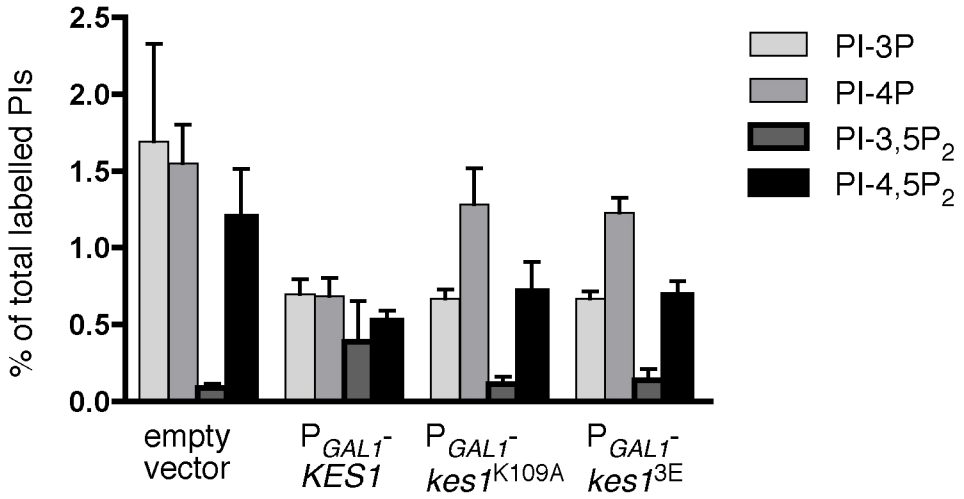


Figure 22 Sterol and PIP binding by Kes1 is required for regulation of PI-4P level. BY4741 [vector, pESCURA], *kes1*Δ [P_{GAL1}-*KES1*-HIS6x-pESCURA], *kes1*Δ [P_{GAL1}-*kes1*^{K109A}-HIS6x-pESCURA] and *kes1*Δ [P_{GAL1}-*kes1*^{3E}-HIS6x-pESCURA] cells were grown to mid-logarithmic phase in SRaf medium at 25°C and transferred to SGal medium for a further 16-hour incubation to induce protein expression. Cells were washed in inositol-free medium and labeled with *myo*-[^{3}H]inositol for 1 hour. Phosphoinositides were extracted, deacylated, separated by high-performance liquid chromatography and quantified using an online radiometric detector. Data is presented as percentage of the total number of counts for inositol-containing phospholipids and are expressed as mean ± SE of a minimum of three separate experiments.

To determine if there is a role for PIP or sterol binding by Kes1 in altering PI-4P availability at the Golgi, I expressed the Kes1 mutants, Kes1^{K109A} and Kes1^{3E}, in cells with an inactivated *KES1* gene, and looked at the localization of the PI-4P reporter. The characteristic intracellular punctate spots were still seen in these cells, suggesting that neither mutant Kes1 protein can bind to Golgi PI-4P at a level that significantly affects PI-4P availability (Figure 23). This is consistent with the increased expression of either of these mutants resulting in only a modest decrease in PI-4P level (see Figure 22). The absence of a strong presence of this reporter at the plasma membrane in these cells may be due to a change in ratio for distribution of PI-4P. An increase in PI-4P at the Golgi, compared to plasma membrane, may cause re-distribution of the reporter to more punctate spots.

3.2.5 Kes1-GFP Localization and Level are Dependent on PI-4P Level at the Golgi

I showed that Kes1 localization at the Golgi is dependent on PIP binding, and previous data demonstrates that PI-4P is required for localization of Kes1 to this membrane (Fairn et al., 2007). I set out to determine if PIPs, other than PI-4P, are required for proper localization of Kes1 to the Golgi. Consistent with previous data, at the non-permissive temperature, cells with a temperature-sensitive allele of *PIK1* (encoding PI-4 kinase at the Golgi) showed no association of Kes1-GFP with the Golgi. Cells with an inactivated *INP51* (encoding PI-4,5P_2 phosphatase) gene, which also exhibit decreased PI-4P levels, also had decreased association of Kes1-GFP with Golgi membranes. Furthermore, I observed that Kes1-GFP was not detectable in cells with an inactivated *SAC1* gene (encoding PI-4P and PI-3P phosphatase) (Figure 24A).

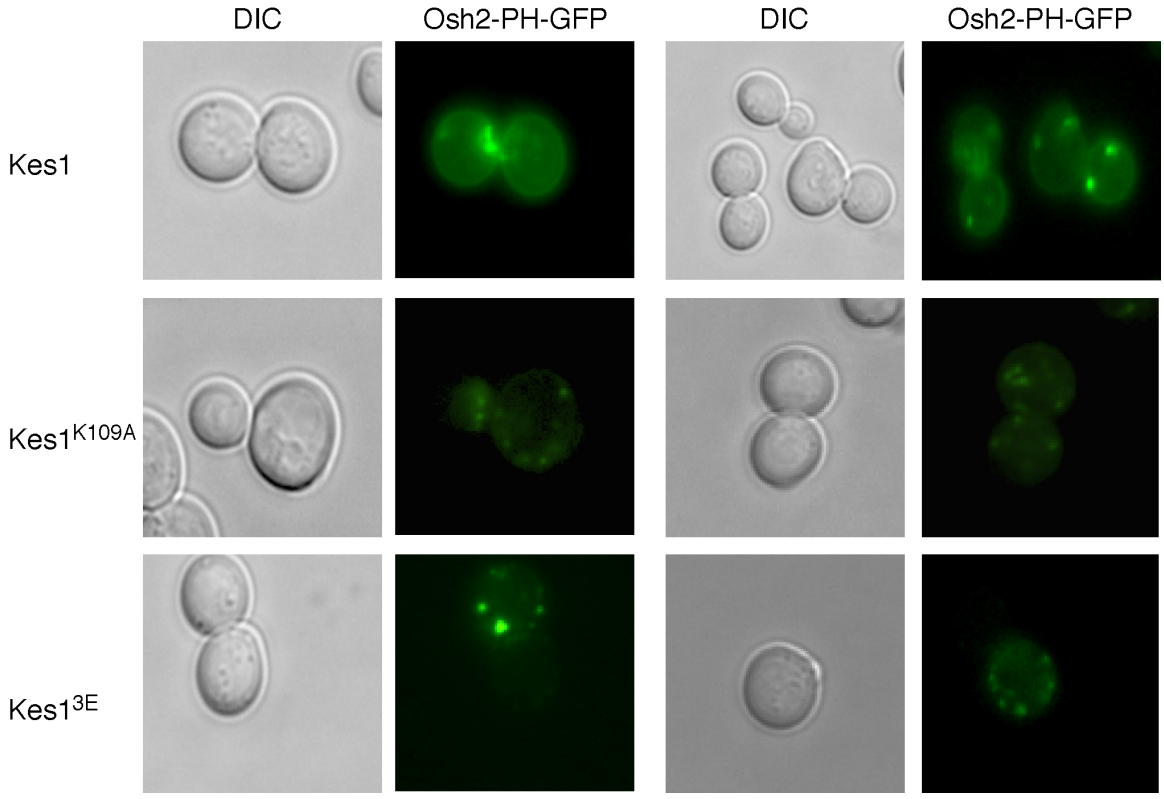


Figure 23 Sterol binding and PIP binding by Kes1 are required to regulate PI-4P availability. *kes1*Δ [*KES1*-pRS415], *kes1*Δ [*kes1*^{K109A}-pRS415] and *kes1*Δ [*kes1*^{3E}-pRS415] cells were transformed with a low-copy plasmid expressing a PI-4P reporter that contains the PH domain of Osh2 fused to GFP. Cells were grown to mid-logarithmic phase in SC medium at 25°C and visualized using fluorescence (GFP) and DIC filters.

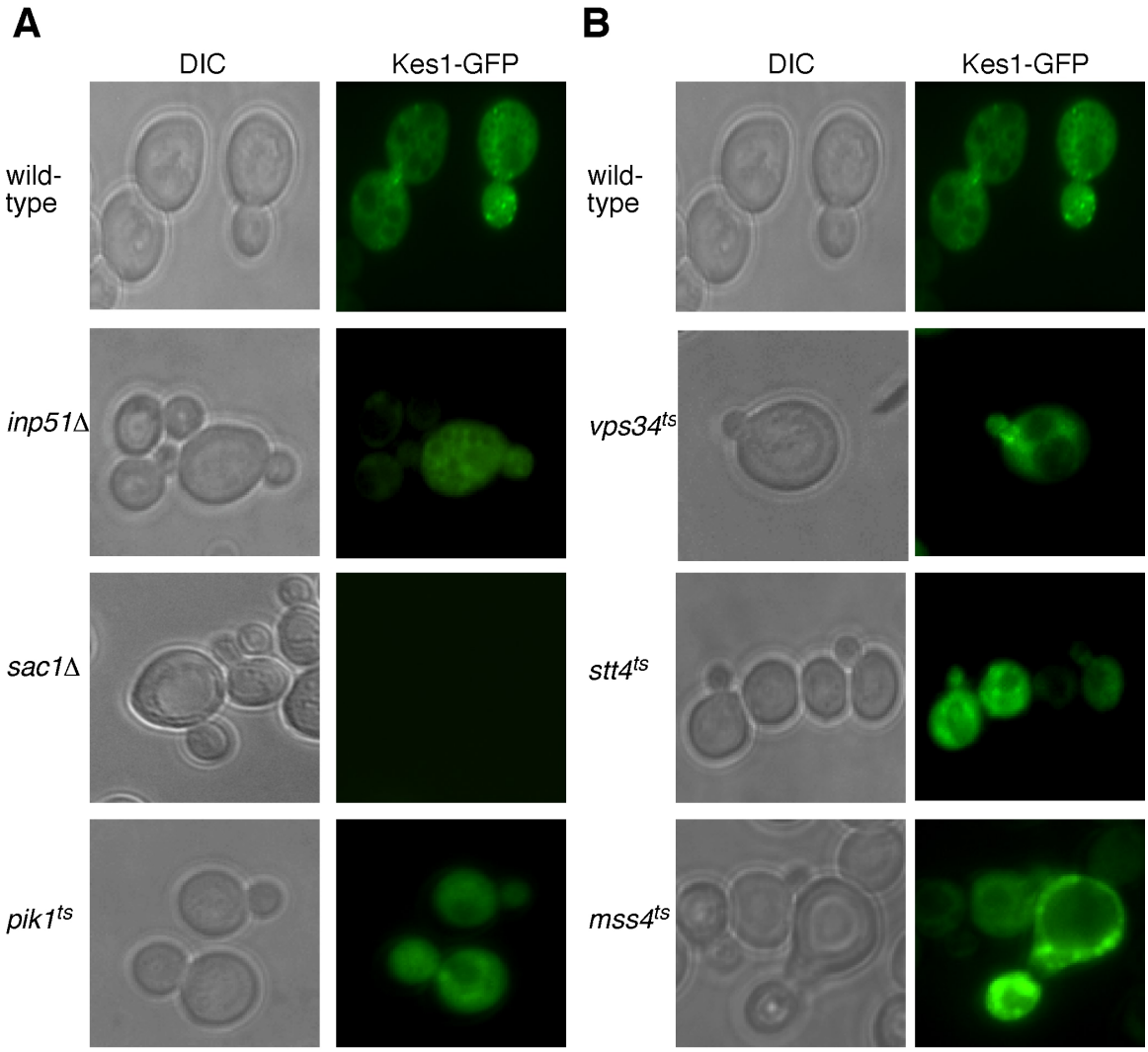


Figure 24 Kes1-GFP localization is dependent on PI-4P levels. [*KES1*-GFP-pRS415] was transformed into BY4741 (wild-type) and A) *pik1*^{ts}, *inp51*Δ and *sac1*Δ cells and B) *stt4*^{ts}, *mss4*^{ts} and *vps34*^{ts} cells. Cells were grown to mid-logarithmic phase in SC medium at 25°C and the temperature-sensitive cells were shifted to 37°C for a 2-hour incubation. Live cells were then visualized using fluorescence (GFP) and DIC filters.

Proper localization of Kes1-GFP appears to be primarily dependent on Pik1-synthesized PI-4P, as cells expressing the temperature-sensitive allele *stt4*^{ts} (affecting PI-4 kinase at the plasma membrane) or *mss4*^{ts} (affecting PI-4,5 kinase at the plasma membrane) displayed some Kes1-GFP localization to the Golgi (Figure 24B). The proper localization of Kes1-GFP also appears to be specific for PI-4P, as *vps34*^{ts} (encoding PI-3 kinase) cells showed association of Kes1 with Golgi membranes at the non-permissive temperature (Figure 24B).

The level of Kes1-GFP also appears to be dependent on proper PI-4P levels at the Golgi. A western blot for GFP determined that Kes1 was undetectable in *sac1*Δ cells (Figure 25A). It appears that Kes1-GFP may be considerably decreased in *pik1*^{ts} and *inp51*Δ cells, and near wild-type levels in *stt4*^{ts}, *mss4*^{ts} and *vps34*^{ts} cells (Figure 25A and 25B). This needs be confirmed and further investigated, as Kes1 protein expression altered by the levels of PIPs has never been previously reported.

3.3 Defects in Distinct Vesicular Transport Pathways

The effects on PIP levels and distribution suggest a role for Kes1 in regulation of PIP-dependent membrane transport pathways. PI-3P is synthesized in endosomal membranes by the PI-3 kinase Vps34, and is required for endosome transport (Schu et al., 1993; Slessareva et al., 2006; Stack et al., 1995; Stack et al., 1993). In contrast, PI-4P is primarily synthesized by two PI-4 kinases, the Golgi-resident Pik1 and Stt4 at the plasma membrane (Audhya et al., 2000; Demmel et al., 2008; Faulhammer et al., 2007; Strahl et al., 2005; Walch-Solimena and Novick, 1999).

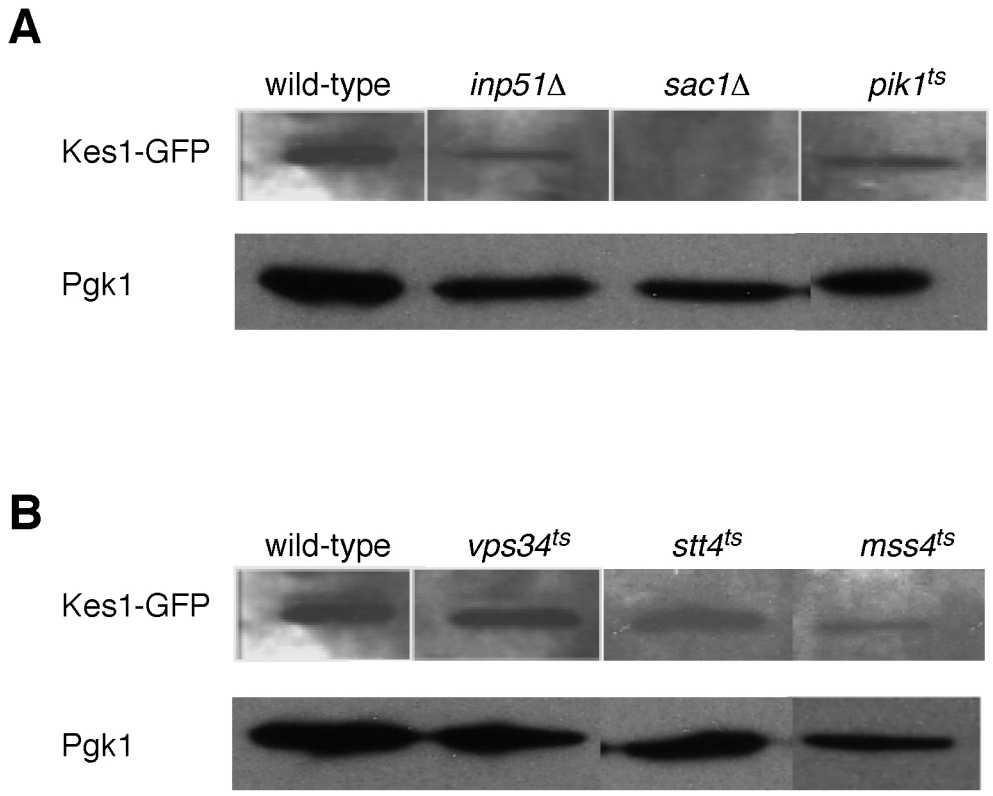


Figure 25 Kes1-GFP levels are affected by the levels of PI-4P and Sac1. [*KES1*-GFP-pRS415] was transformed in BY4741 (wild-type) and A) *pik1*^{ts}, *inp51*Δ and *sac1*Δ cells and B) *stt4*^{ts}, *mss4*^{ts} and *vps34*^{ts} cells. Cells were grown to mid-logarithmic phase in SC medium at 25°C and the temperature-sensitive cells were shifted to 37°C for a 2-hour incubation. Cells were then lysed and lysates were analyzed by SDS-PAGE followed by western blot with anti-GFP antibodies followed by incubation with anti-Pgk antibodies.

3.3.1 Vacuolar Staining with FM4-64

The lipophilic dye FM4-64 can be incorporated into the plasma membrane, from where it travels *via* endosomes to the vacuole (Vida and Emr, 1995). This FM4-64 trafficking was examined in cells with an inactivated *KES1* gene and in cells expressing *KES1* from the *GAL1* promoter. FM4-64 was incorporated into the plasma membrane, traveled through the endosome (as evident by punctate staining) and reached the vacuole (ring-like structure) with similar kinetics regardless of whether the *KES1* gene was inactivated (Figure 26) or over-expressed (Figure 27). Interestingly, although the transit of FM4-64 to the vacuole was not altered, the images did reveal that the vacuole was fragmented, especially upon increased expression of *KES1*. Expression of the *KES1* PIP- and sterol-binding mutants also did not alter the trafficking of the lipophilic dye to the vacuole (Figure 28).

3.3.2 GFP-Snc1 Cycling

Snc1 is a v-SNARE, routinely monitored using GFP-Snc1, that cycles between the *trans*-Golgi and plasma membrane to facilitate continuous rounds of membrane transport and fusion (Curwin et al., 2009). The bulk of Snc1 is normally found on the plasma membrane at the leading edge of the bud. This is where vesicular transport is directed to allow for delivery of components required for membrane expansion and cell growth (Curwin et al., 2009). Transport of Snc1 from the Golgi to the plasma membrane is dependent on PI-4P production in the Golgi. I observed a disruption of GFP-Snc1 trafficking, as indicated through the accumulation of GFP-Snc1 intracellularly, in situations where the *KES1* gene was inactivated (Figure 29) or over-expressed

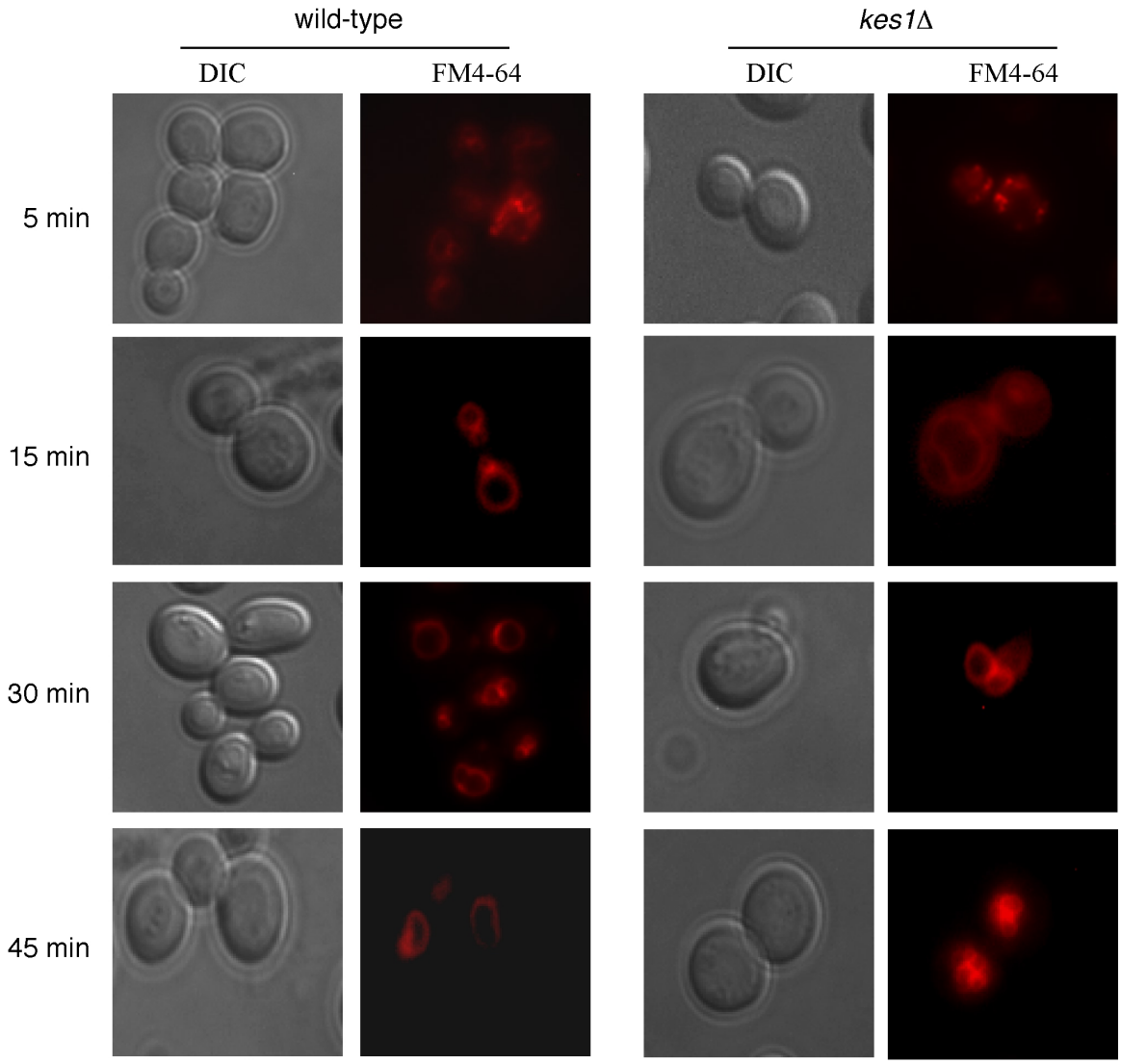


Figure 26 Trafficking of FM4-64 from the plasma membrane to the vacuole is not altered in cells with an inactivated *KES1* gene. BY4741 (wild-type) and *kes1*Δ cells were grown to mid-logarithmic phase at 25° in SC medium. Cells were labeled for 5 min with 40 μM FM4-64, and incubated post-label in fresh medium for the indicated times. Live cells were visualized using fluorescence (RFP) and DIC filters.

