**Members of the OSBP Homologue (Osh) Protein Family in *S. cerevisiae*
Require Specific Lipids to Regulate Polarized Exocytosis**

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Abstract

The Oxysterol Binding Proteins (OSBPs) are an evolutionarily conserved protein family in eukaryotes implicated in many cellular functions. One function, supported by a large body of *in vitro* data is lipid transfer between membranes. In addition, *in vivo* changes in lipid distribution among cellular membranes have been shown to be OSBP dependent, further suggesting a role for OSBPs in lipid homeostasis. Despite these observations, data supporting a role for OSBPs as dedicated lipid transfer proteins *in vivo* is less developed. It is not known whether lipid binding and transfer is the main function of the OSBP family or whether lipid binding and transfer also regulates OSBP activity in other processes. Beyond lipid transfer, in the yeast, *S. cerevisiae*, OSBPs have been found necessary for support of polarized exocytosis, the exocytosis of cellular materials to the site of polarized growth. Because OSBP activity supports polarized exocytosis, I focused on two questions regarding OSBP function: i) is lipid binding by the yeast OSBP Osh4p required for polarized exocytosis and ii) what processes within polarized exocytosis does Osh4p support. In this study, I establish that lipid binding by Osh4p, is required for polarized exocytosis. I also determined that lipid binding by Osh4p is required for exocytic vesicle docking at the plasma membrane and proposed a two-step model of Osh4p function in vesicle docking. These results establish support of polarized exocytosis as the first essential cellular function that has been shown to require lipid binding by a yeast OSBP *in vivo*. These findings describe an essential function for lipid binding by yeast OSBPs which, while not excluding a role for yeast OSBPs as dedicated lipid transfer proteins, do highlight that OSBPs support other essential cellular functions in a lipid binding dependent manner.

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Abbreviations Used

ALPS	ArfGAP1 lipid-packing sensor
BAR	Bin/Amphiphysin/Rvs homology domain
ER	Endoplasmic reticulum
ERMES	ER-mitochondria encounter structure
FFAT	Two phenylalanines in an acidic track
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GOLD	Golgi dynamics
MCS	Membrane contact site
NEM	N-ethymaleimide
NSF	N-ethymaleimide sensitive fusion protein
NVJ	Nuclear vacuolar junction
ORD	Oxysterol binding protein related domain
ORP	Oxysterol binding protein related protein
OSBP	Oxysterol binding protein
PE	Phosphatidylethanolamine
PH	Pleckstrin homology
PI	Phosphatidylinositol
PIP	Phosphatidylinositol Phosphate
PI3,4P₂	Phosphatidylinositol-3,4-Bisphosphate
PI4P	Phosphatidylinositol-4-Phosphate
PI4,5P₂	Phosphatidylinositol-4,5-Bisphosphate

PM	Plasma membrane
PS	Phosphatidylserine
PX	Phox domain
SNARE	Soluble NSF attachment protein receptor
TGN	trans-Golgi network
TM	trans-membrane domain
VAP	Vesicle-associated membrane protein-associated protein

Dedications

This dissertation is dedicated to my wife, Chelsea Smindak, who's been instrumental in my completing this degree. As we say, we got a Ph.D in biology and a MLIS.

I'd also like to dedicate this dissertation to my parents Rich and Toni Smindak, my sister Samantha, my Grandparents Rosario (Sam) and Lucy Castiglia, my Mother and Father-in-law Carol and Doug Rives and Grandparents-in-law Walt and Marie Smith.

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Chapter 1: Introduction

Section 1: Polarized Exocytosis

Cell polarity, the asymmetric organization of a cell, is required for many essential cellular processes. The cellular machinery to establish an axis of polarity is conserved among eukaryotes and dictates the organization of the cell into specialized domains. One of the key regulators of cell polarity is the small GTPase Cdc42p, without which most cells will not polarize (Adams *et al.*, 1990). Polarity establishment in yeast depends on the correct dosage and localization of Cdc42p activity. Hyperactive *cdc42* alleles produce multiple axes of polarity each of which can produce a bud (Caviston *et al.*, 2002). In addition to proteins, membrane lipids such as phosphatidylserine and sterol are required for the development of an axis of polarity (Tiedje, *et al.*, 2007; Fairn *et al.*, 2011). Phosphatidylserine and sterols accumulate at the site of polarity and promote polarity establishment at that site (Bagnat and Simons, 2002; Fairn *et al.*, 2011; Makushok *et al.*, 2016). When properly established, through the combined efforts of polarity promoting proteins and lipids, it is along the axis of polarity that polarized exocytosis, the exocytosis of cellular materials at a defined location, occurs (Macara and Mili, 2008)

In the case of the yeast *S. cerevisiae*, polarized exocytosis is necessary for cell viability (Adamo *et al.*, 2001). The necessity of polarized exocytosis for yeast viability makes analyzing yeast cells for polarized exocytosis defects relatively simple. Simplicity of analysis and ease of genetic manipulation have made yeast a leading model for studying this key cellular process (Longtine *et al.*, 1998).

The importance of polarized exocytosis in other eukaryotes is likewise clear. For instance, in plants, pollen tube formation requires polarized exocytosis. Without polarized exocytosis the pollen tube would not extend and facilitate fertilization of the plant ovum, preventing seeds from being produced (Grebnev *et al.*, 2017).

In addition to plants, polarized exocytosis also plays a major role in multiple aspects of the nervous system. For example neurotransmitter release is dependent on polarized exocytosis to deliver neurotransmitters to the synapse prior to release, an event often altered in neurodegenerative conditions such as Parkinson's disease (Esposito *et al.*,

2011). Furthermore, neurotransmitter receptors have to be trafficked in a polarized manner to the post-synaptic membrane in order to respond to neurotransmitter release from presynaptic cells (Marchand and Cartaud, 2002). Therefore polarized exocytosis is needed for both sides of the synapse to function.

Beyond the nervous system, polarized exocytosis is responsible for the establishment of polarity in epithelial cells (Mostov *et al.*, 2003). For instance, efficient nutrient uptake in the intestine is dependent on the polarized organization of intestinal epithelial cells (Walton *et al.*, 2016). Without apical and basal polarity in intestinal epithelia, nutrient uptake would be inefficient leading to serious malnutrition (Walton *et al.*, 2016). Further, loss of polarity in epithelia often precedes cancer because cell polarity and cell adhesion, which must be maintained to prevent epithelial-mesenchymal transition, are linked (Royer and Lu, 2011). These examples illustrate the importance of polarized exocytosis to many important cellular processes and highlight the dependence of multiple organisms on polarized exocytosis.

Exocytosis in *S. cerevisiae*

In yeast there are at least two distinct post-Golgi exocytic pathways (Fig. 1.1), the non-polarized exocytic pathway marked by the soluble protein cargo invertase (Suc2p), and the polarized exocytic pathway, marked by the soluble protein cargo Bgl2p (Harsay and Bretscher, 1995; Adamo *et al.*, 2001). Non-polarized exocytosis is noteworthy for transporting higher-density vesicles than the polarized pathway and these vesicles are routed from the *trans*-Golgi network to a pre-vacuolar compartment before being delivered isotropically to the plasma membrane (Harsay and Bretscher, 1995; Adamo *et al.*, 2001; Harsay and Schekman, 2002). The higher density vesicle class may be due to an enrichment of highly glycosylated proteins such as invertase and acid phosphatases in the dense vesicle population, as carbohydrates are denser than protein (Gascon *et al.*, 1968; Esmon *et al.*, 1981; Harsay and Bretcher, 1995; Blinnikova *et al.*, 2002).

In contrast, low-density vesicles produced in the polarized exocytic pathway transit directly from the Golgi to the plasma membrane, at either the bud tip or neck. Polarized exocytosis in yeast is directed to the bud tip or bud neck, depending on cell cycle stage, as they are the sites of active exocytosis and polarized cell growth in yeast (Grote *et al.*,

Figure 1.1: Two exocytic pathways in *S. cerevisiae*. 1) Vesicles in the non-polarized pathway, marked by the protein invertase (Inv.), are routed from the TGN to a pre-vacuolar endosome-like compartment before being delivered isotropically to the plasma membrane (Harsay and Bretscher, 1995; Harsay and Schekman, 2002). 2) Vesicles in the polarized exocytic pathway carry cargoes such as the endo-beta-1,3-glucanase (Bgl2p) and are routed directly from the TGN to the site of polarized growth, the bud tip or neck (Vida *et al.*, 1993; Harsay and Bretscher, 1995; Adamo *et al.*, 2001; Harsay and Schekman, 2002).

2000). Polarized exocytosis in *S. cerevisiae* is of particular importance because without effective polarized exocytosis, cell division will fail and the cell will die (Brennwald, 2013).

Polarized Exocytosis

Mechanistically, polarized exocytosis follows the same sequence of events as non-polarized exocytosis with some exceptions, and is detailed below. Polarized exocytosis is differentiated from non-polarized exocytosis because vesicles in the polarized pathway are directed specifically to the bud tip or neck, the sites of polarized cell growth in yeast, rather than randomly directed to the entire plasma membrane (Adamo *et al.*, 2001).

Cdc42p function is necessary for polarized exocytosis and influences polarized exocytosis in two ways. First, Cdc42p organizes actin cables toward the site of polarized growth, along which Myo2p, a type V myosin, moves vesicles of the polarized pathway to the site of polarized growth rather than to other plasma membrane locations (Johnson, 1999; Govindan *et al.*, 1995). It appears that Myo2p is used exclusively by the polarized exocytic pathway; since the absence of functional Myo2p does not effect the non-polarized exocytic pathway (Johnston, 1991; Govindan *et al.*, 1995). Thus, how Myo2p selectively associates with vesicles of the polarized pathway is an important question.

One factor which contributes selectivity is the kinesin-like protein Smy1p, which was identified by its ability to rescue the polarized exocytic defect in cells dependent on the nonfunctional *myo2-66* allele (Lillie and Brown, 1992). In support of Smy1p fulfilling this role, a later study showed that Smy1p enhances the interaction between Myo2p and the post-Golgi vesicle Rab Sec4p, which could serve to enrich Myo2p on vesicles mediating polarized rather than non-polarized exocytosis (Lwin *et al.*, 2016). Cdc42p also influences polarized exocytosis in an actin independent manner by regulating the plasma membrane associated vesicle tethering and docking machinery (Ayscough *et al.*, 1997; Adamo *et al.*, 2001; Zhang *et al.*, 2001). Cdc42p influences the activity of the vesicle tethering complex known as the exocyst (Finger *et al.*, 1998; Wu *et al.*, 2010). Cdc42p interacts with the plasma membrane exocyst subunits, Sec3p and Exo70p, recruiting them to the site of polarized cell growth, and by doing so provides a polarity cue for the exocyst complex (Finger *et al.*, 1998; Wu *et al.*, 2010). By polarizing the

distribution of the actin cytoskeleton and acting as a spatial signal for the exocyst complex localization, Cdc42p mechanistically differentiates polarized from non-polarized exocytosis (Adamo *et al.*, 2001).

Although minor differences in the general mechanisms of exocytosis exist among eukaryotes, key events, of the early exocytic pathway (ER to the *trans*-Golgi network) and late exocytic pathway (*trans*-Golgi network to the plasma membrane) are conserved (Keller and Simons, 1997; Barlow and Miller, 2013). Therefore, exocytosis can be studied in less complex organisms that are easier to genetically manipulate, such as *S. cerevisiae*, while still providing insight into the same processes in more complex organisms such as humans.

In the early exocytic pathway, proteins to be secreted are first cotranslationally translocated into the ER (Hansen *et al.*, 1986). A short peptide sequence on the N-terminus of the nascent secretory protein, still being made by the ribosome, is then bound by the signal recognition particle, which pauses translation (Barlowe and Miller, 2013). The SRP bound ribosome then binds to the translocon, the Sec61 complex in yeast, and the nascent peptide is positioned to enter the translocon, translation resumes and the peptide moves through the translocon into the ER (Barlowe and Miller, 2013).

After entering the ER, secretory proteins must then be exported from the ER and transported to the Golgi. Trans-membrane secretory proteins are recruited into ER to Golgi vesicles by COPII and soluble proteins are thought to enter COPII vesicles both by bulk flow and by binding adaptors which link soluble cargo to COPII binding trans-membrane proteins (Nishimura and Balch, 1997; Belden and Barlowe, 2001; Malkus *et al.*, 2002). COPII also forms the vesicle, which then transits from the ER to the Golgi (Spang and Schekman, 1988; Salama *et al.*, 1997; Barlowe *et al.*, 1994). Once at the Golgi, secretory cargoes stay in the Golgi compartment in which they arrived (Losev *et al.*, 2006; Matsuura-Tokita *et al.*, 2006). Individual cisternae mature by changing the complement of resident proteins they contain at a given time, a process known as cisternal maturation (Losev *et al.*, 2006; Matsuura-Tokita *et al.*, 2006).

After maturation, the compartment in which a secretory protein resides matures into a trans-Golgi network compartment (Barlowe and Miller, 2013). At the trans-Golgi network two main vesicle classes form, dense vesicles which mediate non-polarized

exocytosis and light vesicles which mediate polarized exocytosis (Harsay and Bretscher, 1995; Adamo *et al.*, 2001). The dense vesicles that mediate non-polarized exocytosis are formed by clathrin, whereas the light vesicles are not known to require a coat (Harsay and Schekman, 2002). In the case of light vesicles, segregation of sterols and sphingolipids enriches them with sterols and sphingolipids and is thought to promote vesicle biogenesis at the Golgi (Klemm *et al.*, 2009).

In the late exocytic pathway in *S. cerevisiae*, Golgi-derived vesicles are transported from the mother cell to the site of polarized growth, the bud tip or neck, by the myosin motor Myo2p along actin cables (Fig. 1.2; Govindan *et al.*, 1995). Actin cables are established at the site of polarized growth when the Rho proteins Cdc42p and Rho1p recruit the formin Bni1p (Bi and Park, 2012). Bni1p subsequently nucleates actin filaments, which are bundled into cables, for Myo2p to move along (Bi and Park, 2012). Vesicles are then tethered to the plasma membrane by the exocyst complex with the cooperation of the small GTPase Rab protein Sec4p (Fig. 1.3; Guo *et al.*, 1999). After vesicle tethering to the plasma membrane, the v-SNARE Snc1p or Snc2p assembles with the plasma membrane t-SNAREs Sso1p or Sso2p and Sec9p into a *trans*-SNARE complex (Fig. 1.3; Protopopov *et al.*, 1993; Rossi *et al.*, 1997). The *trans*-SNARE complex scaffolds fusion triggers that promote vesicle fusion with the plasma membrane and the release of vesicle cargo into the extracellular environment (Wickner & Rizo, 2017). The complexes and proteins involved in post-Golgi exocytosis are discussed in more detail below.

Rabs, GTPases related to Ras, are one of the essential protein families required for exocytosis (Guo *et al.*, 1999). Rab proteins, like other small GTPases, are regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) that control the nucleotide bound state of the Rab, thereby dictating Rab activity (Hsu, *et al.*, 2004). Rab proteins act as core regulators and recruiters of downstream exocytic effectors including motor proteins and tethering complexes (He and Guo, 2009). Ypt32p is the first post-Golgi Rab required for exocytosis (Jedd *et al.*, 1997). When the exocytic vesicle exits the Golgi apparatus Ypt32p is present on the vesicle and is required for exit from the *trans*-Golgi network (Jedd *et al.*, 1997). In addition Ypt32p, along with vesicular PI4P, initially recruits the myosin motor Myo2p to the vesicle (Fig 1.2; Ortiz *et*

al., 2002). Later, Ypt32p dissociates from the exocytic vesicle and is replaced with the second post-Golgi Rab, Sec4p, which recruits downstream effectors such as Myo2p and exocyst complex subunits, a process known as vesicle maturation (Mizuno-Yamaskai *et al.*, 2010; Santiago-Tirado *et al.*, 2011). As the vesicle matures, Myo2p continues to interact with PI4P but is now bound to Sec4p (Ortiz *et al.*, 2002; Mizuno-Yamaskai *et al.*, 2010; Santiago-Tirado *et al.*, 2011). Myo2p then moves the vesicle from the mother into the bud along actin cables (Govindan *et al.*, 1995; Santiago-Tirado *et al.*, 2011; Bi and Park, 2012). Interestingly, Myo2p activity is not required for non-polarized exocytosis, however Myo2p is required for polarized exocytosis (Govindan *et al.*, 1995; Donocan and Bretscher, 2015). This finding highlights the unique dependence of polarized exocytosis on actin filaments and type V myosin motors (Govindan *et al.*, 1995; Donocan and Bretscher, 2015). In addition to Myo2p, Sec4p also recruits the exocyst complex subunits to the vesicle. The exocyst complex forms a membrane tether necessary for vesicle docking, however when and where exocyst complex formation occurs is unclear (Fig 1.2 and 1.3; Guo *et al.*, 1999; Boyd *et al.*, 2004; Heider *et al.*, 2016). Interestingly, Rabs do not provide spatial selectivity to exocytosis, however due to the ability of Rabs to recruit tethering complexes they are ultimately required for providing the spatial selectivity that tethering complexes confer to vesicles (Grote and Novick, 1990; He and Guo, 2009).