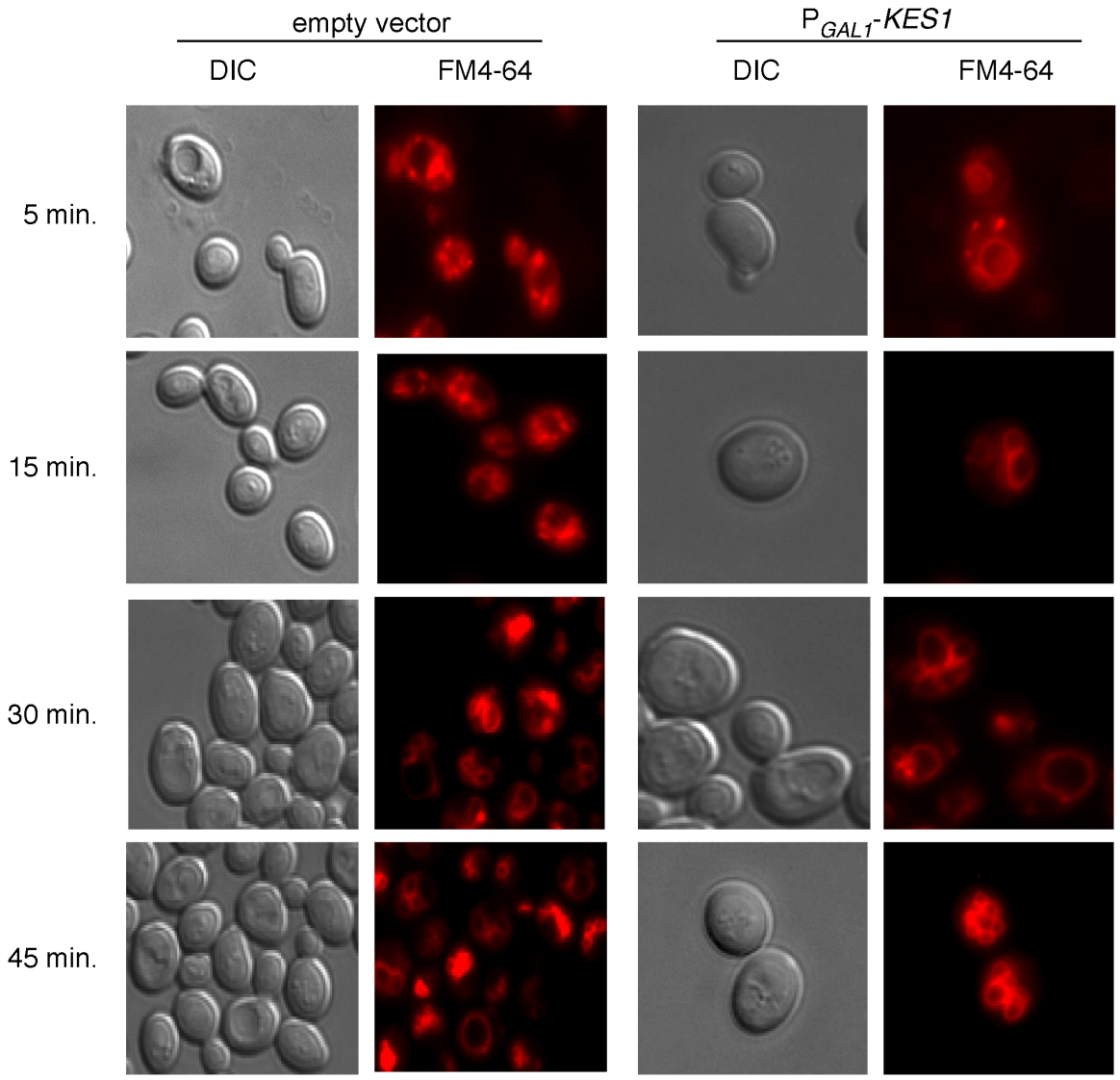


Figure 27 Increased *KES1* expression causes vacuole fragmentation without affecting FM4-64 trafficking from the plasma membrane to the vacuole. BY4741 [vector, pESCURA] (wild-type) and *kes1*Δ [P$_{GAL1}$-*KES1*-HIS6x-pESCURA] cells were grown to mid-logarithmic phase in SRaf medium at 25°C and shifted to SGal medium for 16-hour incubation to allow protein expression. Cells were then labeled for 5 min with 40 μM FM4-64, and incubated post-label in fresh medium for the indicated times. Live cells were visualized using fluorescence (RFP) and DIC filters.

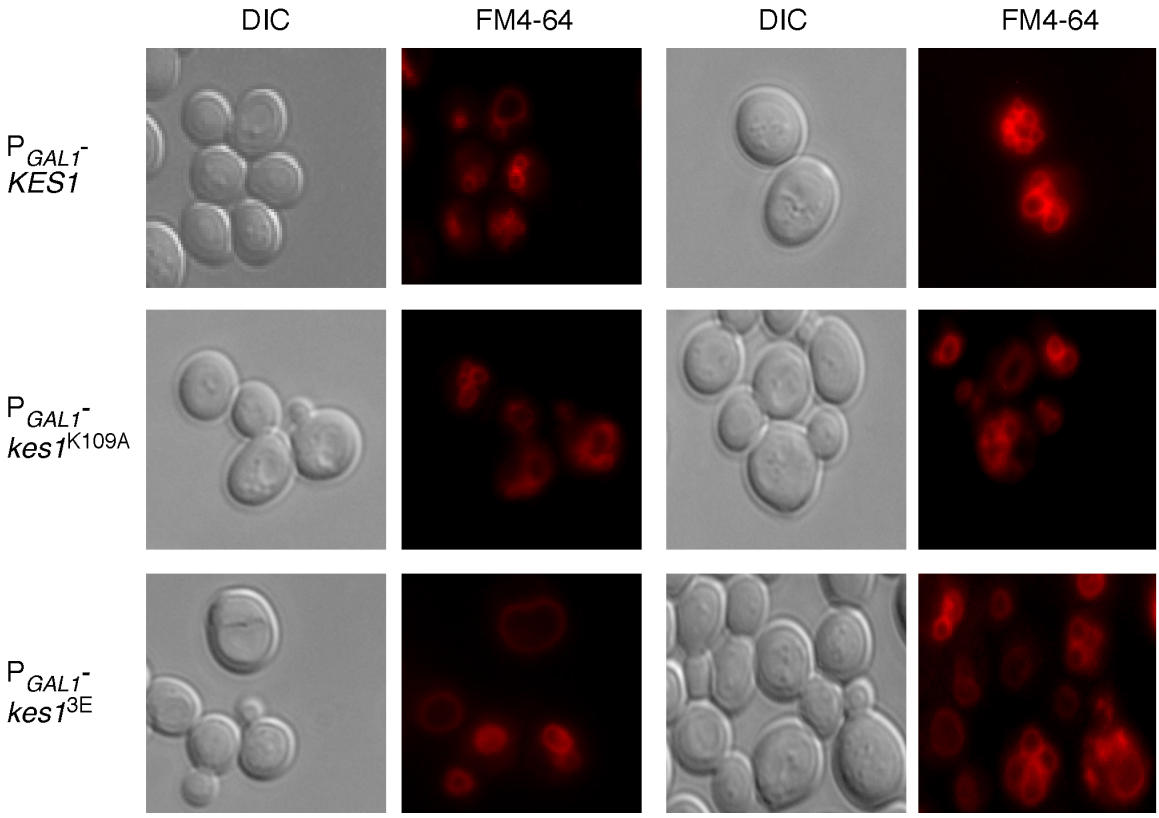


Figure 28 PIP and sterol binding by Kes1 do not regulate plasma-membrane-to-vacuole trafficking. *kes1*Δ [P_{GAL1}-*KES1*-HIS6x-pESCURA], *kes1*Δ [P_{GAL1}-*kes1*^{K109A}-HIS6x-pESCURA] and *kes1*Δ [P_{GAL1}-*kes1*^{3E}-HIS6x-pESCURA] cells were grown to mid-logarithmic phase in SRaf medium at 25°C and shifted to SGal medium for a 16-hour incubation. Cells were then labeled for 5 minutes with 40 μM FM4-64, and incubated post-label in fresh medium containing galactose for 45 minutes. Live cells were then visualized using fluorescence (RFP) and DIC filters.

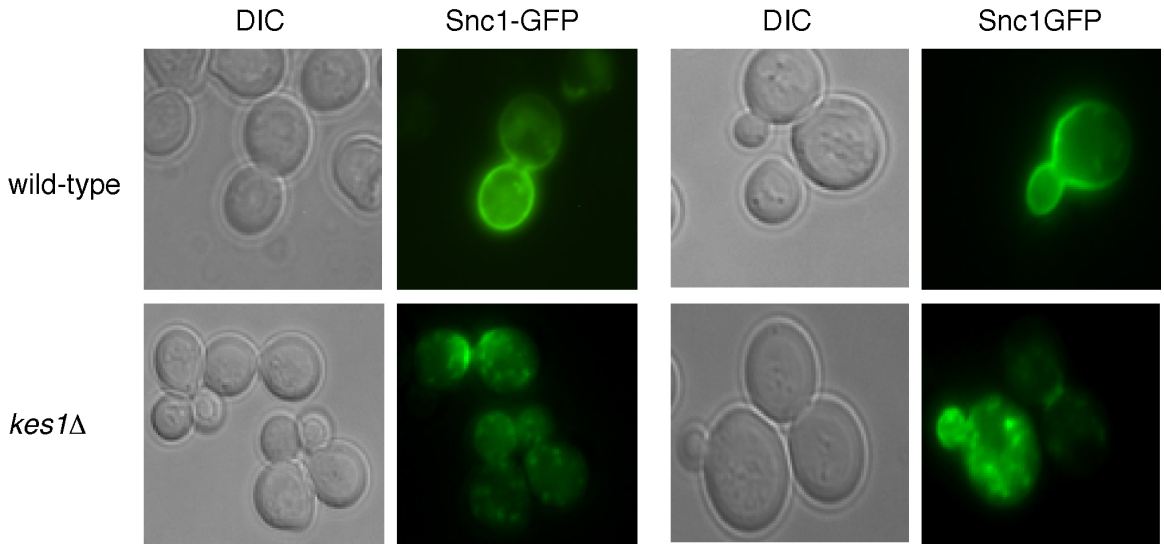


Figure 29 Inactivation of *KES1* impairs Snc1-GFP cycling. BY4741 (wild-type) and *kes1*Δ cells were transformed with a plasmid expressing Snc1 fused to GFP (Snc1-GFP). Cells were grown to mid-logarithmic phase in SC medium at 25°C and imaged using fluorescence (GFP) and DIC filters.

(Figure 30). These trafficking defects within the *trans*-Golgi/endosome pathway are consistent with Kes1 regulation of PI-4P levels.

I report a bi-functional role for Kes1 in the regulation of PI-4P and PI-3P levels. Kes1 binding to sterols and PIPs is required for regulation of PI-4P levels but not PI-3P levels (see Figure 22). Since Snc1-GFP recycling is dependent on proper PI-4P homeostasis, I set out to determine if the ability of Kes1 to bind to sterols and PIPs regulates the recycling of Snc1-GFP. Over-expression of the $Kes1^{K109A}$ (decreased sterol binding) and $Kes1^{3E}$ (no PIP binding) mutants did not affect Snc1-GFP recycling as much as over-expression of wild-type Kes1 did (Figure 31). This observation is consistent with the decreased PI-4P levels upon over-expression of these mutants compared to wild-type Kes1 (see Figure 22). The data imply that the ability of Kes1 to bind to both sterols and PIPs is required to inhibit Snc1 trafficking.

Localization of organellar markers can give insights concerning whether the organelle in question is forming properly. Defects in the *trans*-Golgi/endosome trafficking pathway were already identified; therefore I assessed the localization of different organellar markers in situations where *KES1* gene expression was increased. The organelle markers for the late-Golgi (Chc1-RFP) and endosome (Snf7-RFP) did not appear to localize normally compared to wild-type cells when *KES1* expression was increased (Figure 32). However, organellar markers for lipid droplets (Erg6-RFP), medial Golgi (Anp1-RFP), early Golgi (Cop1-RFP) and ER-to-Golgi vesicles (Sec13-RFP) did appear to localize properly regardless of the level of Kes1 (Figure 33). Additional organellar markers should be used to determine if this is indeed a problem with the organelle morphology or the marker itself. However, this data is consistent with Kes1

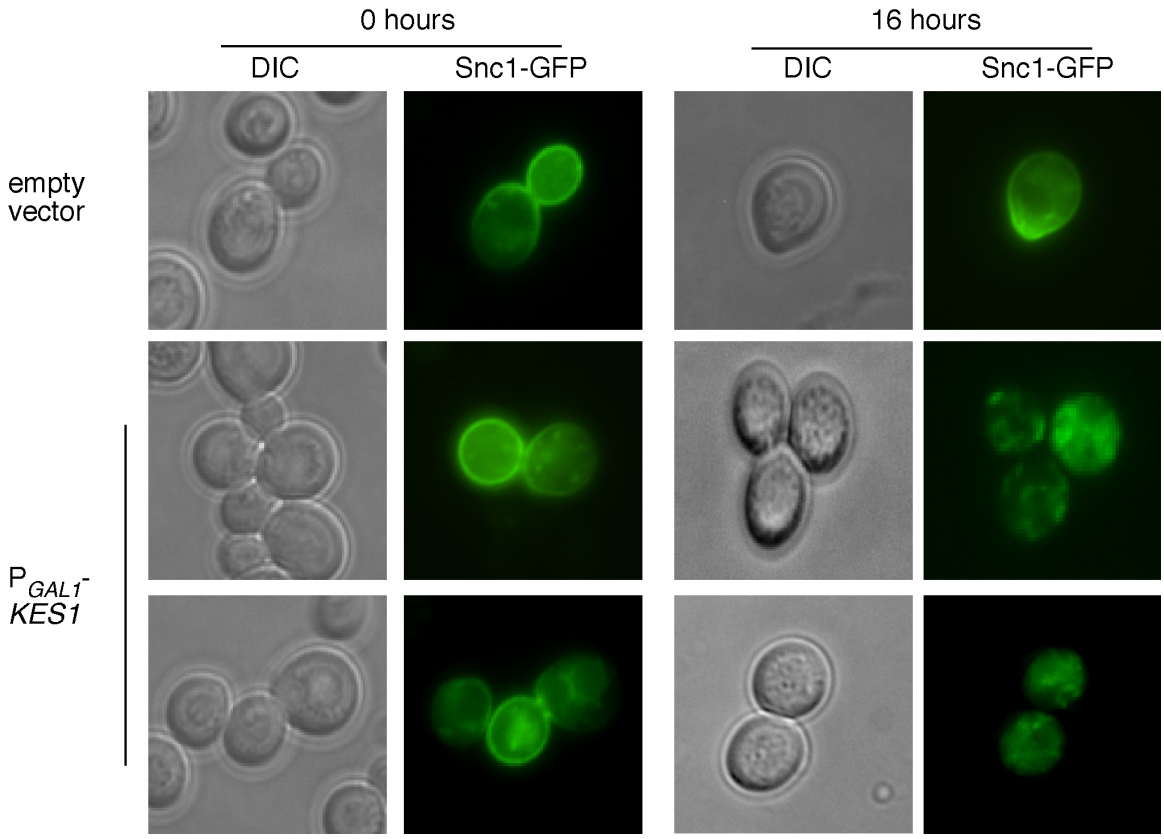


Figure 30 Snc1-GFP accumulates intracellularly in cells over-expressing *KES1*. BY4741 [vector, pESCURA] (wild-type) and *kes1*Δ [P_{GAL1}-*KES1*-HIS6x-pESCURA] cells were transformed with a plasmid expressing Snc1-GFP. Cells were grown to mid-logarithmic phase in SRaf medium at 25°C and shifted to SGal medium for a 16-hour incubation to induce protein expression. Live cells were then imaged using fluorescence (GFP) and DIC filters.

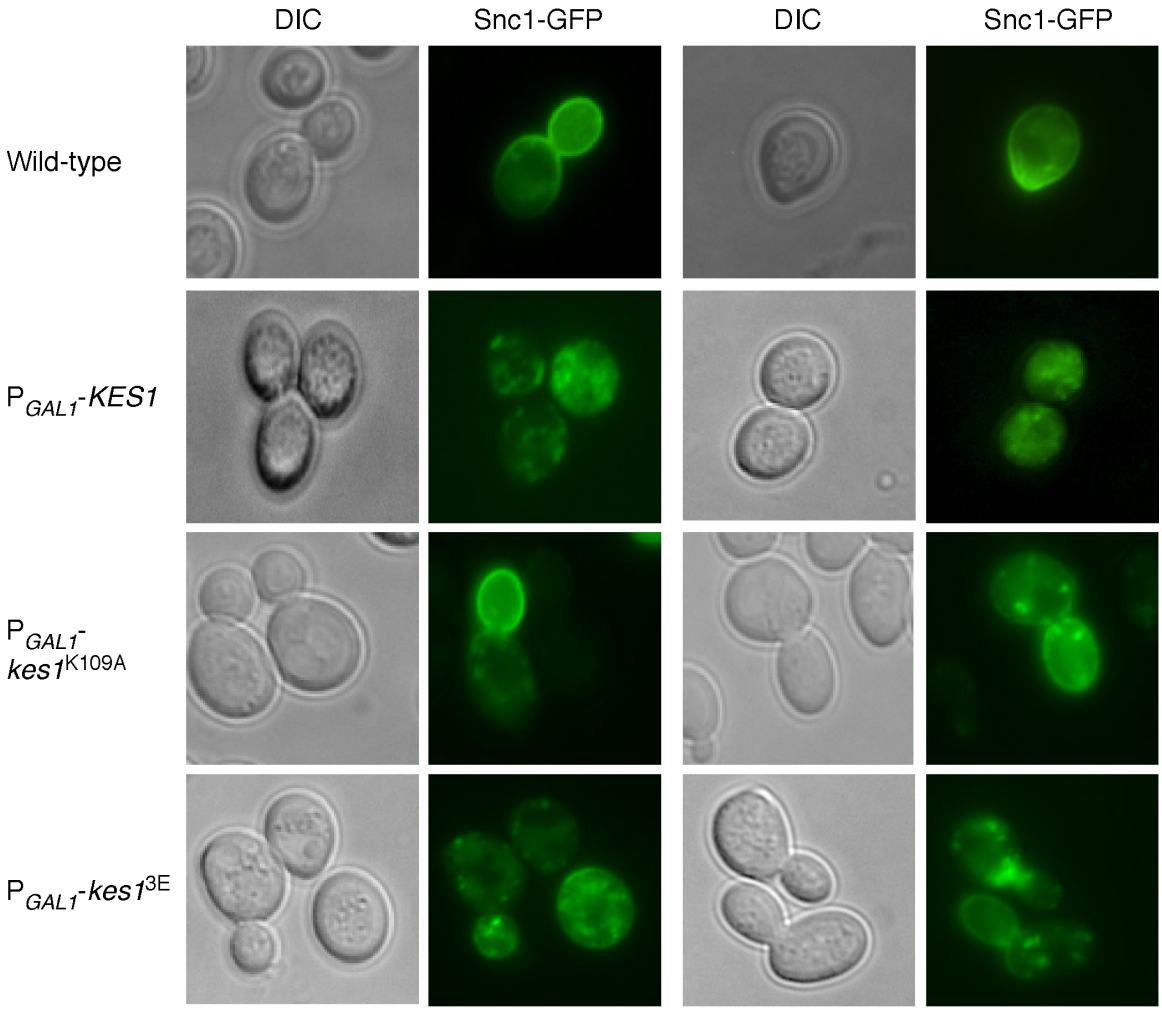


Figure 31 PIP and sterol binding by Kes1 is required to regulate Snc1-GFP cycling. BY4741 [vector, pESCURA] (wild-type), *kes1*Δ [P_{GAL1}-*KES1*-HIS6x-pESCURA], *kes1*Δ [P_{GAL1}-*kes1*^{K109A}-HIS6x-pESCURA] and *kes1*Δ [P_{GAL1}-*kes1*^{3E}-HIS6x-pESCURA] cells were transformed with a plasmid expressing Snc1-GFP. Cells were grown to mid-logarithmic phase in SRaf medium at 25°C when shifted to SGal medium for a 16-hour incubation. Live cells were then imaged using fluorescence (GFP) and DIC filters.