If vesicle maturation occurs, but the vesicles cannot dock and fuse with the PM, vesicles cluster in the cytoplasm (Salminen and Novick, 1989; Rossi and Brennwald, 2011). Formation of vesicle clusters, as reported in the literature, has been shown to be dependent on Sec4p activity and Sec4p localization to the vesicle, however it is possible that vesicle clustering could occur by other mechanisms (Rossi and Brennwald, 2011). Vesicle clustering is suggestive of an accumulation of docking competent vesicles, which cannot dock and fuse because they do not come into close apposition with the plasma membrane. It should be noted that vesicles which can be transported into the mother, but which do not dock at the PM often return to the mother cell (Alfaro *et al.*, 2011).

Figure 1.2

A

B

Figure 1.2: Post-Golgi vesicle maturation in *S. cerevisiae*. After vesicle formation at the Golgi the vesicle must mature to a transport and docking competent form (Mizuno-Yamasaki *et al.*, 2010). When produced, vesicles are enriched with the lipid PI4P and marked by the Rab protein Ypt32p. Ypt32p must be released from the vesicle membrane and replaced with Sec4p. This is dependent on PI4P removal from the vesicle membrane (Mizuno-Yamasaki *et al.*, 2010). After Sec4p is loaded onto the vesicle membrane it recruits Myo2p, which moves the vesicle along actin cables to the site of exocytosis, and seven exocyst complex subunits (He and Guo, 2009; Santiago-Tirado *et al.*, 2011; Heider *et al.*, 2016). The model of exocyst association with the vesicle presented in this figure is based on a recent study that showed all eight subunits assembled and did not identify a six subunit vesicular exocyst subcomplex as suggested in a previous study (Boyd *et al.*, 2004; Heider *et al.*, 2015). Thus the entire exocyst complex is depicted here to illustrate the complex, despite the lack of clarity in regards to exocyst subunit association with the vesicle.

A

B

C

Figure 1.3: Vesicle tethering, docking, and fusion at the plasma membrane in *S. cerevisiae*. Upon arrival at the plasma membrane, the vesicle undergoes a series of steps prior to vesicle fusion. **A)** First, the exocyst complex assembles at the plasma membrane, tethering the vesicle to the plasma membrane. At this stage the vesicle is considered tethered to the plasma membrane (He and Guo, 2009). **B)** Next, the v-SNARE (Snc1/2p) associates with the plasma membrane t-SNARES (Sso1/2p and Sec9p, forming a *trans*-SNARE complex, which docks the vesicle at the plasma membrane. **C)** Finally, the vesicle fuses with the plasma membrane and releases its contents (Grote *et al.* 2000; Wickner & Rizo, 2017).

Another core feature of the post-Golgi exocytic pathway is the exocyst complex. The exocyst complex is a hetero-octameric protein complex, with six subunits (Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and Exo84p) on the vesicle and two on the plasma membrane (Exo70p and Sec3p) (Figs. 1.2 and 1.3; Finger *et al.*, 1998; Boyd *et al.*, 2004; He and Guo, 2009). This model of exocyst assembly has recently been called into question by data showing that octameric assembled exocyst complex can be isolated, but a six subunit subcomplex cannot (Heider *et al.*, 2016). This might suggest that the six vesicular subunits are selectively transported to sites of exocytosis by the vesicle but do not assemble into a complex until their arrival at the site of exocytosis, presumably upon interaction with Sec3p, which does not rely on vesicle trafficking to localize to the site of exocytosis (Finger *et al.*, 1998; Boyd *et al.*, 2004; Heider *et al.*, 2016). It should be noted that while figure 1.2 illustrates the assembled exocyst complex on the vesicle, it is possible that rather, as noted earlier, the exocyst complex assembles on the vesicle upon its arrival at the site of exocytosis (Boyd *et al.*, 2004; Heider *et al.*, 2016).

The exocyst complex is a key determinant of spatial specificity in post-Golgi vesicle trafficking, and tethers exocytic vesicles to the plasma membrane prior to docking and fusion (Fig. 1.3; Grote *et al.*, 2000; Boyd *et al.*, 2004; He and Guo, 2009). An interesting aspect of the exocyst complex is its dependence on the lipid phosphatidylinositol 4,5 bisphosphate (PI4,5P₂) for assembly at the plasma membrane (He *et al.*, 2007). The two plasma membrane associated subunits, Sec3p and Exo70p, interact directly with plasma membrane PI4,5P₂ at sites of exocytosis on the plasma membrane (He *et al.*, 2007; He and Guo, 2009). Additionally, Sec3p and Exo70p are recruited to the plasma membrane by interacting with the small G-protein Cdc42p (Finger *et al.*, 1998; Wu *et al.*, 2010). Therefore, proper localization of Exo70p and Sec3p to the plasma membrane is dependent on coincidental detection of both PI4,5P₂ and Cdc42p which together provide specificity to both the plasma membrane and the site of polarized growth (Ayscough *et al.*, 1997; Finger *et al.*, 1998; He *et al.*, 2007; He and Guo, 2009; Wu *et al.*, 2010). The importance of lipids in exocytosis, and in particular of phosphatidylinositol phosphates (PIPs), in the exocytic pathway raises the question of whether PIPs influence exocytosis in other ways.

A third protein family that is an effector of polarized exocytosis are the SNAREs (SNAP (Soluble NSF Attachment Protein) Receptor)s (Rothman, 1994). V-SNAREs assemble with t-SNAREs, forming a *trans*-SNARE complex, which then orients the opposing vesicle and plasma membranes into close apposition; upon *trans*-SNARE complex formation the vesicle is considered docked (Grote *et al.*, 2000). The docked state enables membrane fusion by scaffolding fusion triggers at that site (Fig. 1.3; Grote *et al.* 2000; Wickner & Rizo, 2017). These fusion triggers are expected to include proteins such as Sec17p, the yeast α -SNAP, and synaptotagmin (Wickner and Rizo, 2017). These proteins have an apolar loop in their structures that can insert into membranes, which may destabilize the membrane at a given point to promote membrane fusion (Wickner and Rizo, 2017). In this context, the SNARE complex may serve to position proteins such as Sec17p at potential sites of fusion (Wickner and Rizo, 2017).

In systems where exocytosis is regulated, rather than constitutive, an additional step, vesicle priming, occurs after vesicle docking (Südhof, 2013). Priming occurs when docked vesicles are made ready to fuse in response to an influx of calcium ions (Südhof, 2013). In the case of neurons, an electrical impulse evokes Ca^{2+} entry and binding to synaptotagmin. Ca^{2+} -bound synaptotagmin then promotes vesicle fusion (Südhof, 2013). Thus, synaptotagmin primes the vesicle for fusion directly in response to stimulation (Südhof, 2013).

In yeast, the post-Golgi v-SNAREs are Snc1p and Snc2p and the t-SNAREs on the plasma membrane are Sec9p and Sso1p and Sso2p (Katz *et al.*, 1998). SNAREs, despite being required for membrane fusion, do not convey spatial specificity to exocytosis, an effect conferred by the exocyst complex (He and Guo, 2009). Interestingly, mismatched *trans*-SNARE complexes will form *in vitro* (Tsui and Banfield, 2000). In addition, a number of mismatched SNARE complexes will support various fusion processes, for instance the SNARE Bet1p supports both ER to Golgi and Golgi to ER vesicle trafficking, demonstrating that one SNARE is required for two directions of membrane traffic (Spang and Schekman, 1998). Collectively, this suggests that factors other the SNAREs, such as membrane tethers like the exocyst, provide spatial selectivity to vesicle trafficking.

Lipids Involved in Polarized Exocytosis

In addition to the many known protein components involved in exocytosis, lipid content adds a second level of regulation that appears selective for specific organelles, in particular, organelles possess a unique PIP signature (Strahl and Thorner, 2007; van Meer *et al.*, 2008). Phosphatidylinositol (PI) is maintained in select membranes by selective lipid transfer and lipid kinase and phosphatase activity (Strahl and Thorner, 2007). It is proposed that the phosphatidylinositol phosphates serve a variety of functions. For instance, in the context of the Golgi, PI4P is proposed to facilitate sterol transfer from the ER to the Golgi (Mesmin *et al.*, 2013). In addition, PI3P is found on yeast vacuoles and contributes to HOPS complex association with vacuoles thereby facilitating vacuolar tethering (Gillooly *et al.*, 2000; Stroupe *et al.*, 2006).

There also are important regulatory events that control the conversion of PIPs from one species to another as a membrane transitions from being part of one organelle to being part of another (Posor *et al.*, 2013). For instance, the plasma membrane is marked by PI4,5P₂, which is predominantly made from PI4P synthesized at the PM (Strahl and Thorner, 2005). However, clathrin coated vesicles formed at the PM require a different lipid, PI3,4P₂, to recruit the PX-BAR containing protein SNX9, which is required to complete endocytic vesicle formation (Poser *et al.*, 2013). Thus, although the plasma membrane is predominantly identified by its PI4,5P₂ content, specialized regions marked by PI3,4P₂ exist to facilitate endocytosis, which acts to selectively recruit cellular factors that separate PI3,4P₂ marked membrane from the greater PI4,5P₂ marked plasma membrane (Posor *et al.*, 2013). This specialized PM region, which is incorporated into the endocytic vesicle, also serves as an intermediate between the PM and endosome from the perspective of PIP content, as endosomes are marked by PI3P, and therefore endocytic vesicle membrane can be converted from PI3,4P₂ to PI3P by removing the 4-phosphate (Gillooly *et al.*, 2000).

In the context of post-Golgi vesicles, the importance of PIPs for the recruitment of exocytic effector proteins to membranes such as the vesicle and Golgi is increasingly recognized. For instance, PI4,5P₂ recruits the plasma membrane exocyst subunits Sec3p and Exo70p to the site of polarized exocytosis and vesicular PI4P content regulates Rab association with exocytic vesicles (He and Guo, 2009; Mizuno-Yamasaki *et al.*, 2010).

Interestingly, while PI4,5P₂ is required for polarized cell growth, it is not restricted to the site of polarized growth but is present throughout the plasma membrane, suggesting that other factors, such as Cdc42p are at play (Finger *et al.*, 1998; Homma *et al.*, 1998; Wu *et al.*, 2010). Thus, the vesicle likely requires coincidental detection of multiple factors in order to dock at the correct site. However, this does not exclude the possibility that PI4,5P is accumulating at the site of polarized cell growth, but under detectable limits or very transiently as suggested in a later study (Yakir-Tamang and Gerst, 2009).

Whether PIPs mediate vesicle docking in ways other than effector recruitment is less clear. Up to this point, beyond selective actin based trafficking of vesicles to the site of secretion, only the exocyst complex has been shown to lend spatial specificity to post-Golgi vesicle docking, however the membrane specificity conferred by the exocyst complex is mediated by the membrane lipid PI4,5P and increasing evidence suggests that the concentration of specific lipid species at the site of polarity is required for polarity establishment (Govindan *et al.*, 1995; He and Guo, 2009; Fairn *et al.*, 2011; Ling *et al.*, 2014; Makushok *et al.*, 2016). For example, in addition to PI4,5P₂, in yeast, phosphatidylserine polarizes to the bud tip and neck and this lipid distribution is required for activated Cdc42p polarity to the bud tip (Fairn *et al.*, 2011). Further, in fission yeast sterol polarity to the poles is required for polarity establishment, independent of Cdc42p activity, and without sterol polarity the cell does not polarize and cell growth occurs at a random location on the fission yeast plasma membrane (Makushok *et al.*, 2016). These data suggest that membrane lipids are in fact key to spatial specificity in membrane-membrane interactions and a closer look at the PIPs, and the proteins that are able to bind to them such as the oxysterol binding proteins is warranted.

Membrane-Membrane Contacts

Membrane contacts sites are areas of membrane apposition between organelles of 30 nm or less (Lediedzinska *et al.*, 2009). There are a variety of membrane-membrane contacts all of which require different membrane associated tethers to hold the membranes in apposition (Lediedzinska *et al.*, 2009). The mechanisms of tethering and the molecular events that occur at membrane contact sites are detailed below.

One membrane contact site of particular note is the contact between the ER and the PM. ER-PM contacts are sites of non-vesicular lipid transfer, at which lipids such as sterols and phosphatidylserine are enriched in the plasma membrane upon production in the ER (Toulmay and Prinz, 2012; Chung *et al.*, 2015). In yeast, 30 to 45 percent of the plasma membrane is associated with ER, known as cortical ER, and the connection between the ER and the PM is formed by the VAP proteins Scs2p, Scs22p, and Ist2p, and the extended synaptotagmins Tcb1p, Tcb2p, and Tcb3p (Stefan *et al.*, 2013). All six proteins are ER localized TM proteins which directly or indirectly bind to PM lipids, with Ist1p binding PM PIPs with its polybasic domain and the extended synaptotagmins binding PM lipids as well (Stefan *et al.*, 2013). Interestingly, the VAP proteins Scs2p and Scs22p do not directly interact with PM lipids, but rather interact with Osh proteins, such as Osh3p, which themselves bind to plasma membrane PI4P (Stefan *et al.*, 2013).

Another membrane contact site, this one particularly important for the transfer of sterol, is the ER-Golgi contact site (Toulmay and Prinz, 2012). Sterol produced at the ER is transported to the Golgi at these sites in order to enrich the Golgi membranes with sterol (Mesmin *et al.*, 2013). It is likely that OSBP is not only the lipid transfer protein at this interface, but also the tether that holds the ER and Golgi together (Mesmin *et al.*, 2013). In this case, OSBP would bind to Golgi PI4P with its PH domain, and to the ER using its FFAT domain to bind ER VAP proteins (Mesmin *et al.*, 2013). Given the similar domain architecture of Osh1p, Osh2p, and Osh3p to OSBP it is conceivable that these yeast ORPs could carry out a similar tethering function at the interface of the ER and Golgi (Fig. 1.4).

ER-mitochondria contact sites are another membrane contact site with important lipid transfer roles (Toulmay and Prinz, 2012). These contacts are tethered together in either of two ways. First ER and mitochondria localized Mitofusion proteins may dimerize and hold the ER and mitochondria in apposition (Brito and Scorrano, 2008). Mitofusion proteins are TM proteins, localized to both the ER and mitochondria, which oligomerize, based on heptad repeat regions, into an antiparallel coiled coil (Koshiba *et al.*, 2004). Secondly the ERMES complex, composed of mitochondria localized Mdm10p and Mdm14p, the ER localized protein Mmm1p, and the cytosolic protein Mdm12p tether the ER to the mitochondria as well (Toulmay and Prinz, 2012). ER-

mitochondrial contact sites are important sites of lipid transfer, because PS is converted to PE at the mitochondria by a serine decarboxylase and this PE is then sent back to the ER to be translocated elsewhere (Vance, 2003). This is the major source of cellular PE because, even though PE can be produced from PS at the vacuolar membrane, the loss of the vacuolar serine decarboxylase does not lead to a significant loss of cellular PE (Trotter and Voelker, 1995).

A fourth membrane contact site is the nuclear-vacuolar junction, which is formed by the interaction of vacuolar Vac8p and the nuclear outer membrane localized Nvj1p (Pan *et al.*, 2000). Nuclear-vacuolar junctions are known to be sites of piecemeal microautophagy of the nuclear membrane, in response to cell growth and starvation in order to provide the cell with macromolecules needed for survival (Roberts *et al.*, 2003). Interestingly, while not required for nuclear-vacuolar junction formation, Osh1p does localize to nuclear-vacuolar junctions and is required to support piecemeal microautophagy of the nuclear membrane (Evam and Goldfarb, 2004).

Section 2: What Essential Functions are Provided by the Oxysterol Binding Proteins?

The OSBPs were first identified in 1985 as cytosolic proteins that bound oxysterols in a bulk affinity assay (Taylor and Kandutsch, 1985). The discovery of the OSBP family led to the initial hypothesis that OSBPs regulated cellular oxysterol levels (Taylor and Kandutsch, 1985). The OSBPs bind to both cholesterol and hydroxysterols such as 25-hydroxycholesterol and are known to bind 25-hydroxycholesterol with higher affinity than cholesterol (10 and 173 nM respectively) (Dawson *et al.*, 1989; Wang *et al.*, 2008). However, it is more likely that cholesterol is the physiological ligand for OSBP, rather than the 25-hydroxysterol, because cholesterol is present at much higher concentrations (3 to 6 orders of magnitude greater) than 25-hydroxycholesterol (Björkhem, 2002). The OSBP family also associates with other lipids, possessing certain key residues that mediate lipid binding, and have been implicated in various cellular functions (Fig 1.5).

Figure 1.4

Figure 1.4: Domain architecture of human OSBP, ORP5, and the yeast Osh family.

In addition to the ORD (Oxysterol binding protein Related Domain), which all OSBPs possess, some OSBP family members, including the yeast Osh proteins have other protein domains (Lehto and Olkonnen, 2003). For instance, human OSBP, and Osh1p, Osh2p and Osh3p all have a PH domain for binding membrane PIPs. In addition, Osh1p and Osh3p have ankyrin repeats and GOLD domain which mediate protein-protein interaction (Anantharaman and Aravind, 2002; Nagae *et al.*, 2016). Further some ORPs such as ORP5 are integral membrane proteins (Chung *et al.*, 2015).