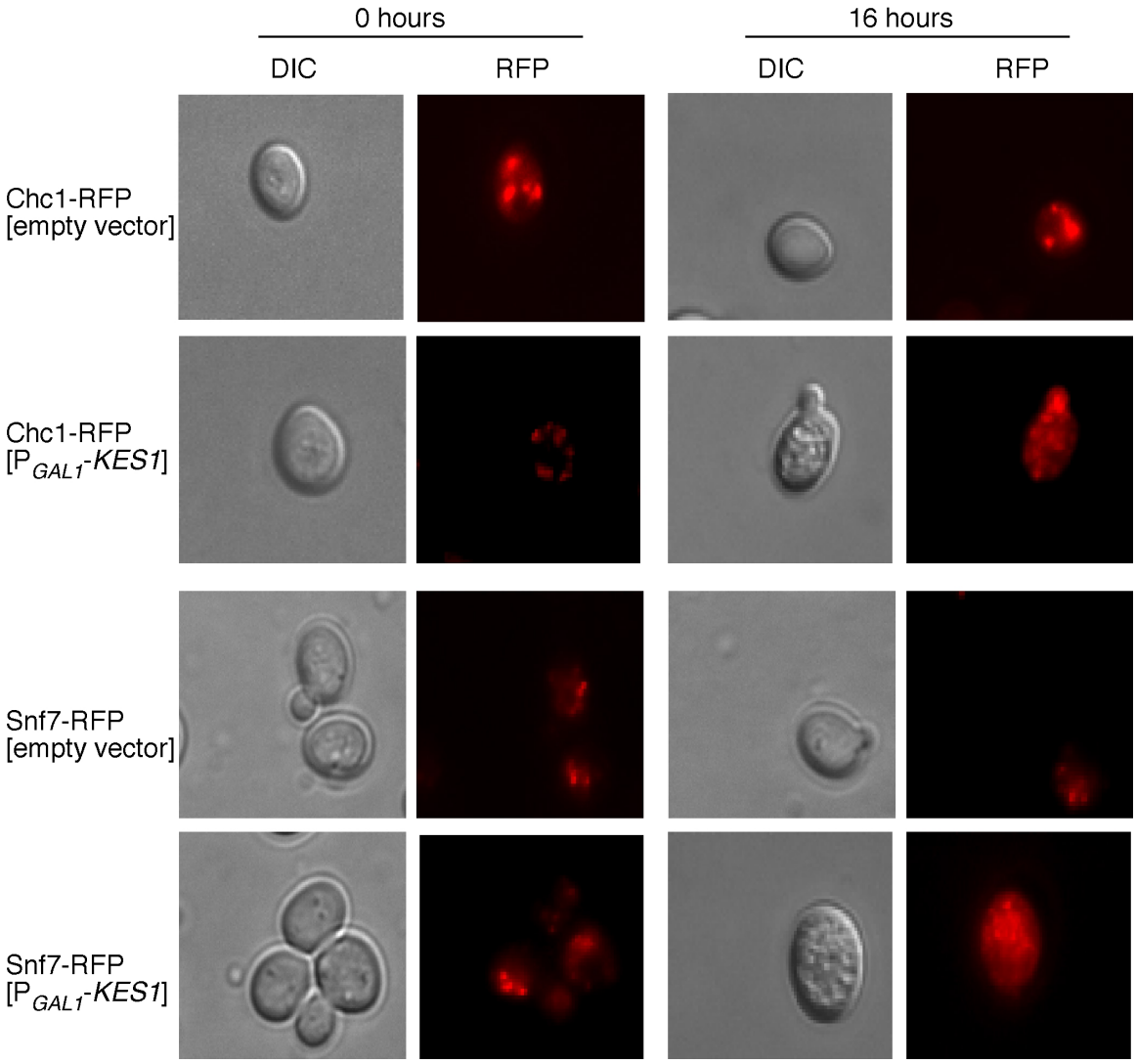


Figure 32 Late Golgi and endosome organelle markers are mis-localized in cells over-expressing *KES1*. [vector, pESCURA] or [P_{GAL1}-*KES1*-HIS6x-pESC-URA] was transformed in yeast strains containing the organellar markers for the *trans*-Golgi (Chc1) or endosome (Snf7) fused to RFP. Cells were grown to mid-logarithmic phase in SRaf at 25°C and shifted to SGal medium for a 16-hour incubation at 25°C for protein induction. Cells were visualized by using fluorescence (RFP) and DIC filters.

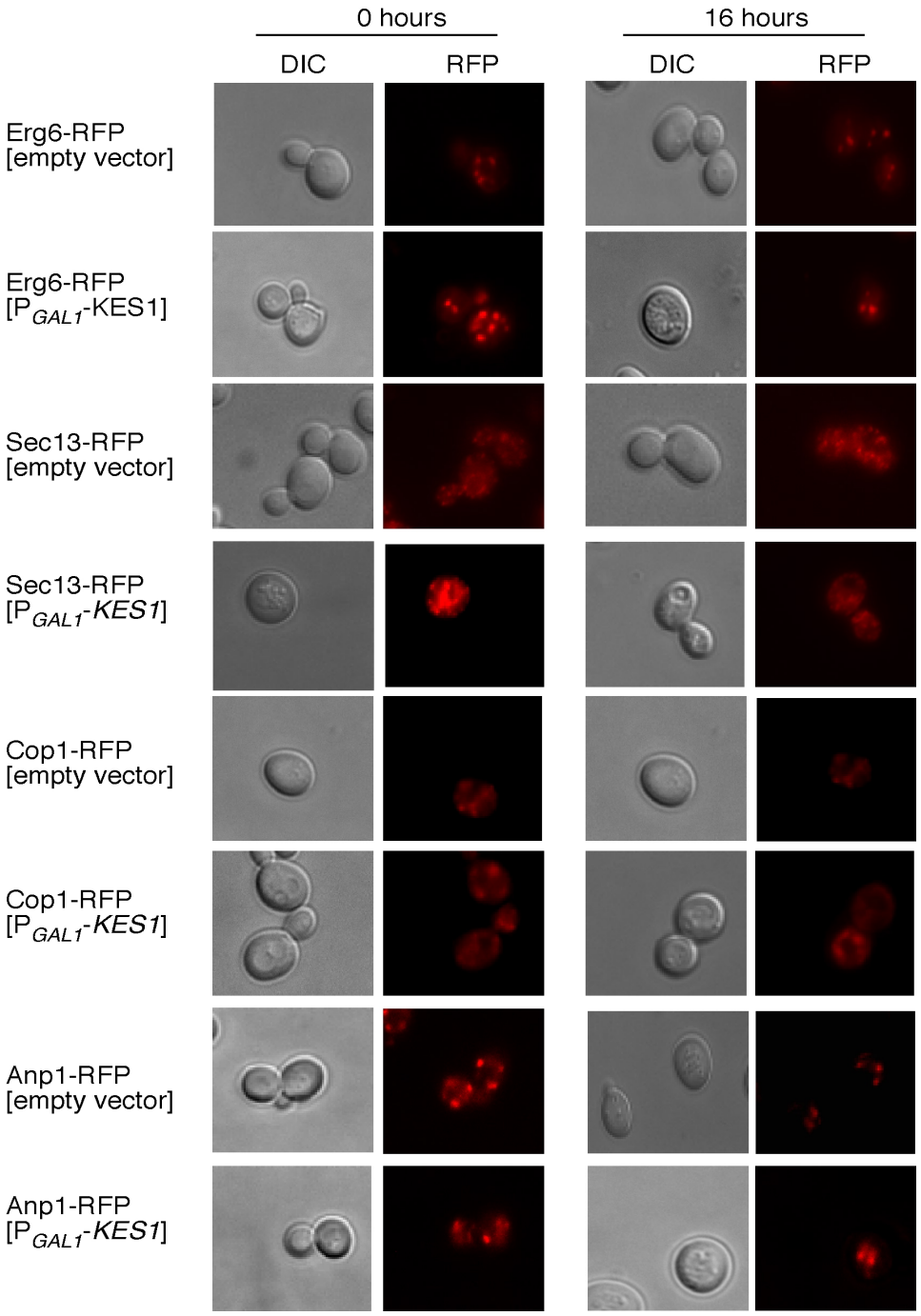


Figure 33 Over-expression of *KES1* does not affect localization of *cis*-Golgi, *medial*-Golgi, lipid particles or ER-to-Golgi (COPII) vesicle markers. [vector, pESCURA] or [P$_{GAL1}$-*KES1*-HIS6x-pESCURA] were transformed in yeast strains containing organellar markers for the *cis*-Golgi (Cop1), the Golgi (Anp1), the lipid particles (Erg6) and ER-to-Golgi COPII vesicles (Sec13) fused to RFP. Cells were grown to mid-logarithmic phase in SRaf at 25°C, then shifted to SGal medium for protein expression for a 16-hour incubation. Cells were then visualized by using fluorescence (RFP) and DIC filters.

regulating *trans*-Golgi and endosome transport pathways, processes that require an appropriate PI-4P level.

3.3.3 Lipid Droplet and Intracellular Membrane Accumulation

To try to clarify where Kes1 may be acting to cause trafficking defects, I looked at electron micrographs (EM) of cells in which Kes1 levels were altered. EM images of cells with an inactivated *KES1* gene showed a clear defect in cell wall morphology and an accumulation of intracellular membranes (Figure 34). EM images after induction of *KES1* expression were also obtained and it was evident that not only was the vacuole fragmented (as was also seen by FM4-64 staining), but membranous structures accumulated in the cytoplasm (Figure 35). After 4 hours of *KES1* expresion from the P_{GAL1} promoter, the membrane-accumulation defect was prominent, and temporally this coincided with the decreased levels of PI-4P and PI-3P. The EM also showed that there appeared to be lipid-droplet accumulation when *KES1* was over-expressesed. Their identity as lipid droplets was confirmed using the neutral-lipid staining dye BODIPY 493/503 (Kurat et al., 2009; Petschnigg et al., 2009) (Figure 36).

3.3.4 SGA Screen Identifies a Role for Kes1 in Autophagy

To attempt to determine the pathway(s) resulting in membrane accumulation in cells that have increased expression of *KES1*, I performed a systematic genetic array screen (Curwin et al., 2009; Sopko et al., 2006; Tong et al., 2001; Tong et al., 2004) versus all (~5000) non-essential *S. cerevisiae* genes. The screen was based on the fact that increased expression of *KES1* from the *GAL1* promoter caused cell growth arrest

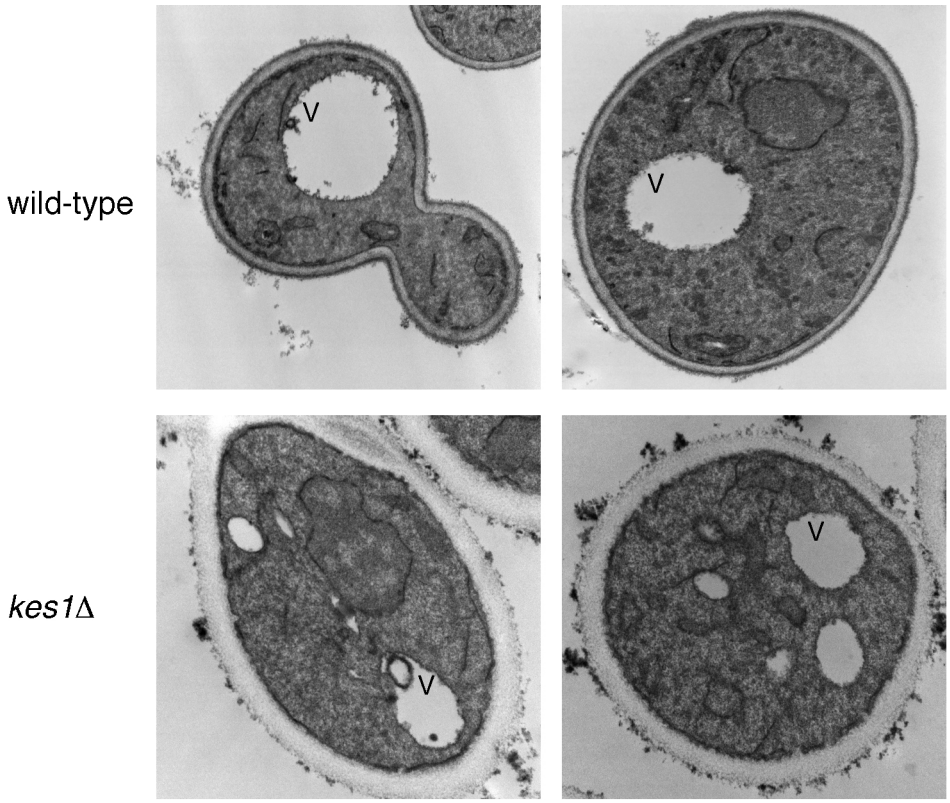


Figure 34 Defects in cell wall morphology and membrane accumulation in cells with an inactivated *KES1*. BY4741 (wild-type) and *kes1*Δ cells were grown to mid-logarithmic phase in SC medium at 25°C. Cells were fixed using $KMnO_4$ (1.5%), 1% sodium periodate and 1% NH_4Cl and processed for electron microscopy. Abbreviations used: V, vacuole.

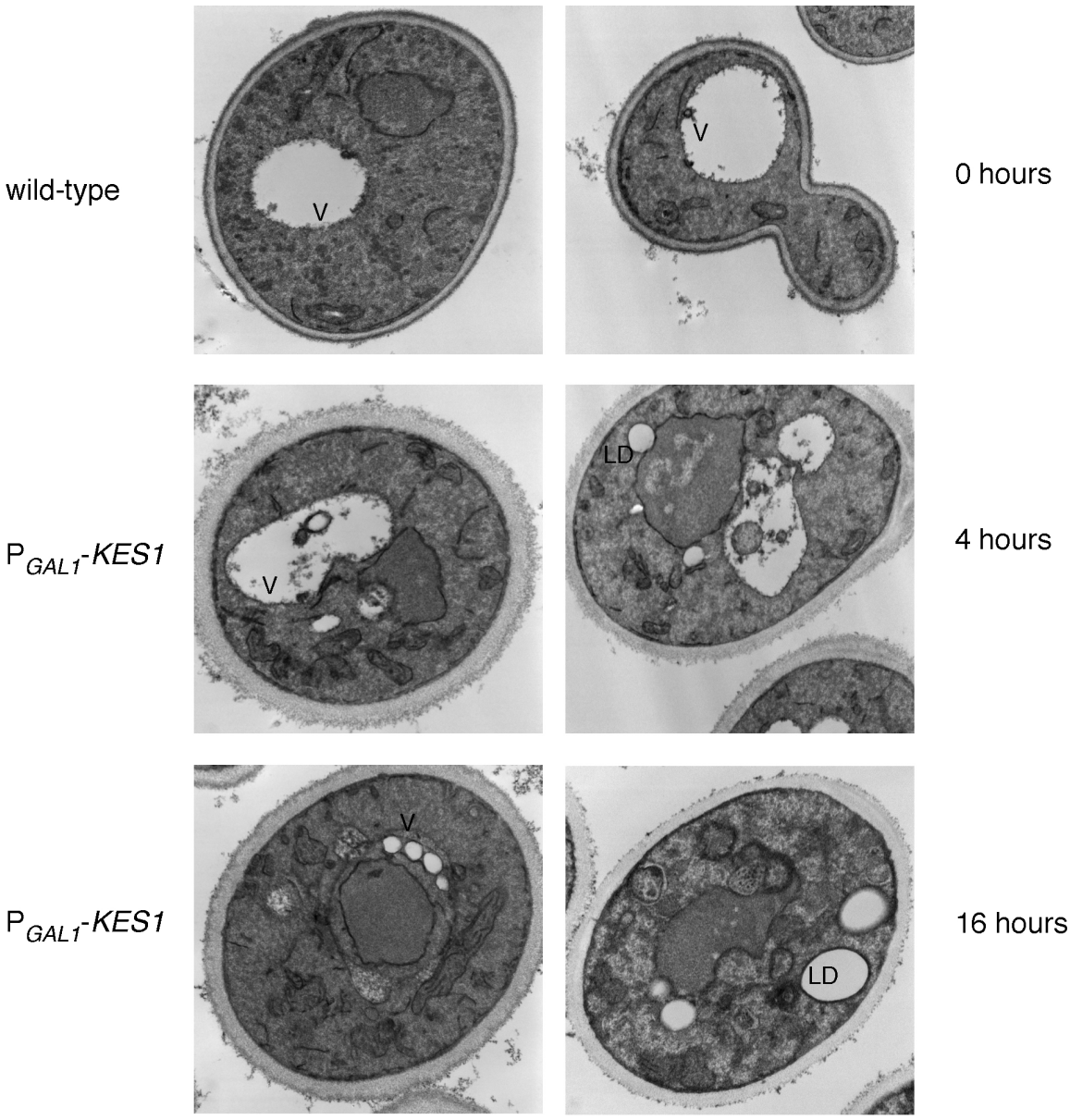


Figure 35 Increased expression of *KES1* causes vacuole fragmentation, lipid droplet formation and intracellular membrane accumulation. BY4741 [vector, pESCURA] (wild-type) and *kes1*Δ [P_{GAL1}-*KES1*-HIS6x-pESCURA] cells were grown to mid-logarithmic phase in SCraf medium at 25°C and shifted to SCgal medium for 0, 4 or 16 hours of incubation to allow Kes1 expression. Cells were then fixed using $KMnO_4$ (1.5%), 1% sodium periodate and 1% NH_4Cl and processed for electron microscopy. Abbreviations used: V, vacuole; LD, lipid droplets.

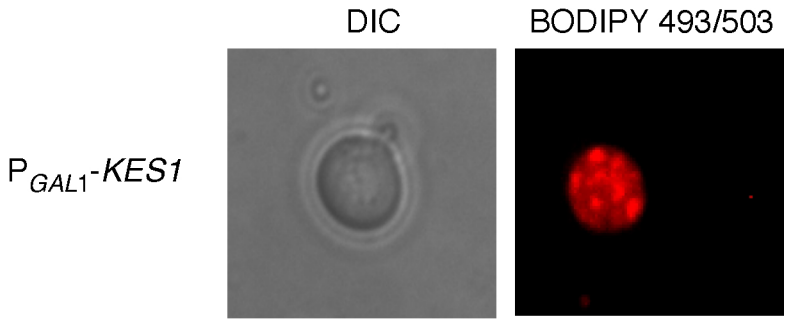


Figure 36 Over-expression of *KES1* causes lipid-droplet formation. *kes1*Δ [P$_{GAL1}$-*KES1*-HIS6x-pESCURA] cells were grown to mid-logarithmic phase at 25°C. Cells were stained with 50 μM Bodipy 493/503 for 30 minutes. Live cells were visualized using fluorescence (RFP) and DIC fliters.

(Figure 37). Therefore, I set out to identify non-essential genes whose inactivation allows a reprieve from the growth inhibition associated with increased expression of *KES1*.

Of genes known to affect membrane metabolism or trafficking, only the deletion of those required for the autophagy and Cvt trafficking pathways restored growth and included: *ATG24*/*SNX4*, encoding a sorting nexin that has a PX domain that binds to PIPs (Teasdale et al., 2001); *ATG9*, encoding a protein involved in membrane delivery to the phagophore assembly site (PAS) (Legakis et al., 2007; Noda et al., 2000; Reggiori et al., 2004; Tsukada and Ohsumi, 1993); and *ATG22*, encoding a vacuolar integral-membrane protein involved in amino acid efflux from the vacuole during autophagy (Suriapranata et al., 2000; Tsukada and Ohsumi, 1993; Yang et al., 2006). The bulk of the remaining genes identified encode transcription factors and proteins involved in protein synthesis and stability, that most likely affect Kes1 abundance (Table 4).

Most components of the autophagy and Cvt pathways are common to both pathways, including the sole yeast PI 3-kinase Vps34. However, some are specific for each pathway (Nair and Klionsky, 2005; Xie and Klionsky, 2007). *ATG24*, identified in the SGA screen, encodes a protein that is thought to be specific for the Cvt pathway. To elucidate if inactivation of autophagy, the Cvt pathway, or both, affects membrane accumulation upon expression of *KES1,* genes known to be specific to each pathway (*ATG17*, *ATG31* and *ATG29* for autophagy, and *ATG11*, *ATG19*, and *ATG24* for the Cvt pathway) were inactivated and EM images of mutant cells were examined. Inactivation of either *ATG17* or *ATG24* ameliorated the membrane-accumulation defect upon increased expression of *KES1* (Figure 38). Inactivation of *ATG11* also appears to ameliorate the

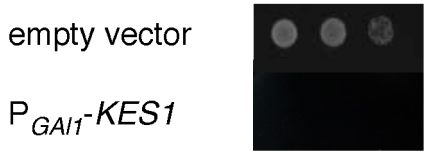


Figure 37 Increased expression of *KES1* causes cell growth arrest. BY4741 [vector, pESCURA] and *kes1*Δ [P$_{GAL1}$-*KES1*-HIS6x-pESCURA] cells were grown to mid-logarithmic phase in SRaf medium at 25°C and shifted to SGal medium for 16 hours of additional incubation to allow for protein expression. Cells were serially diluted on SGal medium to assess for growth.

Table 4. Genes whose inactivation allows for growth of cells with increased Kes1 abundance

Cellular function	Gene name	Function	Reference
Autophagy	*ATG24*	Sorting nexin, has PX domain that binds PIPs	(Hettema et al., 2003; Nice et al., 2002; Teasdale et al., 2001)
	ATG9	Involved in membrane delivery to PAS, interacts with Atg11 directly	(Legakis et al., 2007; Noda et al., 2000; Reggiori et al., 2004; Tsukada and Ohsumi, 1993)
	ATG22	Vacuolar integral-membrane protein, amino acid efflux from the vacuole during autophagy	(Legakis et al., 2007; Noda et al., 2000; Reggiori et al., 2004; Suriapranata et al., 2000; Tsukada and Ohsumi, 1993; Yang et al., 2006)
Lipid Metabolism	*YEH1*	Steryl ester hydrolase, localizes to lipid particles	(Koffel et al., 2005)
	IFA38	Microsomal beta-ketoreductase, has oleate-responsive element in promoter, reduced levels of VLCFA and increased levels of sphingosines and medium-chain ceramides	(Beaudoin et al., 2002; Gopalacharyulu et al., 2009)
	SFK1	Upstream of Stt4	(Audhya and Emr, 2002)
Glycolysis/ Gluconeogen-esis	*PFK1*	Glycolysis, phosphofructokinase subunit, converts fructose-6-P to fructose 1,6-bisphosphate	(Arvanitidis and Heinisch, 1994)
	GID8	Gluconeogenesis, involved in proteosome-dependent inactivation of fructose-1,6-bisphosphate	(Pathak et al., 2004)
	TDH1	Glycolysis/gluconeogenesis, triose-P-dehydrogenase, glyceroaldehyde-3-P to 1,3-bisphosphoglycerate	(McAlister and Holland, 1985a; McAlister and Holland, 1985b)

Cellular function	Gene name	Function	Reference
	IDH2	Isocitrate dehydrogenase, part of TCA cycle in mitochondria, converts isocitrate to alpha-ketoglutarate	(Cupp and McAlister-Henn, 1992)
Amino acid transport/ metabolism	*ODC2*	Mitochondrial membrane protein that exports 2-oxoadipate and 2-oxoglutarate for Lys and Glu synthesis and Lys catabolism	(Palmieri et al., 2001)
	BAP3	Amino acid permease for Cys, Leu, Ile and Val	(Regenberg et al., 1999)
	MET10	Converts sulfite to sulfide for synthesis of sulfur-containing amino acids	(Hansen et al., 1994)
Protein stability/ processing	*PUP2*	20S proteasome subunit	(Georgatsou et al., 1992)
	UBP11	Ubiquitin-specific protease	(Layfield et al., 1999)
	AFG1	Mitochondrial chaperone for degradation of cytochrome C oxidase subunits	(Lee and Wickner, 1992)
	HSP31	Unfolded protein binding chaperone, has Cys protease activity	(Wilson et al., 2004)
	APE2	aminopeptidase	(Garcia-Alvarez et al., 1991)
Transcription / translation	*HAA1*	Transcription activator for genes that respond to membrane and acid stress	(Garcia-Alvarez et al., 1991)
	YLL054C	Similar to Pip2, an oleate response element transcription activator that contains a Zn^{2+}/Cys cluster domain, activates beta oxidation of fatty acids	(Ahmed Khan et al., 2000)
	RDS1	Zi^{2+} cluster transcription factor	(Akache and Turcotte, 2002)
	TIS11	mRNA binding and degradation	(Puig et al., 2005)
	TRZ1	tRNA 3'-end processing	(Chen et al., 2005)
	PAT1	mRNA decapping	(Wang et al., 1999)
	RPF2	Processing of pre-rRNA	(Morita et al., 2002)
	NSA1	Part of 66S pre-ribosomal particle	(Harnpicharnchai et al., 2001)

Cellular function	Gene name	Function	Reference
Chromosome segregation	*CSM1*	Chromosome segregation	(Rabitsch et al., 2003)
	DAD2	Chromosome segregation	(Li et al., 2002b)
DNA repair	*RAD52*	DNA repair	(Adzuma et al., 1984)
	RAD55	DNA repair	(Fortin and Symington, 2002)
	RAD28	DNA repair	(Bhatia et al., 1996)
	RAD27	DNA repair	(Ayyagari et al., 2003)
	HHO1	Histone H1, DNA repair	(Ushinsky et al., 1997)
Others	*PSY4*	Putative regulatory subunit of an evolutionarily conserved protein phosphatase; localization is cell-cycle dependent and regulated by Cdc28 phosphorylation	(Hastie et al., 2006; Kosugi et al., 2009)
	BUD31	Component of the SF3b subcomplex of U2 snRNP; diploid mutants display a random budding pattern instead of the wild-type bipolar pattern	(Ni and Snyder, 2001; Troyanskaya et al., 2003; Wang et al., 2005d)
	PPH21	Catalytic subunit of protein phosphatase 2A	(Sneddon et al., 1990)
	AST1	Interacts with the plasma-membrane H+ ATPase Pma1 for its localization to lipid rafts	(Chang and Fink, 1995)
	OCH1	*Cis*-Golgi mannosyltransferase	(Nakayama et al., 1992)
	CTR1	Plasma-membrane Cu^{2+} transporter	(Dancis et al., 1994)
Unknown function	*FMP27*	Localizes to mitochondria	(Reinders et al., 2006)
	SNZ2	Transcription is increased in stationary phase	(Padilla et al., 1998)
	YNR066C	Unknown	
	YHL017W	Unknown	
	YLR126C	Unknown	
	YLR179C	Unknown	

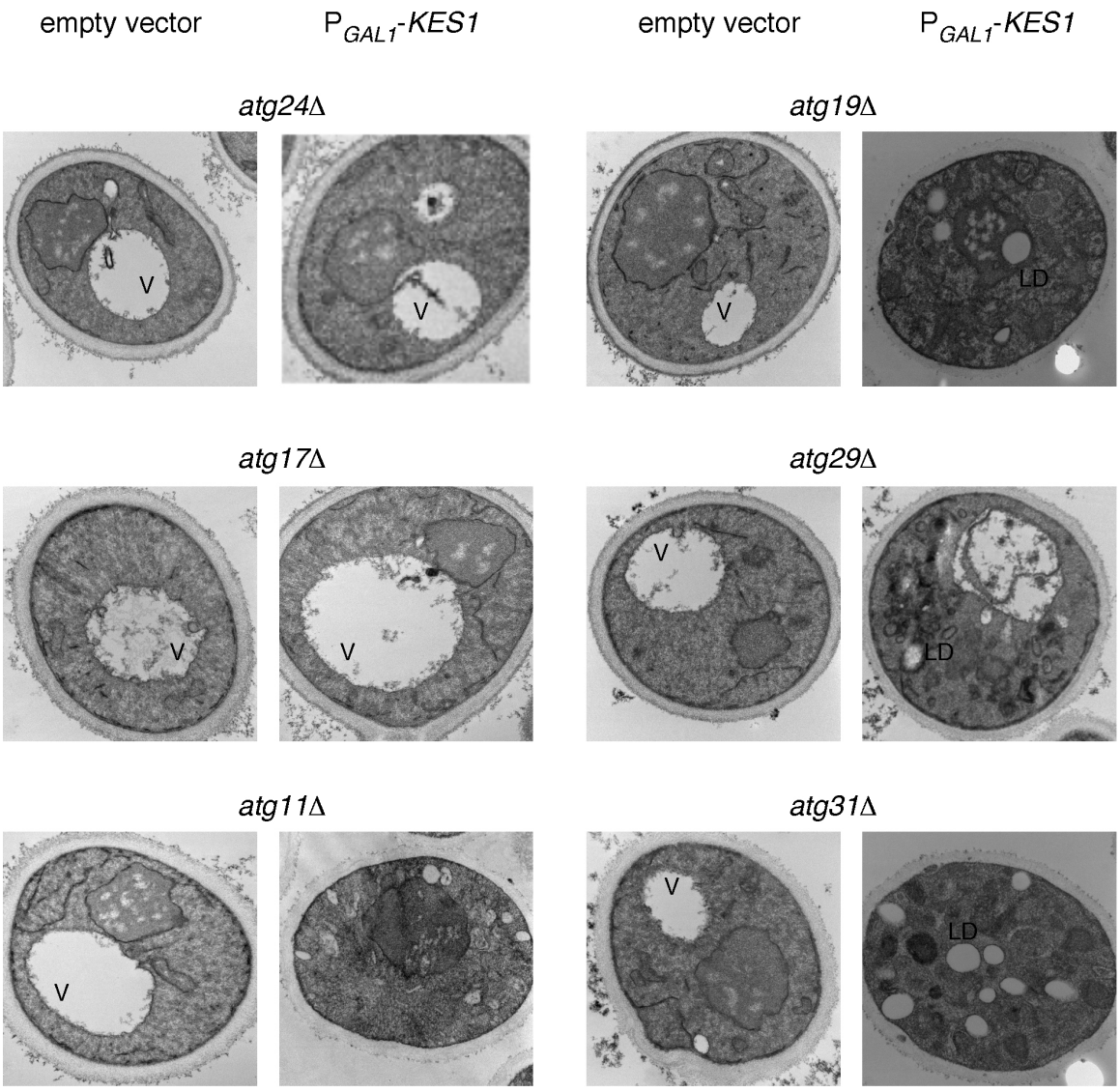


Figure 38 Defects associated with increased expression of *KES1* require certain autophagy components. *atg17*Δ, *atg31*Δ and *atg29*Δ (eliminating components specific to autophagy) and *atg11*Δ, *atg19*Δ and *atg24*Δ (eliminating components specific to the Cvt pathway) cells were transformed with either [vector, pESCURA] or [P$_{GAL1}$-*KES1*-HIS6x-pESCURA]. Cells were grown to mid-logarithmic phase in SRaf medium at 25°C and transferred to SGal containing medium for a further 16 hours of incubation. Cells were then fixed using $KMnO_4$ (1.5%), 1% sodium periodate and 1% NH_4Cl and processed for electron microscopy. Abbreviations used: V, vacuole; LD, lipid droplets.

membrane accumulation defects, although it may not be to the same extent as *ATG17* or *ATG24* inactivation.

The PI-3 kinase dependent step, as well as four of the five genes whose inactivation prevented Kes1 mediated membrane and lipid droplet accumulation, *ATG9*, *ATG11*, *ATG17*, and *ATG24*, are involved in formation of the PAS membrane (or cargo assembly at the PAS) pointing to this step as a possible site of action for Kes1 mediated regulation of this pathway.

3.3.5 The Role of PIP and Sterol Binding by Kes1 in Regulation of the Autophagy/Cvt Pathway(s)

To try to correlate the known lipid-binding characteristics of Kes1 with the membrane and lipid-droplet anomalies, I determined if the lack of ability to bind either of these lipids would cause fewer defects. Increased expression of the *KES1* gene with mutations in either PIP or sterol binding resulted in increased growth relative to the wild-type gene (Figure 39). However, these cells maintained vacuole fragmentation, membrane accumulation and lipid-droplet formation (Figure 40). This is consistent with the bi-functional role of Kes1 where upon increased expression of either of the sterol-or PIP-binding mutants, PI-3P levels remain low. PI-3P is the PIP known to regulate the autophagy/Cvt pathways. Indeed, increased expression of Vps34, the sole PI-3-kinase in yeast, prevented membrane accumulation in cells overexpressing Kes1 (Figure 41A). In contrast, increased expression of Pik1, the PI-4-kinase at the Golgi, had no such effect: membrane accumulation still occurred (Figure 41B). The data imply that the regulation of PI-3P metabolism by Kes1 results in intracellular membrane accumulation.

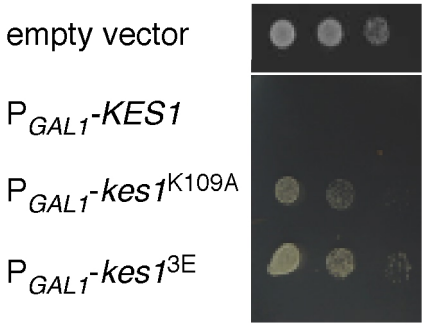


Figure 39 PIP and sterol binding by Kes1 is required to cause cell growth arrest upon increased Kes1 expression. BY4741 [vector, pESCURA], *kes1*Δ [P_{GAL1}-*KES1*-HIS6x-pESCURA], *kes1*Δ [P_{GAL1}-*kes1*^{K109A}-HIS6x-pESCURA] and *kes1*Δ [P_{GAL1}-*kes1*^{3E}-HIS6x-pESCURA] cells were grown to mid-logarithmic phase in SRaf medium at 25°C and shifted to SGal medium for a 16-hour incubation to allow for Kes1 expression. Cells were then spotted on SGal medium to assess for growth.

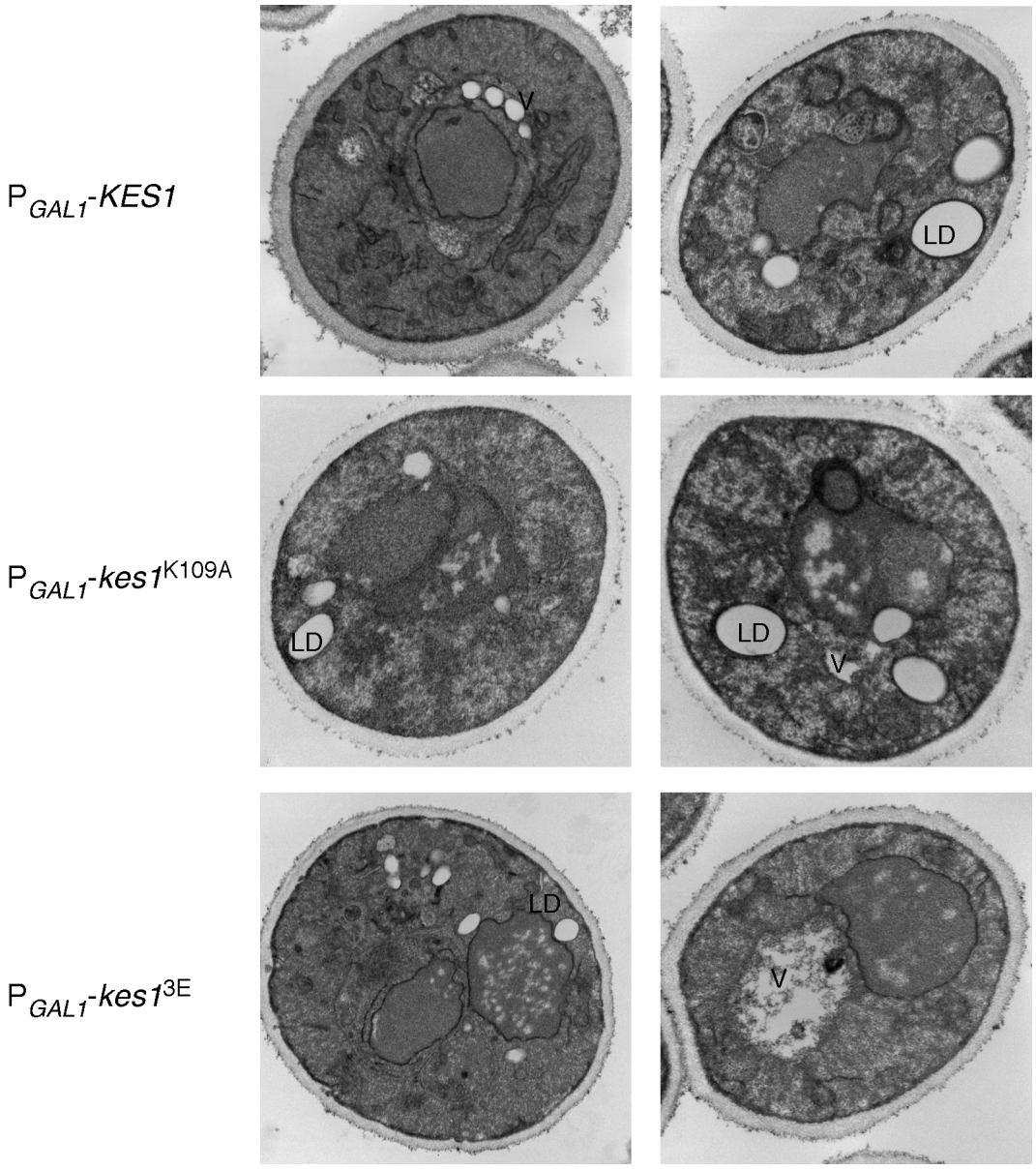


Figure 40 PIP and sterol binding by Kes1 is not required for regulation of membrane homeostasis, vacuole fragmentation and lipid droplet formation. *kes1*Δ [P_{GAL1}-*KES1*-HIS6x-pESCURA], *kes1*Δ [P_{GAL1}-*kes1*^{K109A}-HIS6x-pESCURA] and *kes1*Δ [P_{GAL1}-*kes1*^{3E}-HIS6x-pESCURA] cells were grown to mid-logarithmic phase in SCraf medium at 25°C and shifted to SCgal medium for a 16-hour incubation for Kes1 expression. Cells were then fixed using $KMnO_4$ (1.5%), 1% sodium periodate and 1% NH_4Cl and processed for electron microscopy. Abbreviations used: V, vacuole; LD, lipid droplets.

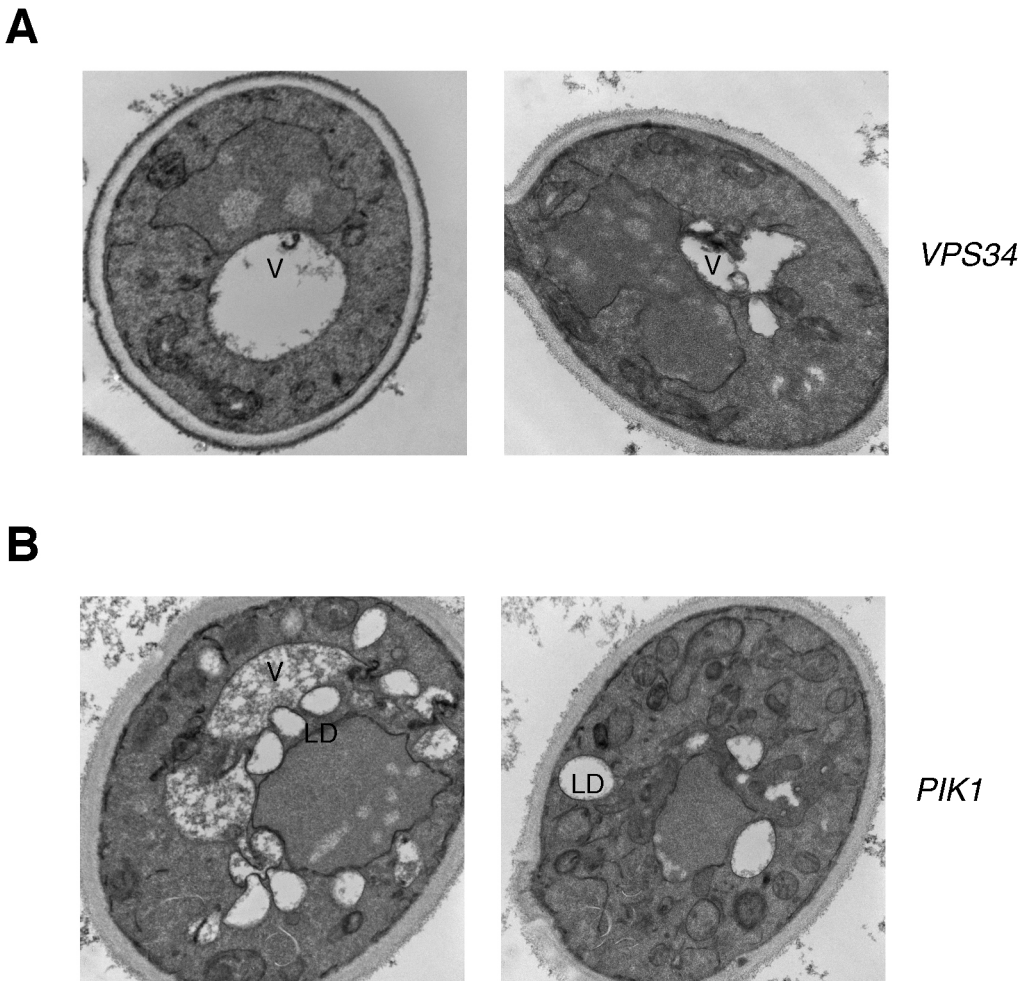


Figure 41 Increased expression of *VPS34* prevents Kes1 mediated membrane accumulation. The PI-3 kinase [*VPS34*-pRS425] or the PI-4 kinase [*PIK1*-pRS425] on high copy plasmids were transformed in *kes1*Δ cells expressing [P_{GAL1}-*KES1*-HIS6x-pESCURA]. Cells were grown to mid-logarithmic phase in SRaf medium at 25°C and transferred to SGal containing medium for a further 16 hours of incubation. Cells were fixed using $KMnO_4$ (1.5%), 1% sodium periodate and 1% NH_4Cl and processed for electron microscopy. Abbreviations used: V, vacuole; LD, lipid droplets.

3.4 A Role for Kes1 and Osh Proteins in Regulation of PIP-to-Sphingolipid Metabolism

3.4.1 Kes1 and Sphingolipid Metabolism

Recently, Sac1 (a PI-3P and PI-4P phosphatase) was shown to be responsible for providing the PI pool for synthesis of the complex sphingolipids inositol-phosphoceramide (IPC), mannose-inositol-phosphoceramide (MIPC) and mannose-(inositol-P)$_2$-ceramide (M(IP)$_2$C) (Brice et al., 2009). Inactivating *SAC1* results in a dramatic decrease in PI-4P metabolism to PI for its subsequent use in the synthesis of sphingolipids (Brice et al., 2009).

Since I saw that Kes1 regulated the level of PI-4P in a manner similar to Sac1, we determined if Kes1 also regulated sphingolipid mass/metabolism. Cells with an inactivated *KES1* gene displayed a 50% decrease in PI and complex-sphingolipid mass (Figure 42). These results are typical of what is observed when *SAC1* is inactivated (Brice et al., 2009).

3.4.2 Kes1 and Lipid Raft Formation

Complex sphingolipids are synthesized in the Golgi and are necessary for lipid-raft formation for delivery to the plasma membrane. We assessed the localization of Pma1, a protein that assembles with lipid rafts in the Golgi, in cells with an inactivated *KES1* gene (Bagnat et al., 2000; Gong and Chang, 2001; Lee et al., 2002; Wang and Chang, 2002). In wild-type cells, a Pma1-RFP chimera was effectively delivered to the cell surface. However, in cells with an inactivated *KES1* gene, Pma1-RFP accumulated in intracellular punctate spots with very little Pma1-RFP at the plasma membrane (Figure 43).

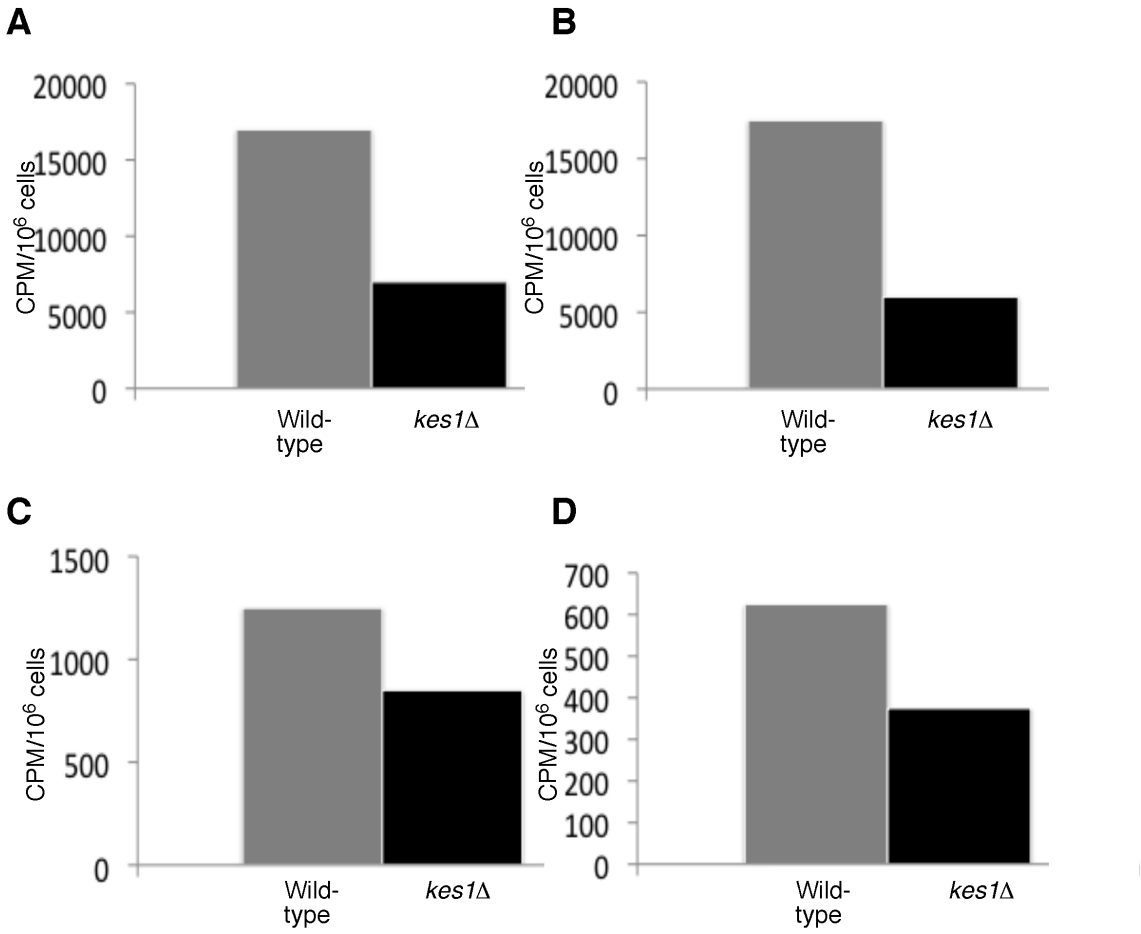


Figure 42 Kes1 regulates the synthesis of PI and complex sphingolipids. BY4741 (wild-type) cells and *kes1*Δ cells were grown to late logarithmic phase and inoculated into medium containing 1 μCi *myo*-[^{3}H]inositol. Cells were then grown for 21 hours to mid-logarithmic phase and lipids were extracted and resolved by TLC. Radioactivity of the resolved lipids was measured for (A) PI, (B) IPC, (C) MIPC and (D) MIP_2C.