Figure 1.5: Crystal structures of Osh4p highlighting residues changed in lipid binding deficient mutants used in this study. A) Crystal structure of wild-type Osh4p bound to ergosterol, residue G183, which is the mutated residue in *osh4-1^{ts}*, is highlighted. B) Crystal structure of Osh4p bound to ergosterol with residue Y97 highlighted. C) Crystal structure of Osh4p bound to PI4P with residues H143 and H144 highlighted. Crystal structures of Osh4p bound to ergosterol in A and B are adapted from Im *et al.*, (2005) and crystal structure of Osh4p bound to PI4P is adapted from de St. Jean *et al.* (2011). In all cases, the left column shows the entire protein bound to its lipid ligand with the residues in question highlighted, the right panel in all cases shows only the lipid ligand and the side chains of the residues in question.

Oxysterol Binding Protein Structure

OSBP family proteins are comprised of a β -barrel structure that is closed at one end and open at the other end, with a “lid” that is closed when the protein is lipid bound and open when lipid free (Im *et al.*, 2005). Lipid binding occurs within the β -barrel (Im *et al.*, 2005). Although OSBPs can only bind one lipid molecule at a given time, most OSBPs can bind more than one lipid species and each lipid species has a mutually exclusive binding site within the β -barrel (Fig 1.5; Im *et al.*, 2005; de St. Jean *et al.*, 2011; Chung *et al.*, 2015).

In addition to the central β -barrel and lid, some of the OSBPs have additional protein domains extending from the lid, usually at one terminus of the protein. A subset of the OSBP family, known as the “long” OSBPs have a pleckstrin homology (PH) domain, phenylalanine in an acidic track (FFAT) sequences, and other domains featured on an extended N-terminus, situated beyond the peptide sequence encompassing the lid, which is discussed below (Fig 1.4; Beh *et al.*, 2012). Human OSBP, a “long” OSBP, has been shown to dimerize and this is anticipated to be required for OSBP function (Mesmin *et al.*, 2013). It is possible that the “long” yeast OSBPs may also dimerize however this has not been demonstrated.

The *S. cerevisiae* OSBP family genes are known as the Oxysterol Binding Protein Homologues, or *OSH* genes (Beh, 2012). There are seven *OSH* genes in yeast, encoding Osh1p, Osh2p, and Osh3p, the “long” Osh proteins and Osh4, Osh5p, Osh6p, and Osh7p the “short” Osh proteins. The family is further divided into four sub-families, consisting of Osh1/2p, Osh3p, Osh4/5p, and Osh6/7p, based on amino acid homology (Olkonnen and Levine, 2004; Beh *et al.*, 2012). The “long” Oshs also possess additional protein domains in their extended N-terminal domains, and are also discussed below. Osh4p, also known as Kes1p, will be referred to as Osh4p in this text. The name Kes1p comes from the original screen in which this protein was identified and stands for Kre11-1 suppressor (Jiang *et al.*, 1994).

Many ORPs, for example OSBP and Osh1p, possess a pleckstrin homology (PH) domain. PH domains bind to select membrane PIPs allowing targeting of PH domain containing proteins to membranes that contain a given PIP (Lemmon 2017). Interestingly, PH domain-PIP interactions tend to be promiscuous with a given PH domain binding

multiple PIP species (Wu *et al.*, 2004). For instance, Osh1p strongly binds PI4P, PI3,4P₂, and PI4,5P₂, suggesting that PH domain PIP binding may often be part of a coincidence detection mechanism (We *et al.*, 2004; Lemmon, 2017).

Another domain shared by various ORPs is the FFAT domain, which binds to VAP proteins in the ER, in that way FFAT domains confer ER localization to a given protein (Kaiser *et al.*, 2005). Human OSBP, and Osh1p, Osh2p, and Osh3p all possess FFAT domains, among other ORPs (Schultz *et al.*, 2009; Mesmin *et al.*, 2013). The combination of the FFAT domain with a PH domain in the same protein has been shown to induce ER to Golgi tethering and therefore the combination of the two domains is essential to OSBP-mediated sterol transfer at ER- Golgi contact sites (Mesmin *et al.*, 2013).

Osh1p, Osh2p, as well as other ORPs contain ankyrin repeats in their N-terminal domains (Ngo *et al.*, 2010; Beh *et al.*, 2012). Ankyrin repeats are protein-protein interaction motifs and have been shown to facilitate protein-protein interactions between ORPs that possess them and other proteins (Mosavi *et al.*, 2004). For instance Osh1p binds to Nvj1p at the NVJ through its ankyrin repeats and mammalian ORP1L binds to Rab7 using its ankyrin repeats (Levine and Munro, 2001).

Osh3p, uniquely, has a GOLD domain (Beh *et al.*, 2012). GOLD domain are predicted to promote protein-protein interactions as well, and further, proteins that contain them have been shown to dimerize (Anantharaman and Aravind, 2002; Nagae *et al.*, 2016). Interestingly, Osh3p does not require its GOLD domain for function and what the Osh3p GOLD domain is required for is unclear (Tong *et al.*, 2013).

Finally, some ORPs such as ORP5, have a transmembrane domain (Ngo *et al.*, 2010). ORP5 is an ER-localized integral membrane protein, held in place on that membrane by its TM domain that is C-terminal of the ORD (Chung *et al.*, 2015). This coupled with the PH domain within its N-terminus allow ORP5 to tether the ER to the PM and transfer PS from the ER to the PM (Chung *et al.*, 2015).

Finally all ORPs possess an N-terminal lid that can close over the open end of the ORD (Im *et al.*, 2005). The lid domain of Osh4p, an ALPS domain, promotes movement of lipid bound Osh4p through the cytoplasm, presumably by shielding the hydrophobic lipid from the aqueous environment of the cytoplasm (Im *et al.*, 2005). ALPS domains

are amphipathic alpha helices that associate with highly curved membranes due to the lipid packing defects associated with such membranes (Bigay *et al.*, 2005; Drin *et al.*, 2007). Notably an ALPS domain often confers Golgi localization to proteins that contain one (Parnis *et al.*, 2006). Not all Osh proteins (or OSBPs) have an ALPS domain, however a lid of some form is present on all Osh proteins (Im *et al.*, 2005).

In addition, Osh4p has a trio of positively charged amino acid residues, R236, K243, and K243 that contribute to TGN and endosomal localization of Osh4p (Li *et al.*, 2002). This cluster of residues was proposed to be a PH domain, however upon determination of the crystal structure of Osh4p it was found that this domain is not a PH domain, however the positively charged nature of this cluster does confer PH domain-like capabilities to Osh4p (Li *et al.*, 2002; Im *et al.*, 2005).

OSBPs in Human Disease

OSBPs have been implicated in human diseases, one of which is viral infection. OSBP activity has been shown to be required for replication of poliovirus and hepatitis C virus, among others (Amako *et al.*, 2009; Arita, 2014). For instance, poliovirus requires OSBP activity for maturation of the viral membrane to its terminal sterol-enriched state (Arita, 2014). Hepatitis C virus also requires OSBP activity for viral membrane maturation, but OSBP is expected to be required for secretion of the hepatitis C virions as well (Amako *et al.*, 2009). Both poliovirus and hepatitis C induce membrane contact site formation to allow OSBP to enrich their membrane with sterol, however poliovirus forms this contact with the Golgi, and hepatitis C virus particles form a contact with the ER (Nagy *et al.*, 2016).

In addition to viral infection OSBP activity has been shown to contribute to cancer pathogenesis. For instance, ORP3, an OSBP homolog, has been shown to influence cell adhesion through an interaction with the small GTPase R-Ras3 (Lehto *et al.*, 2008). ORP3 promotes cell spreading and migration by decreasing cell adhesions in cells with oncogenic R-Ras3, suggesting a role for ORP3 in regulating the epidermal to mesenchymal transition, a key event leading to metastasis (Lehto *et al.*, 2008). Chemical inhibitors of OSBP, such as OSW-1, Schweinfurthin A, and cephalostatin 1, have been shown to specifically target glioblastoma and leukemia cells, rather than non-cancerous

cells, by competitively inhibiting OSBP lipid ligand binding (Burgett *et al.*, 2011). Schweinfurthin A in particular, has been shown to interfere with Rho protein signaling leading to F-actin depolymerization (Turbyville *et al.*, 2010). This is consistent with studies in yeast showing that Osh4p interacts with Rho1p and the loss of polarized actin filament distribution observed in cells lacking Osh protein activity (Kozminski *et al.*, 2006; Alfaro *et al.*, 2011). It is unknown at this time whether these compounds inhibit yeast ORPs. In addition, OSW-1 and cephalostatin 1 have been shown to reduce cellular OSBP levels, suggesting perhaps that certain cancer cell lines have elevated OSBP levels and that elevated OSBP levels contribute to tumorigenesis (Burgett *et al.*, 2011). Collectively, these data suggest that OSBP plays a role in cancer progression and that OSBP could be a cancer drug target.