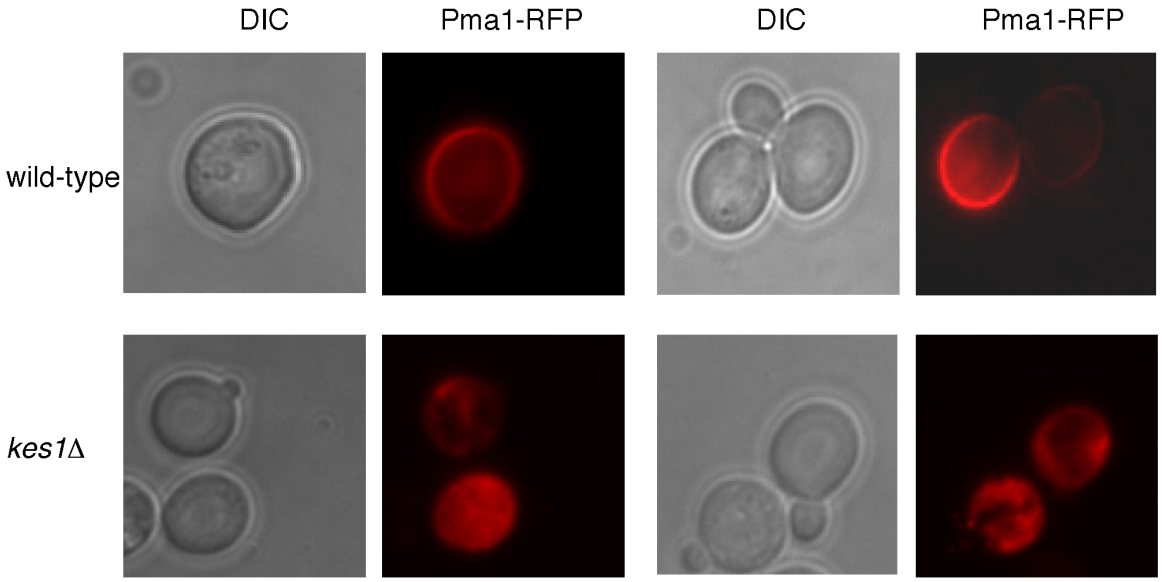


Figure 43 Inactivation of *KES1* hampers Pma1-RFP trafficking at the *trans*-Golgi. SEY6210-PMA1-dsRFP (wild-type) and CMY306 (SEY6210-PMA1-dsRFP, *kes1*Δ) cells were grown to mid-logarithmic phase in SC medium at 25°C. Cells were visualized using fluorescence (RFP) and DIC filters.

Although inactivating *KES1* caused defects in Pma1-RFP delivery to the plasma membrane, and consequently lipid-raft delivery to the plasma membrane, it appears that enough Pma1 reaches the plasma membrane to allow cell viability. Indeed, inactivating *KES1* alone did not cause sensitivity to edelfosine (Figure 44). Edelfosine is an antitumor drug known to modify the biophysical structure of lipid rafts at the plasma membrane (Zaremberg et al., 2005). If lipid rafts are not properly formed at the plasma membrane, cells will be come sensitive to edelfosine.

3.4.3 Regulation of *in vivo* PIP Levels by the Osh Protein Family

To determine if regulation of PIP levels is a general property of the Osh protein family, or is unique to Kes1, I measured *in vivo* PIP levels in cells in which every gene of the *OSH* family (*OSH1-OSH7*, *osh1-7*Δ) is inactivated and the cells are kept alive by the presence of either the wild-type *KES1* gene, or a temperature-sensitive allele of *KES1*, $kes1^{ts}$, carried on a low-copy (1-3 copies per cell) plasmid. At 25°C, the permissive temperature for function of the $kes1^{ts}$ allele, the level of PI-4P was elevated 3-fold in these *osh*Δ cells expressing plasmid-borne wild-type *KES1* or the $kes1^{ts}$ allele. The levels of the other PIPs remained unchanged (Table 5). This observation implies that one or more of the other Osh proteins (Osh1-3 and Osh5-7) can affect the levels of PI-4P. Upon incubation at 37°C for 2 hours, the *osh*Δ cells containing plasmid-borne wild-type *KES1* gene still displayed a PI-4P level 3-fold higher than that of wild-type cells. However, in cells containing the plasmid-borne $kes1^{ts}$ allele, the PI-4P level was 26-fold higher compared to wild-type cells. Kes1 therefore appears to be a major determinant of PI-4P levels, with other Osh proteins also playing a role in the regulation of PI-4P mass.

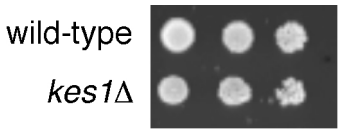


Figure 44 Inactivation of *KES1* does not cause sensitivity to edelfosine. BY4741 (wild-type) and *kes1*Δ cells were grown to mid-logarithmic phase in SC medium at 25°C containing 20 μg/ml edelfosine. Cells were then serially diluted on SC medium with 20 μg/ml edelfosine and incubated at 25°C for 2 days.

Table 5. Percentage of total labeled PIs in Osh-deficient cells

Yeast strain	**PI**	**PI-3P**	**PI-4P**	**PI-3,5P_2**	**PI-4,5P_2**
Wild-type 26°C	93	1.3	1.5	0.3	1.3
***osh1-7Δ* [*KES1*-pRS414] 26°C**	88	1.3	7.2	0.2	1.0
***osh1-7Δ* [*kes1^{ts}*-pRS414] 26°C**	86	1.7	9.6	0.1	0.8
Wild-type 38°C	95	1.0	1.0	0.1	1.2
***osh1-7Δ* [*KES1*-pRS414] 38°C**	93	0.7	3.6	0.1	1.0
***osh1-7Δ* [*kes1^{ts}*-pRS414] 38°C**	73	1.8	23	0.3	0.4

Consistent with the Osh family playing a role in regulation of PI metabolism, I observed that the cells lacking all Osh proteins, but containing the *kes1*^{ts} allele on a low-copy plasmid, were sensitive to edelfosine at the non-permissive temperature (Figure 45). Although I show that Kes1 is the major Osh protein that regulates PI-4P and Pma1-RFP trafficking, the other Osh proteins also collectively play a role in regulation of PIPs and their biological functions.

3.5 SEC14 AND KES1

3.5.1 Lipid Raft Formation in *sec14*^{ts} Cells

Sec14 is an essential PI- and PC- binding protein in *S. cerevisiae*. However, the essential function of Sec14 can be bypassed by the inactivation of the *KES1* or *SAC1* gene (Cleves et al., 1991). Although Sec14 binds PI *in vitro*, it is not known how diminution of Sec14 function affects PI metabolism *per se*. What is clear is that when *sec14*^{ts} cells are grown at 37°C the level of PI-4P is decreased by ~50%, with most, if not all, of this decrease occurring at the Golgi PI-4P pool (Fairn et al., 2007; Li et al., 2002a). Inactivation of *KES1* restores PI-4P levels to near wild-type levels in *sec14*^{ts} cells (Fairn et al., 2007; Li et al., 2002a). Since inactivation of *KES1* can bypass the essential function of Sec14, both Kes1 and Sec14 regulate PI-4P levels and availability at the Golgi. As regulation of PI-4P level by Kes1 and Sac1 results in defects in sphingolipid synthesis and lipid-raft formation, I assessed the ability of lipid rafts to form at the Golgi in cells that possess a *sec14*^{ts} gene. At 37°C, Pma1-RFP was not observed at the plasma membrane in cells containing this temperature sensitive allele of *SEC14* (Figure 46). This observation implies that Sec14 functions in the formation of lipid rafts at the Golgi and/or

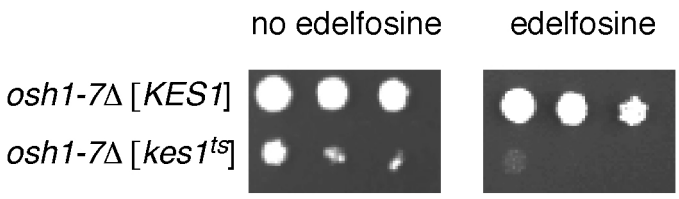


Figure 45 Cells lacking all Osh proteins are sensitive to edelfosine. CBY924 (*osh1-7*Δ [*KES1*-pRS414]) and CBY926 (*osh1-7*Δ [*kes1*^{ts}-pRS414]) cells were grown to mid-logarithmic phase in SC medium at 25°C containing 20 μg/ml edelfosine. Cells were serially diluted on SC medium with and without 20 μg/ml edelfosine and incubated at 37°C for 2 days.

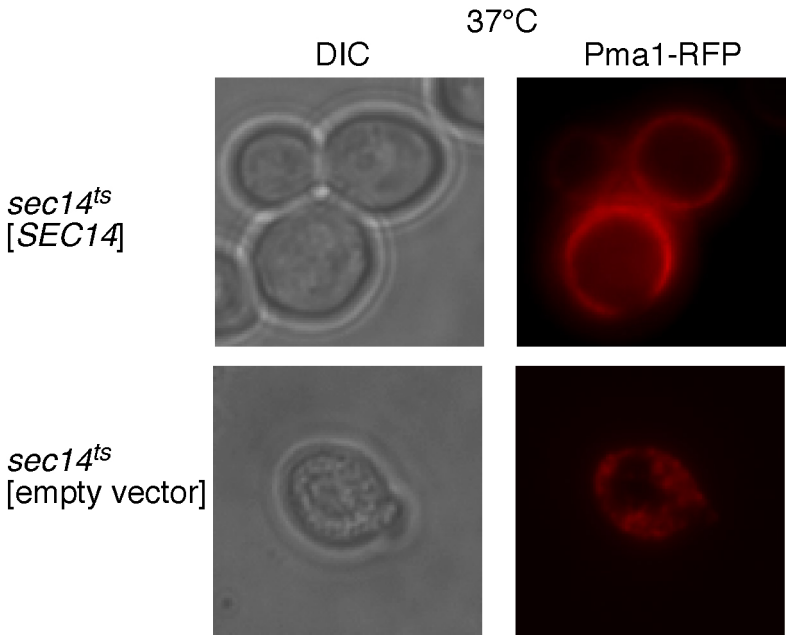


Figure 46 ***sec14*^{ts} hampers Pma1-RFP trafficking at the non-permissive temperature.** CMY307 (SEY6210-PMA1-dsRFP, CMY505) cells transformed with either [*SEC14*-pRS415] or [pRS415] were grown to mid-logarithmic phase in SC medium at 25°C. Cells were then incubated to 37°C for 16 hours and visualized using fluorescence (RFP) and DIC filters.

in the delivery of lipid rafts to the plasma membrane.

3.5.2 Characterization of the *sec14^{ts}* Allele

A recent study from our lab determined that by simply increasing the expression of the *sec14^{ts}* allele, encoding Sec14^{Gly266Asp}, allowed the growth of *sec14^{ts}* cells at the normally non-permissive temperature of 37°C. That study also provided evidence that increased expression of Sec14^{Gly266Asp} does not ameliorate the known PC metabolic or membrane-trafficking defects associated with growth of cells containing the *sec14^{ts}* allele at 37°C (unpublished data from our lab). Since Kes1 and Sec14 both appear to regulate PI-4P levels at the Golgi, I set out to determine if PI metabolism is restored in cells that have increased expression of Sec14^{Gly266Asp}. The results of this experiment could help explain the mechanism by which the increased expression of the *sec14^{ts}* allele allows growth of these cells.

I observed a 50% decrease in PI-4P level after growth of *sec14^{ts}* cells at 37°C for 1 hour at this non-permissive temperature. This was compared to both their growth at 25°C and the growth of cells expressing wild-type *SEC14* on a low-copy plasmid (Figure 47). At 37°C, increased expression of Sec14^{Gly266Asp} still resulted in a 50% decrease in PI-4P level compared to wild-type. At 37°C, *sec14^{ts}* cells also had a 3-fold increase in PI-3P level. This change in PIP levels has never been previously reported.

Since we identified a role for Sec14 in delivery of Pma1-RFP to the plasma membrane, we assessed delivery of Pma1-RFP in cells that have increased expression of the Sec14^{Gly266Asp} mutant. Increasing expression of the Sec14^{Gly266Asp} temperature-sensitive mutant restored most of the Pma1-RFP trafficking to the plasma membrane.

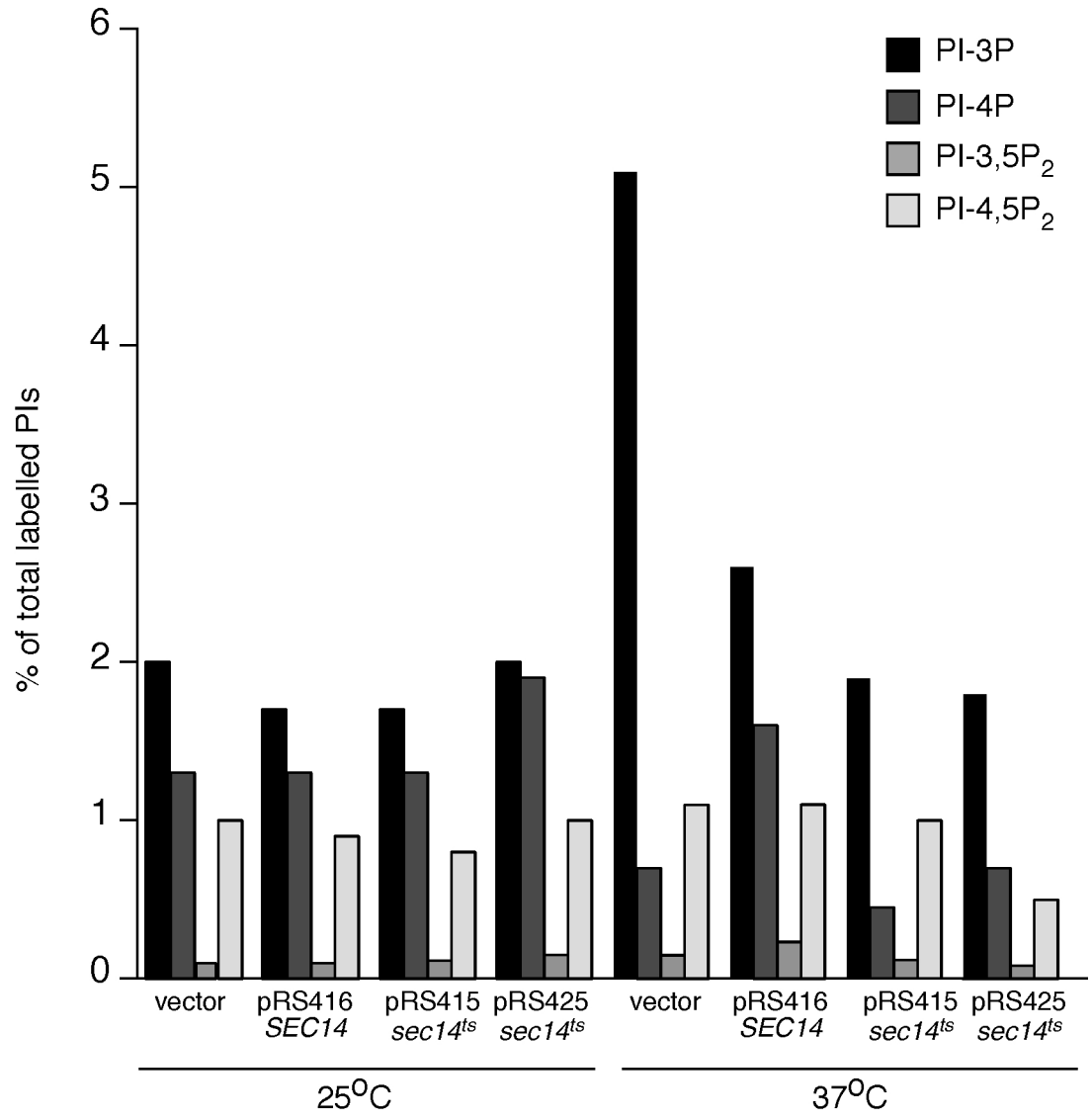


Figure 47 Sec14 regulates PI metabolism. Cells of the *sec14*^{ts} strain CMY505 transformed with the plasmids [vector, pRS415], [*SEC14*-pRS416], [*sec14*^{ts}-pRS415] or [*sec14*^{ts}-pRS425] were grown to mid-logarithmic phase in SC medium at 25°C and shifted to 37°C for a 2-hour incubation. Cells were then washed in inositol-free medium and labeled with *myo*-[^{3}H]inositol for 1 hour. Phosphoinositides were extracted, deacylated, separated by high-performance liquid chromatography and quantified using an online radiometric detector. Data is presented as percentage of the total number of counts for inositol-containing phospholipids and are expressed as mean ± SE of a minimum of three separate experiments.

Even when the level of Sec14^{Gly266Asp} was modestly increased due to expression from a low-copy plasmid (1-3 copies per cell), the trafficking of Pma1-RFP was improved (Figures 48). The increased delivery of lipid rafts to the plasma membrane, monitored by the resident protein Pma1-RFP, correlated with cell growth.

The data clarify a role for Sec14 in the regulation of PI metabolism through regulation of both PI-3P and PI-4P. It also suggests a possible mechanism for how increased expression of Sec14^{Gly266Asp} allows cell growth even at the non-permissive temperature.

3.5.3 Kes1 Bypass of Sec14 Essential Function

I described a role for Sec14 and Kes1 in maintaining the proper balance of PI for synthesis of complex sphingolipids and lipid raft formation. I also know that inactivation of *KES1* allows bypass of the essential function of Sec14 (Cleves et al., 1991). To determine if eliminating the lipid-binding properties of Kes1 are enough to bypass the essential function of Sec14, I introduced a plasmid-borne genes expressing Kes1^{K109A} or Kes1^{3E} in a *sec14^{ts} kes1*Δ yeast strain (Figure 49). The growth of the *sec14^{ts} kes1*Δ cells expressing either of these mutants suggests that the Kes1 mutants are not able to restore enough Kes1 function to kill *sec14^{ts}* cells. The levels of PI-3P and PI-4P are both decreased upon increased expression of wild-type Kes1. When we compare the levels of PI-3P and PI-4P when either the mutant Kes1^{K109A} or Kes1^{3E} protein is expressed, I only see a decrease in PI-3P and not in PI-4P (see Figure 22). Since inactivation of *KES1* causes PI-4P levels to return to wild-type values in *sec14^{ts}* cells, adding wild-type Kes1 causes PI-4P levels to again decrease, presumably causing cell death. However,

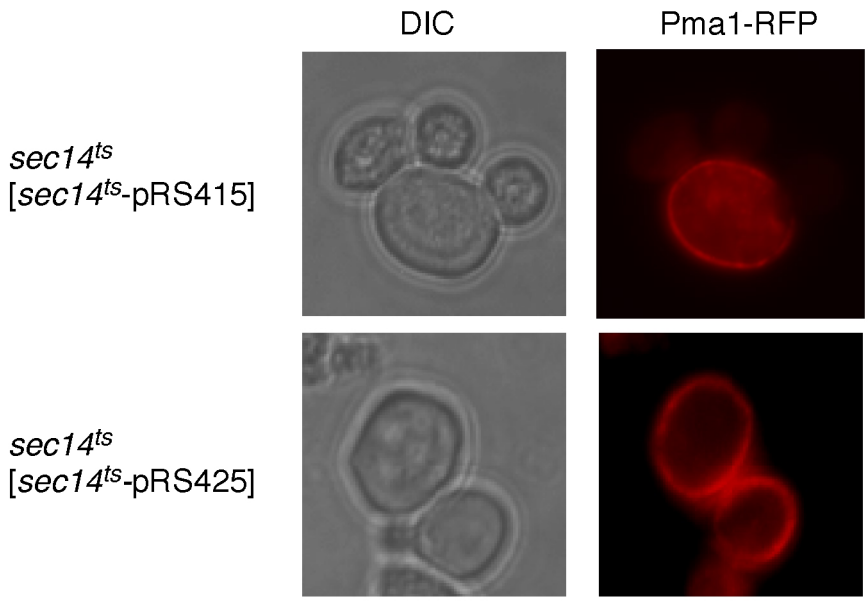


Figure 48 Increased expression of Sec14^{Gly266Asp} improves lipid raft delivery to the plasma membrane. CMY307 (SEY6210-PMA1-dsRFP, CMY505) cells transformed with the plasmids [*sec14^{ts}*-pRS415] or [*sec14^{ts}*-pRS425] were grown to mid-logarithmic phase in SC medium at 25°C. Cells were then shifted to 37°C and incubated for 16 hours. Cells were visualized using fluorescence (RFP) and DIC filters.

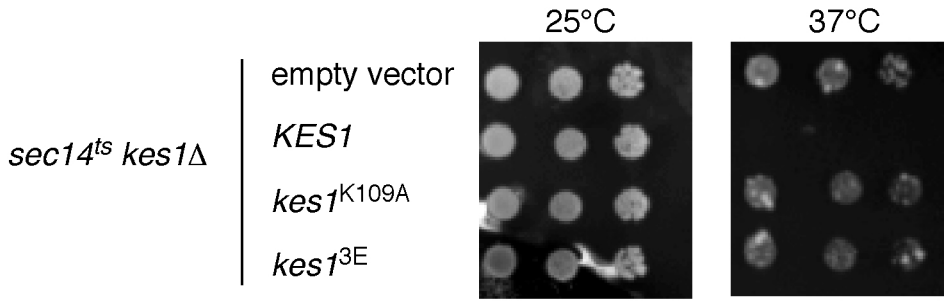


Figure 49 PIP and sterol binding by Kes1 is not required to bypass the essential function of Sec14. CMY136 (*sec14*^{ts} *kes1*Δ*)* cells were transformed with either [*KES1*-pRS415], [*kes1*^{K109A}-pRS415] or [*kes1*^{3E}-pRS415] and grown to mid-logarithmic phase in SC medium at 25°C. Cells were serially diluted on SC solid medium and incubated for 2 days at 25°C and 37°C.

introducing either of the Kes1 mutants in a *kes1*Δ strain did not cause PI-4P levels to drop. This suggests that the function of PIP and sterol binding by Kes1 (which in turn regulates Sac1 and PI-4P levels) and Sec14 could all be functioning along the same pathway.

3.6 A Possible Role for Kes1 in Energy Homeostasis

During the course of this thesis work, genetic and protein-protein screens were performed to try to further elucidate the role of Kes1. At the time these genetic and proteomic screens were performed, the link between the genes/proteins recovered and Kes1 was unclear. Many of these genes we now know are affected during processes affected by Kes1, such as autophagy and sphingolipid metabolism. Autophagy is a response to an imbalance in energy homeostasis and many of the genes from these screens regulate processes that regulate glycolycis/gluconeogenesis and the tricarboxylic acid (TCA) cycle. Sphingolipid metabolism regulates the TOR pathway, which in turn senses nutrient/energy homeostasis. When this is perturbed, autophagy can be induced. Although the link is still unclear, the sections below provide preliminary data that summarizes these findings.

3.6.1 High-Copy Suppressor Screen

The Osh proteins in yeast have a shared overlapping function that has yet to be determined. Inactivation of any six of the seven Osh family members has very little to no deleterious effect on cell growth, although deletion of the seven Osh genes results in inviability (Beh et al., 2001). To gain insight into the process regulated by Osh proteins, high-copy suppressors of a yeast strain lacking all *OSH* genes, but containing the *kes1*^{ts}

allele on a low-copy plasmid, were isolated. A yeast library, containing yeast genes on a high-copy plasmid, was transformed into cells of the yeast strain with every gene of the *OSH* family inactivated but kept alive by the presence of a temperature-sensitive allele of *KES1*. Plasmids containing the genes of interest were isolated from the cells that grew at the non-permissive temperature. The identified genes were subsequently expressed in cells of the above strain lacking all Osh proteins to confirm that growth was due to the plasmid-borne gene. Library plasmids with this activity encoded the proteins Kes1, Utr1, Pdc5, Rps15 and Kgd2 (Table 6). These identified proteins could allow for growth of the cells lacking function of all Osh proteins by i) increasing the function of $kes1^{ts}$ itself, ii) increasing the flux or the function of the pathway that Kes1 takes part in or iii) increasing the function of a parallel pathway to Kes1 that has the same endpoint. The other *OSH* genes were not identified in this selection, suggesting that the results of this screen do not encompass all of the possible high copy suppressors.

3.6.2 Kes1 Interaction Partners

To identify *in vitro* and *in vivo* interaction partners of Kes1-HIS6x, we isolated proteins that purified with Kes1-HIS6x (Table 7) or Kes1-TAP (Table 8), respectively. Proteins were separated by SDS-PAGE followed by silver staining. Bands present in the gel, and corresponding to a protein, were identified by mass spectrometry. The majority of the Kes1 interaction partners identified in these ways are involved in glycolysis/gluconeogenesis and the TCA cycle.

Table 6. High-copy suppressors of the lethality of yeast cells lacking all Osh proteins

Gene name	Function	Reference
KES1	Member of the oxysterol binding protein family, which includes seven yeast homologs; involved in negative regulation of Sec14p-dependent Golgi complex secretory functions	(Fang et al., 1996; Li et al., 2002a; Padilla et al., 1998)
UTR1	ATP-NADH kinase; phosphorylates both NAD and NADH; active as a hexamer; enhances the activity of ferric reductase	(Kawai et al., 2001; Lesuisse et al., 1996; Shi et al., 2005)
PDC5	Minor isoform of pyruvate decarboxylase, key enzyme in alcoholic fermentation	(Seeboth et al., 1990)
RPS15	Protein component of the small (40S) ribosomal subunit; has similarity to *E. coli* S19 and rat S15 ribosomal proteins	(Lecompte et al., 2002)
KGD2	Dihydrolipoyl transsuccinylase, component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes the oxidative decarboxylation of alpha-ketoglutarate to succinyl-CoA in the TCA cycle	(Reinders et al., 2006; Repetto and Tzagoloff, 1990)

Table 7. *In vitro* interaction partners with Kes1-HIS6x

Gene name	Function	Reference
KES1	Member of the oxysterol binding protein family, which includes seven yeast homologs; involved in negative regulation of Sec14p-dependent Golgi complex secretory functions, peripheral membrane protein that localizes to the Golgi complex	(Fang et al., 1996; Li et al., 2002a)
ACS2	Acetyl-CoA synthetase isoform which, along with Acs1, is the nuclear source of acetyl-CoA for histone acetylation; mutations affect global transcription; required for growth on glucose; expressed under anaerobic conditions	(Guranowski et al., 1994; Takahashi et al., 2006; van den Berg et al., 1996)
URA1	Dihydroorotate dehydrogenase, catalyzes the fourth enzymatic step in the *de novo* biosynthesis of pyrimidines, converting dihydroorotic acid into orotic acid	(Guranowski et al., 1994; Takahashi et al., 2006; van den Berg et al., 1996)
PGK1	3-phosphoglycerate kinase, catalyzes transfer of high-energy phosphoryl groups from the acyl phosphate of 1,3-bisphosphoglycerate to ADP to produce ATP; key enzyme in glycolysis and gluconeogenesis	(Blake and Rice, 1981; Hitzeman et al., 1980)
ENO2	Enolase II, a phosphopyruvate hydratase that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis; expression is induced in response to glucose	(Entian et al., 1987; McAlister and Holland, 1982)
ADH1	Alcohol dehydrogenase, fermentative isozyme active as homo- or heterotetramers; required for the reduction of acetaldehyde to ethanol, the last step in the glycolytic pathway	(Bennetzen and Hall, 1982; Dickinson et al., 2003; Young and Pilgrim, 1985)
LYS20	Homocitrate synthase isozyme, catalyzes the condensation of acetyl-CoA and alpha-ketoglutarate to form homocitrate, which is the first step in the lysine biosynthesis pathway; highly similar to the other isozyme	(Ramos et al., 1996)
ALF1	Alpha-tubulin folding protein, similar to mammalian cofactor B; Alf1-GFP localizes to cytoplasmic microtubules; required for the folding of alpha-tubulin and may play an additional role in microtubule maintenance	(Feierbach et al., 1999)

Table 8. *In vivo* interaction partners with Kes1-TAP

Gene name	Function	Reference
KES1	Member of the oxysterol binding protein family, which includes seven yeast homologs; involved in negative regulation of Sec14p-dependent Golgi complex secretory functions, peripheral membrane protein that localizes to the Golgi complex	(Fang et al., 1996; Li et al., 2002a)
PYK1	Pyruvate kinase, functions as a homotetramer in glycolysis to convert phosphoenolpyruvate to pyruvate, the input for aerobic (TCA cycle) or anaerobic (glucose fermentation) respiration	(Pearce et al., 2001)
PDC1	Major of three pyruvate decarboxylase isozymes, key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde; subject to glucose-, ethanol-, and autoregulation; involved in amino acid catabolism	(Kellermann et al., 1986)
PDC6	Minor isoform of pyruvate decarboxylase, decarboxylates pyruvate to acetaldehyde, involved in amino acid catabolism; transcription is glucose- and ethanol-dependent, and is strongly induced during sulfur limitation	(Kellermann et al., 1986)
PDC5	Minor isoform of pyruvate decarboxylase, key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde, regulation is glucose- and ethanol-dependent, repressed by thiamine, involved in amino acid catabolism	(Kellermann et al., 1986)
PGK1	3-phosphoglycerate kinase, catalyzes transfer of high-energy phosphoryl groups from the acyl phosphate of 1,3-bisphosphoglycerate to ADP to produce ATP; key enzyme in glycolysis and gluconeogenesis	(Blake and Rice, 1981; Hitzeman et al., 1980)
ENO2	Enolase II, a phosphopyruvate hydratase that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis; expression is induced in response to glucose	(Entian et al., 1987; McAlister and Holland, 1982)
PGD1	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; essential for basal and activated transcription; direct target of Cyc8-Tup1 transcriptional corepressor	(Papamichos-Chronakis et al., 2000)
ADH1	Alcohol dehydrogenase, fermentative isozyme active as homo- or heterotetramers; required for the reduction of acetaldehyde to ethanol, the last step in the glycolytic pathway	(Bennetzen and Hall, 1982; Dickinson et al., 2003)

Gene name	Function	Reference
ALF1	Alpha-tubulin folding protein, similar to mammalian cofactor B; Alf1-GFP localizes to cytoplasmic microtubules; required for the folding of alpha-tubulin and may play an additional role in microtubule maintenance	(Feierbach et al., 1999)
PSA1	GDP-mannose pyrophosphorylase (mannose-1-phosphate guanyltransferase), synthesizes GDP-mannose from GTP and mannose-1-phosphate in cell wall biosynthesis; required for normal cell wall structure	(Hashimoto et al., 1997)
STM1	Protein required for optimal translation under nutrient stress; perturbs association of Yef3 with ribosomes; involved in TOR signaling; binds G4 quadruplex and purine-motif triplex nucleic acid; helps maintain telomere structure	(Utsugi et al., 1995)
OYE2	Widely conserved NADPH oxidoreductase containing flavin mononucleotide (FMN), homologous to Oye3 with slight differences in ligand binding and catalytic properties; may be involved in sterol metabolism	(Niino et al., 1995)
RHR2	Constitutively expressed isoform of DL-glycerol-3-phosphatase; involved in glycerol biosynthesis, induced in response to both anaerobic and, along with the Hor2/Gpp2 isoform, osmotic stress	(Norbeck et al., 1996)
GPM1	Tetrameric phosphoglycerate mutase, mediates the conversion of 3-phosphoglycerate to 2-phosphoglycerate during glycolysis and the reverse reaction during gluconeogenesis	(Rodicio et al., 1993)
TDH1	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 1, involved in glycolysis and gluconeogenesis; tetramer that catalyzes the reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate; detected in the cytoplasm and cell wall	(McAlister and Holland, 1985a)
TPI1	triose phosphate isomerase, abundant glycolytic enzyme; mRNA half-life is regulated by iron availability; transcription is controlled by activators Reb1, Gcr1, and Rap1 through binding sites in the 5' non-coding region	(Krieger and Ernst, 1994)
FBA1	Fructose 1,6-bisphosphate aldolase, required for glycolysis and gluconeogenesis; catalyzes conversion of fructose 1,6 bisphosphate to glyceraldehyde-3-P and dihydroxyacetone-P; locates to mitochondrial outer surface upon oxidative stress	(Schwelberger et al., 1989)

Gene name	Function	Reference
GND1	6-phosphogluconate dehydrogenase (decarboxylating), catalyzes an NADPH regenerating reaction in the pentose phosphate pathway; required for growth on D-glucono-delta-lactone and adaptation to oxidative stress	(Sinha and Maitra, 1992)
OLA1	P-loop ATPase with similarity to human OLA1 and bacterial YchF; identified as specifically interacting with the proteasome; protein levels are induced by hydrogen peroxide	(Godon et al., 1998; Koller-Eichhorn et al., 2007)
VAT2	Subunit B of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; contains nucleotide binding sites; also detected in the cytoplasm	(Forgac, 1999)

3.6.3 Sensitivity to Rapamycin

Cells with an inactivated *KES1* gene also show sensitivity to rapamycin (Figure 50). The effects of rapamycin mimic the effect of nitrogen starvation. Sensitivity of cells without Kes1 function to rapamycin suggests a role for Kes1 in nutrient/energy homeostasis. Rapamycin also induces autophagy, supporting our theory that Kes1 plays a role in regulation of autophagy.

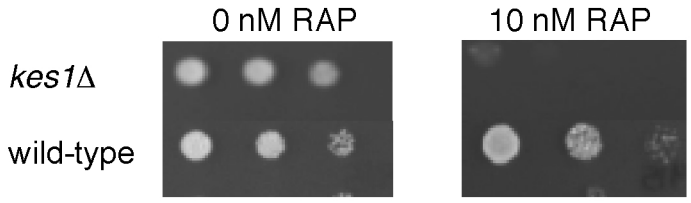


Figure 50 Inactivation of *KES1* causes sensitivity to rapamycin. (A) BY4741 (wild-type) and *kes1*Δ cells were grown to mid-logarithmic phase in SC medium at 25°C. Cells were serially diluted on SC solid medium +/- 10 nM rapamycin and grown for 2 days at 25°C.

CHAPTER 4 DISCUSSION

This thesis clarifies the functions of the known lipid-binding properties of Kes1 in their ability to negatively impact secretion from the *trans*-Golgi. It also identifies a role for Kes1 in the regulation of trafficking to the vacuole through the autophagy/Cvt pathway. Kes1 appears to regulate PI-4P and PI-3P homeostasis by separate mechanisms. Regulation of PI-3P level does not require sterol and PIP binding by Kes1, whereas Kes1 regulation of PI-4P homeostasis is affected by its ability to bind both sterols and PIPs.

Kes1 primarily localizes to the Golgi, and my work suggests Kes1 does so through its ability to bind PI-4P. PI-4P is required for Golgi-derived secretion, and the presence of Kes1 appears to attenuate its production. The production of a Golgi PI pool, from PI-4P, by the PIP phosphatase Sac1, is crucial for the synthesis of complex sphingolipids. These sphingolipids are in turn required for lipid-raft formation at the Golgi for their delivery to the plasma membrane. I identified Kes1 as an activator of sphingolipid synthesis, likely through increasing Sac1 activity. This activity is important for the delivery of the lipid raft resident protein Pma1 to the plasma membrane.

Sec14, a PI-binding protein, also appears to be involved in the regulation of PI and complex sphingolipid synthesis and functions at the Golgi. My results indicate that cells expressing the temperature-sensitive mutant protein, $Sec14^{Gly266Asp}$, have decreased delivery of Pma1 to the plasma membrane at the non-permissive temperature. Kes1 and Sec14 may coordinate PI metabolism at the *trans*-Golgi and therefore regulate synthesis of complex sphingolipids and lipid raft formation/delivery to the plasma membrane.

Finally, my thesis work demonstrates that Kes1 may play a role in energy homeostasis. Many physical and genetic interactions were identified between Kes1 and

members of glycolysis/gluconeogenesis or the TCA cycle. My data suggests a role for Kes1 in energy homeostasis, possibly *via* the TOR pathway and endosome trafficking to the vacuole.

4.1 A Bi-Functional Role for Kes1 in the Regulation of PI-3P and PI-4P Levels

This thesis further defines the role for Kes1 in the regulation of PI-4P and *trans*-Golgi/endosome trafficking pathways, and determines characteristics of Kes1 responsible for its regulation. It also identifies a new role for Kes1 in the regulation of PI-3P level. Regulation of PI-3P level by Kes1 becomes critical for what appears to be formation of pre-autophagosomes in the autophagy/Cvt pathway for delivery to the vacuole (Figure 51).

4.1.1 Rationale for the Creation and Study of Kes1 Sterol-and PIP-Binding Mutants

Characterization of the lipid- and membrane-binding properties of Kes1 in this study was performed using mutant proteins previously identified by crystallization and preliminary structure-function studies (Figure 52) (Im et al., 2005; Li et al., 2002a; Schulz and Prinz, 2007). The core of Kes1 is a central antiparallel β-sheet of 19 strands (residues 115-293) that forms a nearly complete anti-parallel β-barrel (Im et al., 2005). The N-terminal lid consists of residues 1-29 and is required for sequestering sterols within the core of the protein (Im et al., 2005). The lid is also predicted to contain an amphipathic α-helix (Drin et al., 2007). This membrane-binding motif, named ALPS, has a polar face that is rich in serine and threonine (polar and uncharged) residues, allowing membrane association to be driven by both the hydrophobic effect and by electrostatic

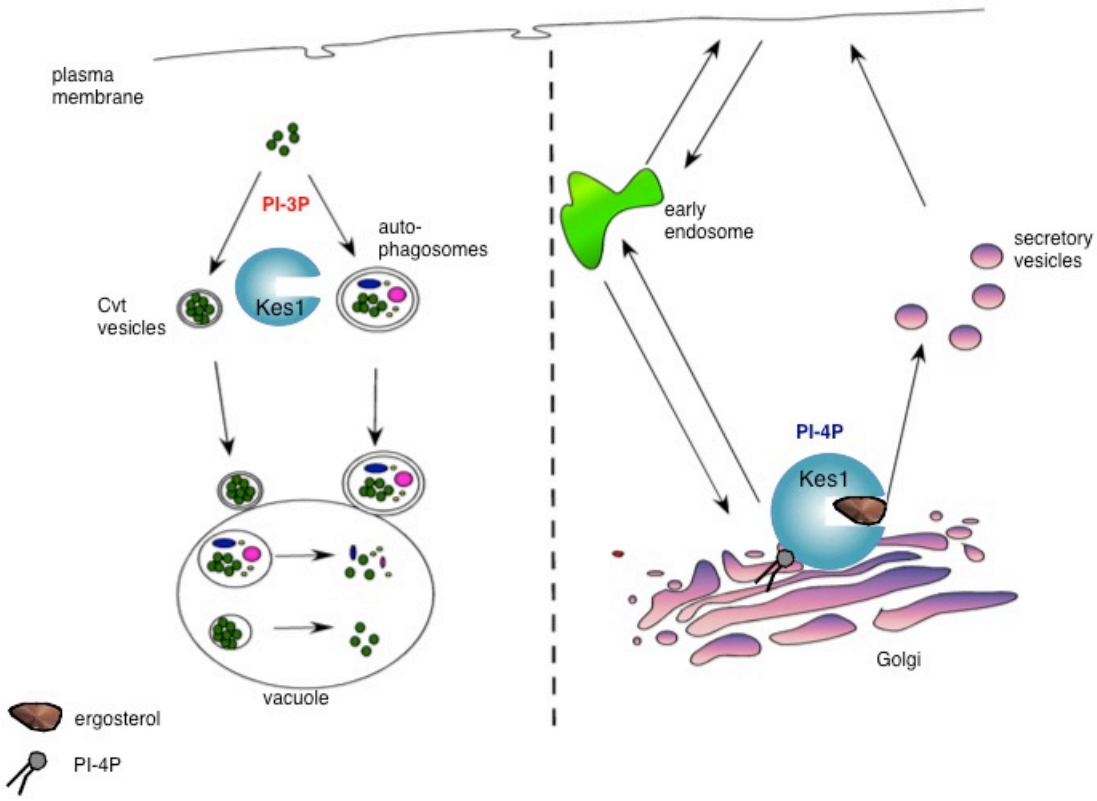


Figure 51 A bi-functional role for Kes1 in the regulation of PI-3P and PI-4P levels and availability. Kes1 regulates both PI-3P (left) and PI-4P (right) homeostasis. Kes1 regulation of PI-3P level is independent of sterol and PIP binding by Kes1 and affects trafficking to the vacuole *via* the autophagy/Cvt pathway. Kes1 regulation of PI-4P level requires PIP and sterol binding by Kes1 and affects *trans*-Golgi/endosome trafficking

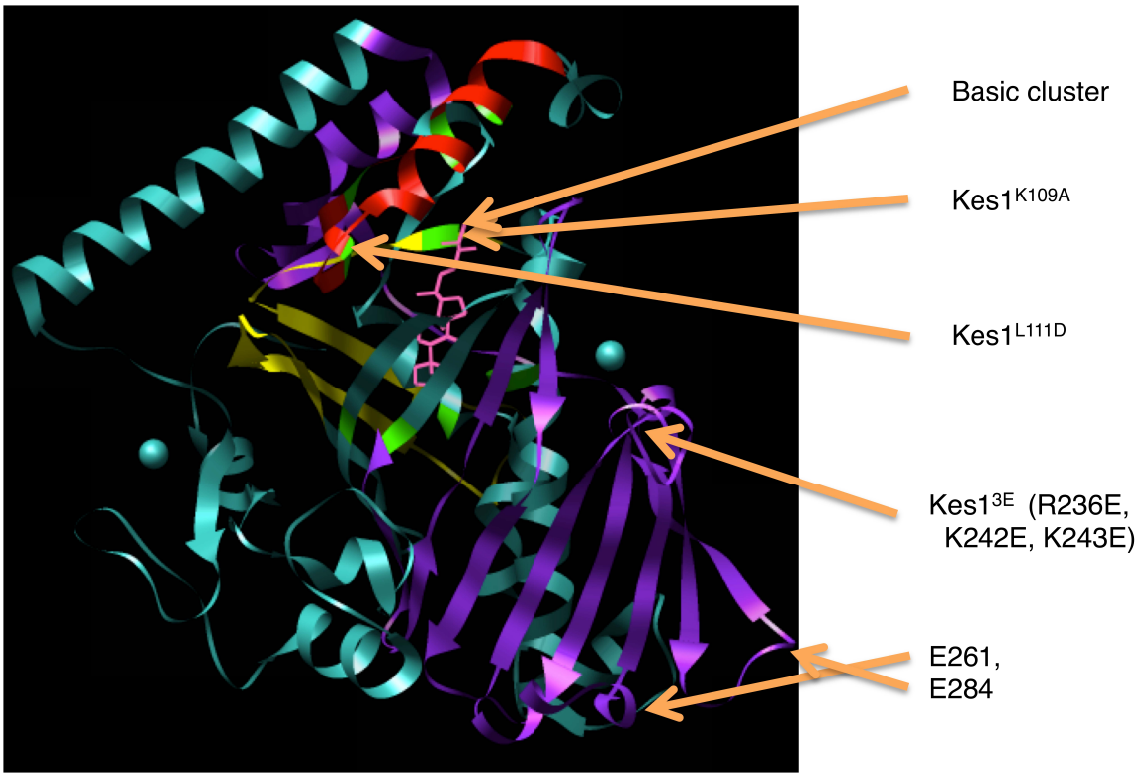


Figure 52 Kes1 mutants discussed in this study. Known domains include the N-terminal lid (amino acid 2-29) (red), the phosphoinositide-binding domain (purple) and the OSBP-related-domain (ORD) domain (yellow). Also shown are amino acids that interact with ergosterol (green) and ergosterol found in the core of the protein (pink). Mutants used throughout the course of this study are identified and include Kes1$^{2\text{-}29\Delta}$ (red), Kes1^{K109A} (decreased sterol binding) and Kes1^{3E} (no PIP binding). Other mutants are also identified here and include Kes1^{L111D} (no sterol binding), the basic cluster near the opening of the tunnel (important for sterol and PIP binding) and the distal membrane-binding site (E261 and E284).

interactions (Drin et al., 2007). This motif can also associate with membranes containing strong positive curvature (Drin et al., 2007). The exterior surface around the lid of the tunnel contains six highly conserved basic residues (Im et al., 2005). Kes1 can bind lipids other than sterol in its core, mainly PIPs on a discrete surface, whereupon its PIP-binding properties are required for Kes1 localization to Golgi membranes (Fairn et al., 2007; Li et al., 2002a).