Lipid Binding and Transfer Activity of the OSBPs

Despite the existence of seven Osh proteins in yeast, any single functional *OSH* gene is sufficient for viability, raising the question of what is the essential function shared by the seven Osh proteins (Beh *et al.*, 2001). The Osh proteins all bind PI4P, but vary in the other lipid they bind, with Osh1p, Osh2p, Osh4p/Kes1p, and Osh5p binding sterol, Osh6p and Osh7p binding phosphatidylserine, and with Osh3p only known to bind PI4P, however that does not preclude Osh3p binding other lipids (Im *et al.*, 2005; Maeda *et al.*, 2013; Tong *et al.*, 2013).

Despite the ability of ORPs to bind two different lipids, they only bind one lipid at a time, and each lipid has an exclusive binding site within the lipid-binding pocket (de St. Jean *et al.*, 2011). This implies that at any given moment, ORPs should be carrying out either a sterol or PS specific function, depending on binding capability, or a PI4P specific function (Im *et al.*, 2005; Maeda *et al.*, 2013; Tong *et al.*, 2013). For instance, in the context of vesicle maturation, sterol bound Osh4p should be restricted to targeting PI4P enriched membranes to facilitate PI4P removal and sterol enrichment of that membrane (Ling *et al.*, 2014). In contrast PI4P-bound Osh4p should be specifically primed to target a sterol-enriched membrane, although PI4P bound Osh4p does interact with sterol poor membranes if PI4P concentration is high in that membrane, to perform a function there

such as sterol extraction for the purpose of non-vesicular sterol transfer (de St. Jean *et al.*, 2011; von Filseck *et al.*, 2015).

Based on their ability to bind sterol, it was hypothesized that the Osh proteins were dedicated sterol transfer proteins, which maintain cellular sterol distribution (Beh *et al.*, 2001; Beh and Rine, 2004; Im *et al.*, 2005). This hypothesis was further bolstered by studies showing that both Osh4p and mammalian OSBP could transfer PI4P and sterol between membranes *in vitro*, leading to an extension of the existing sterol transfer hypothesis, that suggested that transfer of PI4P from the *trans*-Golgi to the ER drove the transfer of sterols from the ER to other membranes such as the *trans*-Golgi (de St. Jean *et al.*, 2011; Mesmin *et al.*, 2013; von Filseck *et al.*, 2015). This idea is supported by the observation that *in vitro*, Osh4p can transfer sterol from areas of low concentration to areas of high concentration, so long as the area of low sterol concentration is also low in PI4P, and the area of high concentration is enriched in PI4P, a condition which would mimic the lipid gradient between the Golgi and the ER (von Filseck *et al.*, 2015). It should be noted that Osh4p transfers lipids between membranes at lower rates in this condition than when it can move both sterol and PI4P lipids from areas of high concentration to areas of low concentration (von Filseck *et al.*, 2015). The authors found that, given a constant gradient of PI4P, when sterol concentration in the PI4P containing liposomes was 0% (acceptor) and the non-PI4P containing liposomes contained 10% sterol (donor), the rate of lipid transfer was approximately 20 molecules per minute per Osh4p molecule (von Filseck *et al.*, 2015). If the PI4P gradient remained the same, but the PI4P containing liposome also contained 10% sterol (donor), and the PI4P free liposome contained 20% sterol (acceptor), Osh4p was able to transfer sterol and PI4P between the two sets of liposomes at a rate of approximately 10 molecules per minute per Osh4p (von Filseck *et al.*, 2015). The transfer of sterol from areas of low concentration to areas of high concentration is PI4P gradient dependent (von Filseck *et al.*, 2015). If the PI4P phosphatase Sac1p is available to metabolize PI4P to PI on the acceptor membrane, and thus keep PI4P levels on the acceptor membrane low, sterol transfer occurs more efficiently (von Filseck *et al.*, 2015). In addition, the distribution of cellular PI4P changes when OSBP is overexpressed in HeLa cells, providing *in vivo* support to these *in vitro* observations (Mesmin *et al.*, 2013). Despite these observations, several

issues with the hypothesis that dedicated lipid transfer is an essential function of the Osh protein family have not been addressed.

It should be noted that the question is not whether ORPs can exchange lipids between membranes. It is clear that this protein family possesses that capacity (de St. Jean *et al.*, 2011; von Filseck *et al.*, 2015). The question is whether dedicated lipid transfer, to maintain cellular lipid asymmetry, is the essential function of ORPs or if lipid exchange by ORPs regulates other processes.

The first issue is that lipid transfer by an OSBP has never been observed *in vivo*, as it has been *in vitro* (de St. Jean *et al.*, 2011; von Filseck *et al.*, 2015). Due to resolution and sensitivity issues of observing lipids and single protein molecules *in vivo* this is by nature a difficult event to observe *in vivo*. However, changes in cellular PI4P, dehydroxyergosterol, and phosphatidylserine distribution occur when exogenous OSBP or ORP5 is expressed in cells expressing endogenous OSBP or ORP5 or when ORP5 and ORP8 are depleted (Chung *et al.*, 2015). These observations are consistent with a role for ORPs as dedicated lipid transfer proteins (Mesmin *et al.*, 2013; Chung *et al.*, 2015). Nevertheless, changes in cellular lipid distribution could be consistent with roles for ORPs in other processes. For instance, OSBPs could influence lipid distribution by promoting membrane contact site formation, sites at which lipid transfer is known to occur (Prinz, 2014).

Second, sterol binding and transfer by the Osh proteins cannot be essential. Any single Osh protein is sufficient for cell viability, however all Osh proteins do not bind sterol (Beh *et al.*, 2001; Maeda *et al.*, 2012 Tong *et al.*, 2013; von Filseck *et al.*, 2015). Therefore dedicated sterol transfer by Osh proteins cannot be an essential function. In fact, other data, showing normal PM sterol levels in the absence of Osh protein function, suggest that Osh proteins are not required for sterol transfer between the ER and plasma membrane, further suggesting that dedicated sterol transfer by Osh proteins is not essential (Georgiev *et al.*, 2011). The same logic applies to PS transfer; Osh6p and Osh7p bind and transfer PS, but not sterol, and this Osh protein subfamily is not specifically required for viability, therefore PS transfer by an Osh protein also cannot be an essential function.

Finally it is unlikely that PI or PI4P transfer is the main Osh protein function for the following reasons. First, *S. cerevisiae* have other phosphatidylinositol transfer proteins such as Sec14p. Sec14p is a PI/phosphatidylcholine transfer protein which can transfer PI among organelles, this PI can then be converted to a given PIP based on lipid kinases present on that organelle (Bankaitis *et al.*, 1990; Bankaitis *et al.*, 2005; Strahl and Thorner, 2007). Therefore, Osh proteins are not likely required for PI transfer among membranes or production of PI4P in the Golgi or plasma membrane (Audhya *et al.*, 2000). This idea is supported by the observation that the absence of functional Osh proteins causes an accumulation of PI4P on the plasma membrane (Stefan *et al.*, 2011). PI4P accumulation on the plasma membrane could only occur if PI or PI4P was continuously transferred to the plasma membrane and then converted to PI4P, in the case of PI, and/or not metabolized from PI4P to PI by Sac1p (Stefan *et al.*, 2011). This same study found that Osh3p and Osh2p have a role in this process, supporting ER-PM MCSs so that the ER-associated PI4P phosphatase Sac1p can metabolize PM PI4P to PI (Stefan *et al.*, 2011). These data suggest that Osh proteins have a role in the distribution or turnover of PI4P, but though regulating the metabolism of PI4P by Sac1p rather than transferring PI4P among membranes (Stefan *et al.*, 2011).

These observations suggest that lipid binding by the Osh proteins regulate a non-lipid transfer based function, in addition to their proposed role in lipid transfer, and that PI4P binding is central to the shared essential function of the Osh proteins. Because both sterol and PS are enriched in high concentrations at sites of polarized exocytosis in yeast, the Osh family may play a general role in regulating polarized cell growth (Beh and Rine, 2004; Kozminski *et al.*, 2006; Fairn *et al.*, 2011; Makushok *et al.*, 2016). Despite the shortcomings of the lipid transfer hypothesis, Osh proteins can transfer lipids between membranes, at least *in vitro* (de St. Jean *et al.*, 2012), but the biological significance of this activity is unclear.