Kes1 residues near the tunnel entrance are part of a basic cluster. Mutations in some of these residues, including that in Kes1^{K109A}, result in loss of function *in vivo* as determined by their inability to restore life to cells lacking all other Osh proteins (Im et al., 2005). *In vitro*, this mutant shows a significant decrease in basal cholesterol transport by Kes1 (Raychaudhuri et al., 2006), with cholesterol binding diminished to approximately 50% (Im et al., 2005). One Kes1 mutant with a complete defect in sterol binding is Kes1^{L111D}. Residue L111 is situated in the structural core near the tunnel, and, upon mutation of this residue, sterol binding and transport is abolished *in vitro*. *In vivo*, Kes1^{L111D} cannot support growth in the absence of all other Osh proteins (Im et al., 2005). Other mutants that abolish binding of Kes1 to sterols exhibit similar phenotypes (Im et al., 2005). This strongly suggests that binding to sterol is required for function of Kes1 in cells. However, other mutants in this basic cluster do not result in decreased function *in vivo*, even though they cause a decrease in transport of sterols *in vitro* (Im et al., 2005). These results indicate that although the major biochemical defect seen when residues are mutated within the basic cluster is a defect in sterol transport, it does not always correlate with a reduced function in cells. To study the role of lipid binding on Kes1 function, I chose the lid mutant Kes1$^{2\text{-}29\Delta}$, thought to sequester sterols in the core

and predicted to contain an ALPS motif. The lid mutant has been reported to be unable to bind cholesterol *in vitro* and to have no *in vivo* function (Im et al., 2005). I chose the Kes1^{K109A} mutant to study sterol binding as its function is, reportedly, significantly reduced, but not eliminated, *in vitro* (Im et al., 2005). As Kes1 undergoes conformational change upon sterol binding, there was concern that a mutant completely defective in sterol binding could be locked in the open conformation. That being the case, Kes1 may not be able to be released from membranes since binding to sterols, and the conformational change that is accompanied with, hides the membrane binding site near the tunnel entrance (Im et al., 2005).

The Kes1^{3E} PIP-binding mutant was suggested by structure analysis of Kes1, which identified a variable loop region with basic residues involved in binding inositide headgroups (Li et al., 2002a). This is the same region identified by my lab, through deletion mutagenesis of Kes1, as being required for PIP binding (Fairn et al., 2007). The mutant that eliminates PIP-binding by Kes1 is R236E K242E K243E. The substitution of these three basic residues to acidic residues causes a defect in PIP binding, as assessed by an inability of excess Kes1^{3E} to prevent cross-linking of a PI-4,5-P_2 probe to wild-type Kes1 protein (Li et al., 2002a). Recently, cross-linking studies were used in an attempt to map the surface of Kes1 that associates with membranes. Two sites were identified; wherein one contains residues within the PIP-binding site previously identified by deletion mutagenesis, and the other is a region near the sterol tunnel entrance (Schulz et al., 2009). That study hypothesized that the two membrane-binding sites could simultaneously allow Kes1 to bind to two membranes in *trans*, suggesting a mechanism

by which Kes1 could potentially transfer sterol between membranes *in vitro* (Schulz et al., 2009).

A major determinant of Kes1 function appears to be binding to membranes. However, the role of sterol and PIP binding in membrane association, and how these affect each other, has not been precisely determined. The function of the lid in sequestering sterols, and the targeting of Kes1 to membranes with high curvatures, was also previously unclear. One of the goals of this thesis was to delineate the role of the various lipid- and membrane-binding properties on Kes1 function.

4.1.2 Kes1 Requires PIP Binding to Associate with Golgi Membranes

The structure-function studies of Kes1 in this thesis determined that a major determinant for *in vitro* liposome association by Kes1 is the presence of a free phosphate group on the membrane surface. PI is modified by the addition of phosphate groups at three different positions along the inositol ring (PIPs), giving the lipid additional negative charge. Kes1 only bound liposomes containing PIPs or PA, but not other phospholipids. Vesicle size or the presence of ergosterol in liposomes had limited effects on membrane association by Kes1. The use of Kes1 derivatives supported this concept, where it was found that the basic residues on the β-sheet surface of Kes1 play a major role for Kes1 association with membranes. A Kes1 mutant unable to bind PIPs could not associate with liposomes of any lipid composition, while a Kes1 mutant with a decreased ability to bind sterols, or lacking the N-terminal lid, associated with membranes in a manner similar to wild-type Kes1. *In vivo*, the Kes1 mutant unable to bind PIPs redistributed to the cytoplasm while the mutant with impaired sterol binding still associated with intracellular membranes.

Another Golgi-associated protein, ArfGAP1, associates with curved membranes, over flat lipid membranes, *via* its unusual amphipathic α-helix (ALPS motif). Other ALPS motifs identified in the yeast proteome include one predicted for the N-terminal lid of Kes1 (Drin et al., 2007). My results suggest that the predicted ALPS motif does not play a major role in promoting Kes1 binding to membranes of high curvature. When the lid was deleted from Kes1, Kes1 association with membranes of higher curvature (50 nm vs. 200 nm) was only decreased by a marginal amount. At least *in vitro*, the ALPS motif predicted for the lid of Kes1 does not appear to be a major determinant for Kes1 association with membranes.

The role of sterol binding in the function of Kes1 is still not clear. Ergosterol binding by two other yeast Osh proteins, Osh6 and Osh7, plays a role in regulating their association with the AAA ATPase Vps4 (Wang et al., 2005b). Sterol binding by Kes1 could also mediate its interaction with other proteins, but further studies on this subject await the confirmation of such proteins.

4.1.3 Kes1 Affects PI-4P Level and Availability and Regulates *trans*-Golgi/Endosome Trafficking Pathways

Kes1 primarily associates with Golgi membranes, and my results predict that this association is likely due to the ability of Kes1 to bind PI-4P. PI-4P is enriched at the Golgi, where it plays a role in recruiting proteins that allow maintenance of Golgi integrity (eg. Golgins). Decreasing the synthesis of PI-4P at the Golgi results in a redistribution of Kes1 from the Golgi to a more cytoplasmic localization (Fairn et al., 2007). I went on to determine that Kes1 has a function in the regulation of PI-4P metabolism and availability. I demonstrated that increased expression of *KES1* results in

a decrease in the level of PI-4P in the cell, and a decreased ability of a PI-4P-binding probe to associate with Golgi membranes. Additionally, when *KES1* was inactivated, PI-4P levels were increased and the PI-4P-binding probe remained at the Golgi.

The decreased ability of the PI-4P-binding probe to bind to membranes in situations when *KES1* expression is increased could be explained by two different, and not mutually exclusive, reasons. The PI-4P-binding probe could have less PI-4P to bind (since we know that the PI-4P level is decreased), or Kes1, being a PIP-binding protein, could be binding the bulk of the available PI-4P. The available PI-4P for binding by other PI-4P-binding proteins is thus decreased. Indeed, other proteins have been observed to behave in this fashion. Expression of the exogenous mammalian PH domain of FAPP1 sequesters all the available PI-4P for binding by other PI-4P effectors at the Golgi (Godi et al., 2004). A competition assay between a PI-4P-binding probe and Kes1 may prove useful in identifying the precise role of Kes1 in the regulation of PI-4P availability at the Golgi.

Information on the role of PI-4P in recruiting protein effectors and in affecting their function at the Golgi is sparse. The identities of *in vivo* Golgi PI-4P-binding proteins whose functions and/or localization are affected by Kes1 need to be determined. One potential effector of PI-4P by Kes1 is the TRAPPII complex, a 10-subunit protein complex that appears to act as a gatekeeper for exit from the *trans*-Golgi (Morozova et al., 2006; Sacher et al., 2001). The TRAPPII complex possesses a PIP-binding protein in Bet1, and acts as the GTP-exchange factor for the Rab GTPase Ypt31 and the highly similar Ypt32 (Jones et al., 2000; Morozova et al., 2006). *YPT31* and *YPT32* comprise an essential gene pair that regulates vesicle export and import at the *trans*-Golgi (Benli et al.,

1996). Studies identified that Ypt31/TRAPPII function is downstream of Kes1, implying that Kes1 is an inhibitor of this complex (Fairn et al., 2007). We know that PI-4P is required for cell viability, which renders imperative the identification of other genes, and their protein products, which are affected by Kes1 function and regulate PI metabolism at the *trans*-Golgi.

PI-4P synthesis at the Golgi is required for vesicular transport from this organelle, and I observed that the v-SNARE Snc1 was unable to transit between the Golgi and the plasma membrane upon increased expression of *KES1*. Expression of the Kes1 mutants defective in PIP- or sterol-binding did not regulate the PI-4P level and availability at the Golgi or the GFP-Snc1 transport as much as wild-type Kes1 did. The data imply that both the PIP- and sterol-binding functions are required for Kes1 to inhibit transport at the *trans*-Golgi (see Figure 51). My results also showed that increased expression of these Kes1 mutants did not result in cell growth arrest, unlike wild-type Kes1 increased expression. This is likely a result of PI-4P levels only being modestly decreased when either mutant of Kes1 is over-expressed.

A surprising and unexpected result was the observation that GFP-Snc1 trafficking was also impaired when the *KES1* gene was inactivated. In these cells, the level of PI-4P was increased above wild-type values. Thus, it appears that an appropriate level of PI-4P needs to exist to allow for effective vesicular transport. A defect in PI-4P homeostasis, either up or down, results in the misregulation of GFP-Snc1 trafficking.

My combined data suggests a role for Kes1 as an inhibitor of vesicular transport within the *trans*-Golgi/endosome trafficking pathway, with both its regulation of the PI-4P level and binding as contributing factors. This model is in line with previous studies

which found that inactivation of the *KES1* gene relieves the defects in growth, PIP levels, and *trans*-Golgi-to-plasma membrane vesicular trafficking associated with decreased function of both the Golgi-resident PI-4 kinase Pik1 and the PI/PC-transfer protein Sec14 (Fairn et al., 2007; Li et al., 2002a). We therefore know that as Golgi PI-4P level and availability becomes critical, as when the Sec14 or Pik1 function is decreased, the role of Kes1 in regulating PI-4P level and/or its capacity to bind PI-4P appears to be the cause of its inhibition of vesicular transport (Fairn et al., 2007).

One line of research that needs to be further explored, and which has been opened up by the results of this thesis, is the mechanism by which Kes1 regulates PIP levels. Sterol and PIP binding by Kes1 contributes to its regulation of the PI-4P level, and these properties could contribute to the ability of Kes1 to directly affect the activity of an enzyme that metabolizes PI-4P. Candidate processes include inhibiting the PI-4 kinase Pik1 and activating the PIP phosphatase Sac1. Sac1 is a PI-4P phosphatase that converts PI-4P to PI in the ER and Golgi. Sac1 also plays a role in the regulation of vesicular trafficking from the Golgi apparatus, as inactivation of the *SAC1* gene relieves the vesicular transport defect due to the absence of *SEC14*. Inactivation of *KES1* also alleviates this same vesicular transport defect due to the absence of *SEC14*. A personal communication from Dr. Chris Stefan (Cornell University) informed me that *in vitro*, Kes1 and other Osh proteins activate Sac1 phosphatase activity. Sterol and PIP binding by Kes1 are both important in the regulation of Sac1 activity by Kes1, as Kes1 mutants defective in these functions have a decreased capacity to activate Sac1, all of which is consistent with the results presented in this thesis.

4.1.4 Kes1 Regulates PI-3P Level and Autophagy

Prior to the work presented in this thesis, there were no reports of a role for Kes1 in the regulation of either PI-3P level or the autophagy/Cvt pathway. I found that upon increased *KES1* expression, the level of PI-3P was decreased; conversely, upon inactivation of *KES1,* the level of PI-3P was accordingly increased. Increased expression of *KES1* resulted in phenotypes associated with the induction of autophagy. Cellular defects included membrane accumulation, vacuole fragmentation and lipid droplet formation, and they were observed by EM. Increased expression of the sterol- and PIP-binding mutants of Kes1 also resulted in similar autophagy phenotypes. This correlates well with my observation that the decrease in PI-3P level, due to increased Kes1 expression, did not require the sterol- or PIP-binding functions of Kes1.

Consistent with a role for Kes1 in induction of autophagy were the findings of an SGA screen I performed against the entire non-essential yeast genome. The results revealed that the inactivation of a subset of genes required for the Cvt/autophagy pathway restores growth to cells that are over-expressing *KES1*. These cells also showed a decrease in membrane and lipid-droplet accumulation, both of which are phenotypes associated with the induction of autophagy. The membrane accumulation defects were also minimized by increased expression of the PI-3 kinase Vps34, but not the Golgi-resident PI-4 kinase Pik1. This implies that the reduction in PI-3P level observed upon increased production of Kes1, at least in part, mediates Kes1 induced autophagy. PI-3 kinase activity, as well as three of the four genes whose inactivation prevented Kes1 mediated membrane and lipid droplet accumulation – namely *ATG9*, *ATG17* and *ATG24*

– are all involved in formation of the PAS membrane (Figure 53). The data points to this step as a possible site of action for Kes1 mediated regulation of autophagy. Kes1 inhibition of the autophagy/Cvt pathway could be *via* Vps34 assembly with protein partners, or Vps34 activity itself. Vps34, assembled with protein partners, drives both autophagy and Vps34 PI-3 kinase activity. Both properties of Vps34 are known to be necessary for PAS membrane assembly.

4.2 A Role for Kes1 in Sphingolipid Synthesis at the *trans*-Golgi and Lipid Raft Formation/Delivery to the Plasma Membrane

4.2.1 Kes1 Regulates the Synthesis of Complex Sphingolipids by Regulating PI-4P Levels

Recent studies determined inactivation of the *SAC1* gene, or the use of a catalytically inactive mutant, results in an increase in the level of early intermediates in the sphingolipid biosynthetic pathway, and a decrease in the levels of complex sphingolipids (Brice et al., 2009). I sought to investigate whether Kes1 regulates sphingolipid synthesis in a manner similar to Sac1. The lines of logic that drove this interest were the fact that Sac1 and Kes1 can both localize to the Golgi, both share genetic interactions, Sac1 metabolizes PI-4P to PI for use in sphingolipid synthesis and, finally, Kes1 is a PI-4P-binding protein. Similarly to the inactivation of *SAC1*, the inactivation of *KES1* resulted in a large decrease in PI level and a concurrent decrease in the level of inositol-containing (complex) sphingolipids. The biochemical basis for the regulation of sphingolipid synthesis by Sac1 is the channeling of the PI produced by Sac1 for use by Aur1 to convert ceramide to sphingolipids (Brice et al., 2009). This is consistent with the role proposed in this thesis for Kes1 activating Sac1.

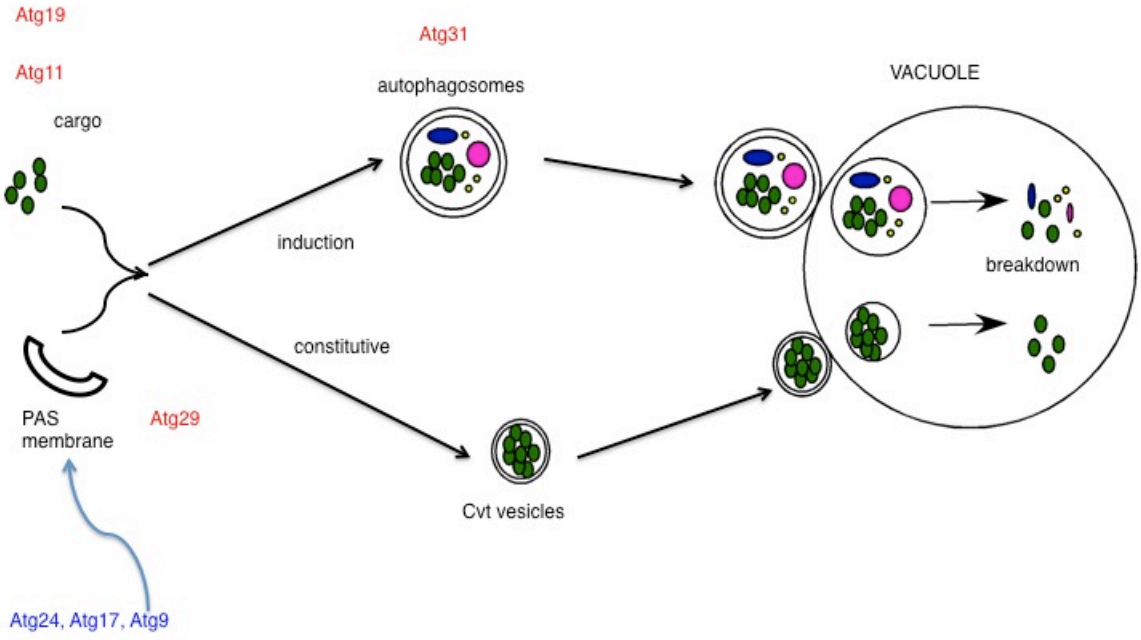


Figure 53 PAS assembly as a possible site for Kes1-mediated regulation of autophagy. Components of the autophagy pathway studied are shown in blue and red. The blue components represent gene products that were identified to have a genetic interaction with Kes1, in which inactivation of the gene alleviates the cellular defects associated with increased expression of *KES1*. These gene products include 1) Atg17, a scaffold protein responsible PAS organization; 2) Atg24, a sorting nexin involved in retrieval of late-Golgi SNAREs in the Cvt pathway; and 3) Atg9, a transmembrane protein involved in forming Cvt and autophagic vesicles, and which cycles between the PAS and other cytosolic punctate structures. Other genes tested for genetic interactions with *KES1* are shown in red and include 1) Atg19, a receptor protein specific for the Cvt pathway that delivers cargo proteins aminopeptidase I (Lap4) and alpha-mannosidase (Ams1) to the PAS for packaging into Cvt vesicles; 2) Atg11, an adapter protein for the Cvt pathway that directs receptor-bound cargo to the PAS for packaging into vesicles and is required for recruiting other proteins to the PAS; 3) Atg29, an autophagy-specific protein that is required for recruitment of other Atg proteins to the PAS; and, 4) Atg31, an autophagy-specific protein required for autophagosome formation that may form a complex with Atg17 and Atg29 that localizes other proteins to the PAS.

Recently, a role for the Orm protein family as homeostatic regulators of sphingolipid metabolism emerged (Breslow et al., 2010; Han et al., 2010). These are ER-localized transmembrane proteins conserved from yeast to humans. Similarly to the inactivation of *SAC1*, the loss of Orm functions results in increased sphingolipid synthesis and in an accumulation of toxic sphingolipids, as determined by yeast studies. Orm proteins bind directly to the enzyme that carries out the first and rate-determining step in the synthesis of sphingolipids – serine palmitoyltransferase – and are part of what has been termed the SPOTS complex: serine palmitoyltransferase, Orm1/2, Tsc3 (a regulatory subunit of serine palmitoyltransferase) and Sac1. Deletion of *ORM1* and *ORM2* is synthetic lethal with deletion of *SAC1*, consistent with their regulating sphingolipid synthesis as part of a protein complex. Whether Kes1 associates with the SPOTS complex has yet to be determined.

4.2.2 Kes1 Regulates Delivery of the Lipid-Raft-Associated Protein Pma1 to the Plasma Membrane

A decrease in the synthesis of complex sphingolipids can lead to functional consequences *in vivo*. One known role for sphingolipids is their involvement in the formation of lipid rafts in the Golgi. Lipid rafts are dynamic nanometer-sized membrane domains primarily consisting of sterols, sphingolipids, saturated glycerophospholipids and proteins (Hancock, 2006; Klemm et al., 2009; Simons and Vaz, 2004). Lipid rafts are enriched at the plasma membrane, where they are thought to serve as points of convergence for the recruitment of signaling complexes. The need to coordinate sphingolipid, sterol and phospholipid synthesis with lipid raft assembly is essential. In addition to improper raft formation, misregulaton of sterol and sphingolipid synthesis

leads to the build up of sphingolipid pathway intermediates, many of which have signaling properties and/or are toxic at high levels (Hancock, 2006; Klemm et al., 2009; Simons and Vaz, 2004). Information regarding how the Golgi apparatus senses its lipid composition, and in turn coordinates lipid metabolism with vesicular trafficking to the plasma membrane, is limited.

A previous genome-wide screen attempting to shed light on these processes determined that yeast mutants defective in sterol and sphingolipid synthesis are unable to assemble lipid rafts at the Golgi properly, and raft-associated proteins are no longer routed to the plasma membrane (Proszynski et al., 2005). That study also observed that inactivation of the *KES1* gene prevented an engineered protein, which has to associate with lipid rafts to travel from the Golgi to the plasma membrane, from doing so. The data implied that Kes1 plays a role in the assembly of proteins into lipid rafts at the Golgi. I determined that Kes1 regulates sphingolipid synthesis at steps that take place in the Golgi, and is required for the delivery of Pma1, a natural lipid raft associated protein, to the plasma membrane. Such results suggest that Kes1 is required not only for sphingolipid synthesis at the Golgi, but also for the proper assembly of proteins into lipid rafts. However, even though the absence of Kes1 inhibits the progression of Pma1 to the plasma membrane, the cells remain viable. This suggests that a sufficient amount of Pma1 must reach the plasma membrane to allow for viability.