One possibility is that selective transfer of sterols from the ER to the Golgi by Osh proteins is required to enrich sterol in the Golgi, relative to the plasma membrane (de St. Jean *et al.*, 2012; Mesmin *et al.*, 2013). The enrichment of sterol in the Golgi is then required for enriching sterol in Golgi-derived vesicles, which plays a role in sorting exocytic cargo proteins into their respective vesicle classes (Klemm *et al.*, 2009). The

enrichment of Golgi sterol into Golgi-derived vesicles would also enrich sterol in the plasma membrane (Klemm *et al.*, 2009). Alternatively, the yeast StART proteins could contribute to sterol transfer in yeast and have been shown to contribute to plasma membrane to ER sterol transfer (Gatta *et al.*, 2015). However, this protein family has not been implicated in ER to Golgi sterol transfer, therefore while the StART family could account for enrichment of sterol in the plasma membrane, it is unlikely that StART proteins contribute to enrichment of sterol in the Golgi (Gatta *et al.*, 2015).

Other Functions of the Oxysterol Binding Protein Family

To consider OSBP family protein function as limited to lipid transfer presents a limited view of the effects of OSBP function *in vivo*. In fact many non-lipid transfer based activities have been assigned to OSBPs.

A 2005 study showed that OSBP regulate the assembly of the tyrosine phosphatase PTPBPS and serine/threonine phosphatase PP2A and that sterol binding by OSBP supported assembly of this complex (Wang *et al.*, 2005). Removal of cholesterol causes the phosphatase complex to dissociate, thus blocking ERK1/2 dephosphorylation (Wang *et al.*, 2005). This complex plays an essential role in regulating EGF signaling by dephosphorylating the MAPK ERK1/2 (Wang *et al.*, 2005). Without OSBP-based stabilization of the phosphatase complex, overactive ERK1/2 signaling could promote cellular over-proliferation leading ultimately to cancer formation (Wang *et al.*, 2005). This finding established sterol binding by OSBP as a key factor in regulating a signaling complex *in vivo* (Wang *et al.*, 2005).

Lipid binding can also regulate the localization of OSBPs within cells (Ridgeway *et al.*, 1992). However, it should be noted that in this study, cells were challenged with 1 $\mu\text{g/mL}$ 25-hydroxycholesterol, a ligand not usually present in high concentrations, so the dramatic translocation from the cytoplasm to the Golgi apparatus observed may not occur at the low concentrations found *in vivo* (Ridgeway, 1992). It is possible that the translocation to the Golgi of OSBP upon treatment with 25-hydroxysterol is due to OSBP attempting to exchange its bound 25-hydroxysterol for Golgi PI4P. However, due to the greater affinity of OSBP for 25-hydroxysterol relative to cholesterol, exchange cannot occur at normal rates and OSBP becomes enriched on the Golgi membrane.

Nevertheless, association of OSBPs with different organelles may be directed by lipid binding.

Another signaling role for OSBPs came from a study in yeast that showed *OSH6* increased replicative lifespan by promoting vacuolar fusion (Gebre *et al.*, 2012). Vacuolar fusion had previously been shown to increase replicative lifespan in yeast, and while the mechanisms underlying vacuolar fusion are well studied, the cellular factors, protein and lipid, regulating fusion is less clear (Tang *et al.*, 2008; Wickner, 2010). Finding that *OSH6* promotes vacuolar fusion suggested that Osh6p binds to vacuolar membrane lipids to regulate the fusion processes (Gebre *et al.*, 2012). Osh6p, being a PS binding protein, may be responsible for transporting PS to the endosome where a resident phosphatidylserine decarboxylase, Psd2p, may convert this PS to PE (Trotter and Voelker, 1995; Maeda *et al.*, 2013). PE is known to support vacuolar fusion, as absence of PE inhibits homotypic vacuolar fusion *in vitro* and loss of proper PE asymmetry leads to vacuolar fragmentation *in vivo* (Mima and Wickner, 2009; Wu *et al.*, 2016). Alternatively, Osh6p may promote loading of the vacuolar Rab Ypt7p onto vacuoles in a similar manner to how Osh4p may promote Sec4p loading on to exocytic vesicles, as discussed below (Wickner *et al.*, 2010; Ling *et al.*, 2010). However, Osh proteins have not been shown to support such a mechanism in regards to vacuoles and further analysis would be required to validate this hypothesis,

Among the non-lipid transfer functions of the OSBPs, Osh proteins are potent regulators of polarized cell growth (Kozminski *et al.*, 2006). The first evidence that the *OSH* family regulates polarized cell growth came from a genetic screen that showed *OSH4* dosage suppresses *cdc42^{ts}* polarity defects, including supporting the proper polarization of Cdc42p at the bud tip (Kozminski *et al.*, 2006). The ability of Osh4p to promote Cdc42p polarity suggests Osh4p is required to maintain the site of polarized growth.

Oxysterol Binding Protein Activity in Polarized Exocytosis

Despite studies identifying non-lipid transfer based roles for OSBP *in vivo*, how Osh proteins act under the umbrella of polarized cell growth is still unknown. After determining that Osh protein activity supports polarized cell growth, it was found that

Osh protein activity supports polarized exocytosis and that Osh4p is present on exocytic vesicles (Kozminski *et al.*, 2006; Alfaro *et al.*, 2011). Thus, it is predicted that Osh4p may regulate exocytosis from the platform of the exocytic vesicle. It is unknown whether other Osh proteins localize to the vesicle in the absence of Osh4p. Further, in the absence of Osh protein function, exocytic vesicles dwell at the plasma membrane longer than when functional Osh protein is available, suggesting that Osh proteins perform a function at the interface of the exocytic vesicle and the plasma membrane (Alfaro *et al.*, 2011). It should be noted, that increased dwell time in the absence of Osh protein activity suggests vesicles exocytose less efficiently, rather than not at all (Alfaro *et al.*, 2011). A role for Osh proteins in supporting polarized exocytosis at the plasma membrane would be in addition to the role of Osh protein activity in supporting the polarized distribution of actin filaments, along which exocytic vesicles of the polarized pathway are transported by Myo2p to the site of polarized growth (Govindan *et al.*, 1995; Kozminski *et al.*, 2006; Alfaro *et al.*, 2011).

Although Osh proteins were found to support polarized exocytosis, the specific molecular mechanism for their function in polarized exocytosis remained unknown (Kozminski *et al.*, 2006; Alfaro *et al.*, 2011). In addition, these earlier studies did not address whether Osh protein function in polarized exocytosis is dependent or independent of lipid binding (Kozminski *et al.*, 2006; Alfaro *et al.*, 2011). A later study conducted by Peter Novick and colleagues made important observations that shed light on this issue when they showed that PI4P concentration in the exocytic vesicle membrane regulates exocytic vesicle Rab association with the vesicle (Mizuna-Yamaski *et al.*, 2012). As mentioned above, when the exocytic vesicle exits the Golgi apparatus the Rab Ypt32p, rather than the Rab Sec4p, is present on the vesicle. Ypt32p is required for exit from the *trans*-Golgi network (Jedd *et al.*, 1997). Novick and colleagues showed that removal of PI4P from the exocytic vesicle is required to replace the Rab Ypt32p with the terminal post-Golgi Rab Sec4p, which is required for exocytic vesicle docking at the plasma membrane (Guo *et al.*, 1999; Mizuna-Yamaski *et al.*, 2012). However this study did not determine how PI4P was removed from the vesicular membrane.

In a subsequent study, Novick and colleagues proposed that Osh4p removes PI4P from the vesicle membrane (Ling *et al.*, 2014). They showed that Osh protein activity is

required to prevent Ypt32p from being transported to sites of polarized growth, consistent with a requirement for Osh protein activity in removing vesicular PI4P for the purpose of Ypt32p release and subsequent Sec4p loading onto the vesicle (Ling *et al.*, 2014). In addition, Novick and colleagues provided data suggesting Osh4p removes PI4P from vesicular membranes (Mizuno-Yamasaki *et al.*, 2012; Ling *et al.*, 2014). This model of Osh4p function is consistent with Osh family protein lipid binding capabilities, as they all bind PI4P (de St. Jean *et al.*, 2012; Maeda *et al.*, 2013; Tong *et al.*, 2013). However, while vesicle maturation by way of vesicular PI4P removal is an intriguing model of Osh protein function, several issues with that study need to be resolved to confirm the role of Osh proteins in vesicle maturation (Ling *et al.*, 2014). First, while data is presented in that study were consistent with and suggestive of a role for Osh proteins in vesicular PI4P removal, quantification of vesicular PI4P changes in the presence and absence of Osh protein activity was not shown (Ling *et al.*, 2014). Vesicles from strains with and without Osh protein activity will need to be isolated and analyzed for PI4P content to validate this hypothesis. Further, while the initial post-Golgi Rab Ypt32p polarized to the site of polarized growth more in the absence of Osh4p than in its presence, direct demonstration of Osh4p dependent Sec4p loading on to vesicles will greatly support this hypothesis (Ling *et al.*, 2014).