4.2.3 A Role for the Osh/ORP Superfamily in Regulation of Sphingolipid Metabolism

To determine if the ability to affect PIP and sphingolipid metabolism is shared by other members of the Osh protein family, and to assess the contribution of Kes1 to this

process, I determined the levels of PIPs and the ability to transport Pma1 to the plasma membrane in yeast cells in which every *OSH* gene is inactivated. These cells were kept viable with a low-copy plasmid expressing wild-type *KES1* or a temperature-sensitive allele of *KES1*. Kes1 was a major determinant in the regulation of PI-4P and PI metabolism. I observed a 26-fold increase in PI-4P level after shifting the cells containing the temperature-sensitive allele of *KES1* to the non-permissive temperature, compared to a three-fold increase in cells containing wild-type *KES1*. This translated into a defect in lipid raft formation, as indicated by the observation that cells lacking all Osh protein function are sensitive to edelfosine, a drug that kills cells with improperly formed lipid rafts (Zaremberg et al., 2005). It also supports the idea that all Osh proteins affect the regulation of PIP conversion to PI for subsequent use for sphingolipid synthesis. However, this has yet to be formally proven through metabolic studies of sphingolipid synthesis in the *OSH* defective cells.

A relationship between members of the oxysterol binding protein family and sphingolipid metabolism appears to be an emerging theme. The founding member of the superfamily, mammalian OSBP, localizes to the Golgi *via* its PI-4P-binding PH domain and to the ER by its FFAT motif that facilitates interaction with the ER-resident VAMP-associated protein (VAP) (Levine and Munro, 2002; Perry and Ridgway, 2006). These studies suggest that since sterol and sphingolipid synthesis are closely linked, OSBP may act as a bridge to regulate synthesis of these two classes of lipids by acting as a sterol sensor (Perry and Ridgway, 2006). Sterol sensing by OSBP appears to stimulate CERT-mediated ceramide transport from the ER to the Golgi, which in turn regulates sphingolipid synthesis at the Golgi (Perry and Ridgway, 2006).

I propose a model whereby Kes1 functions as a sterol-sensor protein at the Golgi (Figure 54). Kes1 localizes to the Golgi through its ability to bind PI-4P. Kes1 integrates the levels of sterol with the metabolism of phospholipid to sphingolipid and coordinates the assembly of lipid rafts at this organelle. This is through the activation of Sac1 for the conversion of PI-4P to PI and its subsequent use as a substrate for sphingolipid synthesis and assembly into lipid rafts. In the absence of Kes1, other Osh proteins allow for the conversion of PI-4P to PI for the synthesis of complex sphingolipids, albeit at a decreased rate, but one that is sufficient for cell viability.

4.3 SEC14 AT THE *TRANS*-GOLGI

Sec14 is a PI- (and PC-) binding protein in *S. cerevisiae*, with its function linked to Kes1 by presumably aiding in maintaining an appropriate lipid balance for vesicular transport in and out of the Golgi (Fairn et al., 2007). Relevant to the regulation of PIPs by Kes1, research has shown that the inactivation of genes that affect PI-4P level (*KES1* or *SAC1*) allow cells with either the $sec14^{ts}$ allele, or with the *SEC14* gene completely inactivated, to grow. Inactivation of genes required for PC synthesis (encoding the enzymes of either the CDP-choline or PE methylation pathways) also allows these cells to grow (Cleves et al., 1991; Fang et al., 1996). Growth is accompanied by the relief of known vesicular-trafficking defects normally associated with the loss of function of Sec14. Since the inactivation of either the CDP-choline enzyme pathway or Kes1 alleviates the essential nature of Sec14, Sec14 can be viewed as opposing the negative regulation of secretion by either the CDP-choline pathway or Kes1. Mutants of Kes1 that are unable to bind PIPs or sterols are non-functional when it comes to causing the death

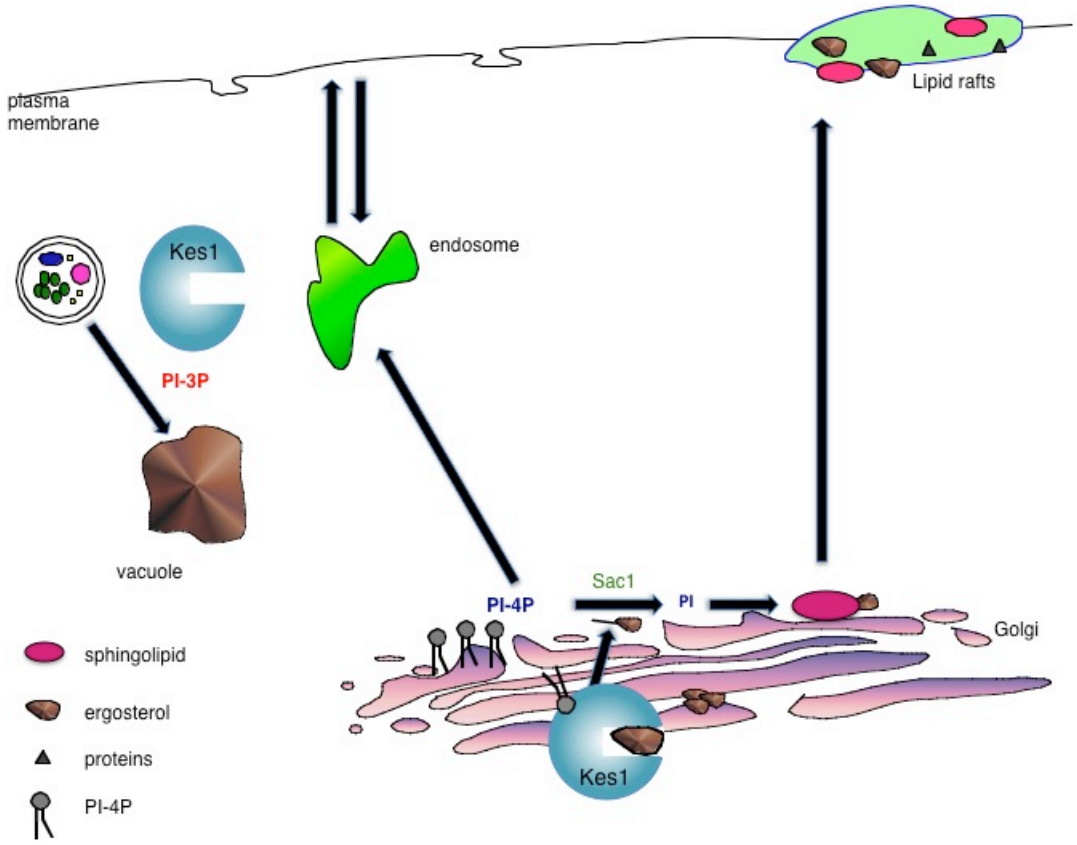


Figure 54 Kes1 functions at the *trans*-Golgi as a sterol sensor to integrate sterol levels with PI and sphingolipid metabolism. Kes1 localizes at the Golgi through its ability to bind PIPs, where it can regulate the transition from phospholipid metabolism to sphingolipid metabolism. This is through modulation of the conversion of PI-4P to PI by Sac1 for subsequent use as substrate for sphingolipid synthesis and assembly of lipid rafts in the Golgi. When Kes1 is not bound to sterols or PIPs, it regulates the formation of PI-3P and the autophagy trafficking pathway to the vacuole.

of *sec14*^{ts} *kes1*Δ cells (or to restoring life to Osh deficient cells). These results indicate that the regulation of Sac1 by Kes1, which requires both sterol and PIP binding, as well as PI metabolism, may be opposed by Sec14 function and thus function along the same metabolic pathway.

That said, although decreasing the Sec14 function results in a decrease in rates of transport by a subset of vesicular trafficking pathways in the cell, and suppressor and bypass mutants correlate with the restoration of these pathways, it is still unclear why cells defective in Sec14 function cease growth and eventually die.

4.3.1 Sec14 Regulates the Delivery of the Lipid Raft Associated Protein Pma1 to the Plasma Membrane

I suggest that Kes1 and Sec14 coordinately regulate the availability of PI at the *trans*-Golgi for the synthesis of complex sphingolipids. In addition, I believe that it may be an inability to coordinate PIP metabolism with sphingolipid synthesis that causes cells with defective Sec14 function to stop growing.

Evidence for this model was gained through my observation that inhibition of Sec14 function impaired delivery of the lipid raft protein, Pma1, to the plasma membrane. This is indicative of non-functional lipid rafts exiting the Golgi and reaching the plasma membrane. Although the lipid rafts are not being delivered to the plasma membrane properly, the profile of complex sphingolipids present in these cells remains to be identified.

Further evidence is gained from my observation that increased expression of the temperature-sensitive mutant protein, Sec14^{Gly266Asp}, allows cell growth even at the non-permissive temperature. However, under these conditions, none of the known vesicular-

trafficking defects associated with *sec14*^{ts} (Curwin et al., 2009) were eliminated (our lab's unpublished data). It was also determined that the known alterations in PC metabolism were not minimized even when Sec14^{Gly266Asp} levels were increased at 37°C. Since we showed that Pma1 association with the plasma membrane is defective in *sec14*^{ts} cells, I assessed the association of Pma1 with the plasma membrane in cells that have increased expression of Sec14^{Gly266Asp}. Lipid raft delivery to the plasma membrane was considerably improved in cells with an increased expression of Sec14^{Gly266Asp}. This was the only known cellular defect that was restored upon an increased expression of Sec14^{Gly266Asp}. Since both Kes1 and Sec14 can regulate lipid raft delivery to the plasma membrane, and both proteins are thought to oppose PI-4P metabolism at the Golgi, these results strongly suggest that both Kes1 and Sec14 regulate the available PI for synthesis of complex sphingolipids.

The data indicates that the function of Sec14 in PI homeostasis appears to be beyond simply maintaining a proper PI-4P pool for Golgi secretion. The PI-4P level is around 50% of wild-type values in *sec14*^{ts} cells and may explain the Golgi-derived-secretion defects seen in these cells. However, it does not explain how cells with an increased expression of the Sec14^{Gly266Asp} mutant protein are now alive, since these cells still do not possess normal PI-4P levels. The apparent positive regulation of Sec14 in the synthesis of complex sphingolipids, and in due course of the synthesis of lipid rafts, may explain the cellular defects associated with cell growth arrest in *sec14*^{ts} cells. In return, an increased expression of the temperature-sensitive mutant, Sec14^{Gly266Asp}, results in more Pma1 at the plasma membrane, implying proper lipid-raft composition and trafficking from the Golgi to the plasma membrane.

A previous study determined that Sfh2 and Sfh5, two Sec14 homologues, are involved in the regulation of the PI-derived PI-4P pool by Stt4 at the plasma membrane (Routt et al., 2005). It was also reported that inactivation of the *SFH2* or *SFH5* gene causes cell death in the otherwise viable *sec14*Δ *kes1*Δ cells (Fairn et al., 2007). These results are indicative of the role of Sfh2 and Sfh5 in providing a PI-4P pool that is critical for cells that already have a decreased PI-4P level at the Golgi. Here, I suggest that Sec14 may be acting in a similar process to regulate PI metabolism at the Golgi and provide a PI pool for synthesis of complex sphingolipids.

4.3.2 Sec14 and Autophagy

Through measurements of *in vivo* PIPs, I determined that PI-3P level increases in *sec14*^{ts} cells, but are restored near wild-type levels in *sec14*^{ts} cells with an increased expression of the Sec14^{Gly266Asp} mutant protein. As previously noted, Vps34, the sole PI-3 kinase in yeast, can assemble into two macromolecular complexes, of which one is required for PAS formation and progression to autophagy. A link between Sec14 and autophagy has never been reported; however, recent studies identified a role for *trans*-Golgi trafficking in synthesis of autophagosomes.

The role for the Golgi in autophagy was proposed when it was observed that the activation of the small G proteins Arf1 and Arf2 by the GEFs Sec7, Gea1 and Gea2 is essential for autophagy (van der Vaart et al., 2010). However, the biogenesis of COPI and clathrin-coated vesicles, also regulated by Arf1 and Arf2 activity, does not play a critical role in autophagy (van der Vaart et al., 2010). Cells lacking Sec7 function can undergo autophagy under starvation conditions, indicating that the autophagy machinery can be assembled at the PAS. Nevertheless, the expansion of the precursor membrane cisterna

from the PAS to autophagosomes is impaired. This suggests that the Golgi complex provides lipids for the biogenesis of autophagy vesicles (van der Vaart et al., 2010).

Other work has linked aspects of vesicular trafficking with autophagy. A third form of the TRAPP complex, containing Trs85 that is a GEF for Ypt1, localizes to the PAS and plays a direct role in the Cvt and autophagy pathways (Lynch-Day et al., 2010; Nazarko et al., 2005). This TRAPPIII complex targets its GEF activity for Ypt1 at the PAS, indicating that certain TRAPP subunits can direct core GEF components to different part of the cell (Lynch-Day et al., 2010). As previously noted, PI-4P at the *trans*-Golgi is crucial for the downstream TRAPP II complex, which Kes1 regulates (Fairn et al., 2007). One possibility is that this PI-4P pool regulated by Kes1 may also be important for the TRAPPIII complex.

Since *sec14*^{ts} cells have trafficking defects at the *trans*-Golgi, they may cease growth because autophagy is induced. The accumulation of PI-3P observed in these cells could be related to a block in autophagosome formation. A block in autophagy could be due to the defects in *trans*-Golgi export in *sec14*^{ts} cells, and therefore a lack of lipid bilayers available for synthesis of these vesicles and/or a lack of TRAPP III GEF activity for Ypt1. Increasing the abundance of Sec14^{Gly266Asp} would allow sufficient *trans*-Golgi export to occur, leading to the formation of autophagosomes and the use of PI-3P.

Sec14 regulation of PI metabolism appears to be, at least in part, linked to Kes1. Maintaining a proper PI-4P and PI pool for synthesis of complex sphingolipids at the *trans*-Golgi remains a critical component for the cell. How both of these proteins can coordinate the regulation of this pool remains to be identified. A role for Sec14 in

autophagy is proposed, which may be linked to defects in *trans*-Golgi export pathways leading to the lack of available lipids for biogenesis of autophagy vesicles.

4.4 A ROLE FOR KES1 IN ENERGY HOMEOSTASIS AND NUTRIENT SENSING

Cells lacking Kes1 activity are also sensitive to rapamycin, a drug that mimics nutrient starvation and inhibits the target of rapamycin (Tor) pathway. Tor kinase has been implicated in controlling cell growth by coordinating metabolism with nutrients and stresses (Wullschleger et al., 2006). Cells treated with rapamycin experience various physiological changes, including G1 cell cycle arrest, protein synthesis inhibition, glycogen accumulation and induction of autophagy. These apparent starvation responses support the notion that Tor is activated by amino-acid derived signals and therefore positively regulates numerous anabolic processes, including translation, transcription, ribosome biogenesis and actin polarization to sites of active cell growth. Conversely, Tor negatively regulates catabolic processes such as protein degradation, mRNA destabilization and autophagy (De Virgilio and Loewith, 2006).

Initially, it was thought that the two Tor protein complexes in yeast, TORC1 and TORC2, were functionally different, with TORC1 rapamycin sensitive and TORC2 rapamycin insensitive (Wullschleger et al., 2006). However, recent reports suggest that each Tor complex may not be as independent as originally thought. Genetic interactions between TORC1 and a network of genes involved in actin polarization and cell wall integrity provides evidence that the TORC1 pathway may regulate processes thought to be regulated by TORC2 (Aronova et al., 2007). This was also observed in cells that have actin organization defects and are rapamycin sensitive (Wang and Jiang, 2003). These findings provide evidence for a functional overlap between TORC1 and TORC2.

Synthesis of *de novo* ceramide synthesis and complex sphingolipids is regulated by the TORC2 complex, which has been shown to control the activity of ceramide synthase (Aronova et al., 2008). Defects associated with decreased ceramide activity include defects in cell wall integrity and in actin polarization. Recent evidence also links *trans*-Golgi network and endosomal trafficking with ceramide homeostasis and Tor signaling in yeast (Mousley et al., 2007). This is consistent with growing evidence that components of the Tor pathway are part of the *trans*-Golgi/endosome trafficking pathway, or is at least compromised in situations where membrane trafficking is defective in these pathways (Aronova et al., 2007; Bertram et al., 2000; Chen and Kaiser, 2003; Puria et al., 2008; Rohde et al., 2008; Wedaman et al., 2003). Elevated ceramide levels, associated with defects in *trans*-Golgi/endosome trafficking, may contribute to the defects in Tor signaling (Mousley et al., 2007).

Defects associated with the lack of Kes1 functions include *trans*-Golgi and endosome trafficking, sphingolipid metabolism and rapamycin/Tor signaling. The data imply that Kes1 plays a major role in the regulation of membrane homeostasis and cell growth. The defects in sphingolipid metabolism may lead to defects in Tor signaling, which clarifies many of the as-yet-unexplained genetic and physical interactions pointing to a role for Kes1 in energy homeostasis.

4.5 FUTURE DIRECTIONS

This work has provided fundamental information on the properties of Kes1 required for its association with membranes, and the role of lipid binding in the regulation of specific trafficking pathways by Kes1. It also clarifies a role for Sec14 in

trans-Golgi trafficking and links Kes1 and Sec14 in the regulation of available PI at the *trans*-Golgi.

Although this study identifies a role for Kes1 in regulation of PI-4P levels at the *trans*-Golgi, the identities of *in vivo* Golgi PI-4P-binding proteins that are affected by Kes1 still need to be determined. As we know that PI-4P is required for cell viability, the identification of genes, and their protein products, that regulate PI metabolism at the *trans*-Golgi is also important. Information about PI-4P-binding proteins is sparse, but the identification of PI-4P-binding proteins affected by Kes1 could lead to the understanding of how Kes1 mediates secretion from the *trans*-Golgi.

From a biochemical standpoint, much needs to be examined in regards to the Kes1-Sac1 interaction. How Kes1 regulates Sac1 phosphatase activity, and in turn coordinates assembly of proteins into lipid rafts at the *trans*-Golgi, remains unclear. I propose Kes1 acts as a sterol sensor to link sterol content with PI metabolism for synthesis of sphingolipids. However, it remains to be determined if sterol binding by Kes1 affects its capacity to activate Sac1 activity and/or association with Sac1.

My thesis identifies a role for Kes1 in PI-4P and sphingolipid homeostasis. By determining the sphingolipid profile in cells lacking all Osh proteins, this may shed light on whether this is a shared function of the Osh protein superfamily. Indeed, the Osh protein family is linked by an as-yet undetermined function, presumably through the ORD domain that is highly conserved among all family members. If Osh proteins have the ability to act as sterol sensors at different cellular organelles, they may be able to coordinate the assembly of highly specialized structures such as lipid rafts and/or signaling complexes that use PIPs as scaffolds or produce second messengers.

Before my work, it had not been reported that Kes1 regulates either PI-3P level or the autophagy/Cvt pathway. It remains to be determined exactly where and how Kes1 is involved in maintaining PI-3P mass. I identified that Kes1 regulation of PI-3P level is independent of PIP and sterol binding, and therefore presumably *via* a separate mechanism than Kes1 regulation of Sac1 activity. How, in turn, Kes1 affects the function of PI-3P in regulation of autophagy also needs to be further defined.

Studies on Sec14 and how it regulates *trans*-Golgi trafficking remain somewhat of a mystery. Here I found that defects in Sec14 function decrease the association of Pma1 with the plasma membrane, likely through decreasing its assembly into lipid rafts at the Golgi. As this process is mediated by sphingolipids, there is presumably a defect in sphingolipid metabolism associated with decreased Sec14 function. However, this aspect of Sec14 function has also not yet been explored. If the defect in sphingolipid metabolism is apparent in cells with decreased Sec14 function, one would need to go on to determine how Kes1 and Sec14 coordinately regulate the available PI/PIP pool at the *trans*-Golgi to subsequently control complex sphongolipid synthesis.

Sec14 is also a PC-binding protein, and it is thought that when Sec14 is bound to either PC or PI it may regulate PC or PI metabolism, respectively. DAG is consumed through synthesis of PC by the CDP-choline pathway, and produced when complex sphingolipids are synthesized. Since inactivation of the CDP-choline pathway restores growth to cells with decreased Sec14 function, it would be interesting to determine whether alterations in cell growth and alleviation of vesicular-trafficking defects are due to alterations in DAG homeostasis. In this way, the function of Sec14 in binding PI and

PC and in regulating PI and PC metabolism may be linked to the regulation of sphingolipids at the *trans*-Golgi.

It also remains to be determined if Sec14 is linked to the regulation of autophagy. Whether this would be a consequence of the accumulation of PI-3P seen in *sec14*^{ts} cells or due to the defects in *trans*-Golgi trafficking would need to be determined. Since it is known that PI-4P is required for TRAPPII GEF activity on Ypt31, it would be interesting to see if PI-4P is also required for TRAPPIII GEF activity toward Ypt1, as Ypt1 is required for autophagy progression.

Previous SGA analyses of Sec14 and Kes1 improved our understanding of how these two proteins coordinately regulate vesicular transport at the *trans*-Golgi through modulation of Golgi PI-4P level. Future use of SGA could provide further insight, when coupled with biochemical advancements, for our understanding of these lipid-binding proteins. For example, Kes1 is thought to bind lipids by three mechanisms: (i) sterol binding in the core of the protein, (ii) sequestration of sterols in the core of the protein by the N-terminal lid, and (iii) binding to PIPs on the surface of the protein. Although biochemical data will be of obvious importance for understanding the function of sterol binding, PIP binding, and the N-terminal lid of Kes1, using these mutants in SGA analysis could also prove beneficial. SGA screens could indicate whether one or more specific lipid-binding properties of Kes1 are required for the participation of Kes1 in its various functions, and how each is regulated.

My study has increased our understanding of Kes1 and the role lipid binding has in its function. It also identified new roles for Kes1 in the cell and linked its function at the *trans*-Golgi to that of another phospholipid-binding protein, Sec14. Nonetheless,

there are many avenues for continuing research on Kes1 that will undoubtedly reveal further aspects of its function.

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