Finally, the exocyst subunit Sec15p was shown to be less polarized in the absence of Osh4p, however the meaning of this result is unclear, especially considering that there is not a vesicular exocyst subcomplex (Boyd *et al.*, 2004; Ling *et al.*, 2014; Heider *et al.*, 2016). The lack of an vesicular subcomplex could mean that Sec15p localization to the vesicle is not Sec4p dependent (Boyd *et al.*, 2004). To address this, Sec4p localization should be analyzed rather than Sec15p. More broadly, in the case of Ypt32p, Sec4p, and even Sec15p, localization to the vesicle, rather than the site of polarized growth, should be analyzed (Ling *et al.*, 2014).

While the model proposed by Novick and colleagues for Osh4p function in exocytosis is attractive, there are a number of observations that suggest additional roles for Osh4p in the exocytic pathway beyond vesicle maturation (Ling *et al.*, 2014). First, GFP-Sec4p puncta accumulate at restrictive temperature in cells of an *osh4-1^{ts}* background carrying only temperature sensitive *osh4-1^{ts}*, suggesting that GFP-Sec4p is loaded onto

the vesicle and that PI4P is removed from the vesicle membrane in this condition (Alfaro *et al.*, 2011). In the same study, and in the work described herein, vesicles often, but not always, transit into the bud in the absence of Osh protein activity (Alfaro *et al.*, 2011). Vesicle transit into the bud can only occur after Sec4p loading onto exocytic vesicles, because of the dependence of Myo2p association with exocytic vesicles on Sec4p (Govindan *et al.*, 1995; Alfaro *et al.*, 2011; Santiago-Tirado *et al.*, 2011). Despite vesicle delivery into the bud in the absence of Osh protein activity in many cases, Osh proteins also support polarization of the actin cytoskeleton at the site of polarized growth, an event necessary for the polarized delivery of vesicles (Kozminski *et al.*, 2006). This suggests that the defect in polarized exocytosis observed in *osh1* cells may be due in part to the inability of vesicles mediating polarized exocytosis to transit into the bud (Kozminski *et al.*, 2006). However, another role, in addition to promoting the formation of polarized actin cables and vesicular maturation, is likely because vesicles often do transit into the bud in the absence of Osh protein activity and are Sec4p positive (Alfaro *et al.*, 2011).

These data collectively indicate a role for Osh protein function in the later steps of polarized exocytosis, beyond the earlier step in vesicle maturation previously described by Novick and colleagues (Ling *et al.*, 2014). Further, the increased dwell time of exocytic vesicles at the plasma membrane in the absence of Osh protein activity suggests this late function may regulate either vesicle docking or fusion at the plasma membrane (Alfaro *et al.*, 2011). I propose that vesicle-associated Osh4p exchanges its bound PI4P for plasma membrane derived sterol to mediate docking between exocytic vesicles and the plasma membrane, leading to the formation of a *trans*-SNARE complex. In the research presented in this dissertation, I tested this hypothesis.

An interesting and untested hypothesis is whether dedicated lipid transfer by Osh proteins is the Osh protein function which is required to support polarized exocytosis (Im *et al.*, 2005; Kozminski *et al.*, 2006). While a possibility, I think this is unlikely for the following reasons. First, because the absence of Osh proteins leads to a selective block in Bgl2p exocytosis, but Bgl2p marked vesicles still accumulate in the cytoplasm, vesicle formation at the TGN appears to be unimpaired (Beh and Rine, 2004; Kozminski *et al.*, 2006; Alfaro *et al.*, 2011). Since the formation of vesicles mediating polarized

exocytosis depends on sterol segregation at the Golgi, it would appear that there is enough sterol in the TGN in the absence of Osh protein activity to support vesicle formation, however Golgi sterol levels will need to be analyzed to confirm this hypothesis (Kozminski *et al.*, 2006; Klemm *et al.*, 2009; Alfaro *et al.*, 2011). Second, because invertase exocytosis occurs at normal levels, it can be assumed that plasma membrane lipid content is fusion competent in the absence of Osh protein activity. Thirdly, based on the translocation of vesicles in the bud and the formation of vesicle clusters I have observed herein it appears that Sec4p is loaded onto exocytic vesicles in the absence of Osh protein function (Alfaro *et al.*, 2011). Sec4p loading onto vesicles implies that PI4P can be removed from the vesicle membrane in this condition, and therefore the PI4P dependent Rab cascade proposed to control vesicle maturation is intact in the absence of Osh protein activity (Ortiz *et al.*, 2002). Thus PI4P is present in an appropriate concentration to support this process (Ortiz *et al.*, 2002; Mizuno-Yamasaki *et al.*, 2010). Once again this will require further analysis to confirm if Ypt32p dissociation and Sec4p loading onto the vesicle is Osh protein dependent (Mizuno-Yamasaki *et al.*, 2010). Thus, while Osh proteins may separately carry out a dedicated lipid transfer role I hypothesize a role of Osh4p in polarized exocytosis that is distinct from dedicated lipid transfer.

Nevertheless, it is possible that dedicated lipid transfer is required for polarized exocytosis, such as for the selective enrichment of sterol into vesicles mediating polarized exocytosis in order to facilitate vesicle maturation (Klemm *et al.*, 2009; Mizuno-Yamasaki *et al.*, 2010; Ling *et al.*, 2014). Also, while vesicle transport into the bud often occurs in the absence of Osh protein activity, this is not always the case and polarized distribution of actin filaments depends on Osh protein activity (Kozminski *et al.*, 2006). Perhaps, Osh4p regulates the polarized distribution of actin filaments through regulating plasma membrane PI4P levels (Ling *et al.*, 2014). Osh protein activity is required to decrease plasma membrane PI4P levels, and it is possible that increased PM PI4P in the absence of Osh protein activity may lead to more PM PI4,5P₂ and change the polarized organization of the actin cytoskeleton, in a manner similar to those which stem from a lack of the PI4P phosphatase Sac1p (Yoshida *et al.*, 1994; Foti *et al.*, 2001; Kozminski *et al.*, 2006; Stefan *et al.*, 2013).

My work establishes that Osh4p is a lipid-regulated protein that regulates vesicle docking at the plasma membrane by at least two mechanisms. I demonstrate for the first time that lipid binding by an OSBP is required for an essential cellular process *in vivo*. I also show that lipid binding directs Osh4p localization, and presumably Osh4p activity, to different cellular membranes. Finally I suggest that specific lipid interactions regulate Osh protein function at the site of exocytosis.

Chapter 2: Lipid-Dependent Regulation of Exocytosis in *S. cerevisiae* by OSBP Homologue (Osh) 4

Chapter 2 has been submitted for publication. The figures and tables have been renumbered to maintain sequence with other figures and tables in this dissertation.

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Author Contributions:

Supraja Chittari, confirmed that *OSH4* is not specifically required for Bgl2p exocytosis and produced EM micrographs (Figure 2.1 and 2.2)

Marissa Hand, developed a chitin accumulation assay (not shown)

Dylan Hyatt, excluded use of a Sec1p-GFP polarity assay to test for vesicle docking defects

Grace Mantus, developed plasmids and strains for the fluorescent microscopy studies in this dissertation (Figure 2.7 and 2.9)

William Sanfelippo, produced EM micrographs (Figure 2.2)

Keith Kozminski, discussion of data and editing of manuscript

Abstract

Polarized exocytosis is an essential process in many organisms and cell types for proper cell division or functional specialization. Previous studies established that Oxysterol Binding Protein (OSBP) homologues in *S. cerevisiae*, which comprise the Osh protein family, are necessary for efficient polarized exocytosis by supporting a late post-Golgi step of the process. Here we define that step as the docking of a specific sub-population of exocytic vesicles with the plasma membrane. We also show that Osh4p can regulate this process in a lipid ligand-dependent manner and propose a two-step mechanism of regulation. This study describes a specific *in vivo* role for lipid ligand binding by an OSBP in an essential cellular process and guides our understanding of where and how OSBPs may function in more complex organisms.

Introduction

Polarized exocytosis is a key cellular process among eukaryotes by which membrane and proteins are delivered to a defined point on the plasma membrane. This process can facilitate the elaboration of specialized structures at the cell surface such as the apical and basolateral domains of intestinal epithelial cells or the formation of a bud in *S. cerevisiae* (He and Guo, 2009; McCaffrey and Macara, 2011; Bi and Park, 2012). If this process becomes unregulated, polarized cell growth fails, leading to a disruption in processes supported by these specialized structures such as nutrient absorption in the intestine or daughter cell growth in *S. cerevisiae*.

Although minor differences in the mechanics of polarized exocytosis exist among eukaryotes, key events, divided between the early exocytic pathway (ER to the trans-Golgi network) and late exocytic pathway (trans-Golgi network to the plasma membrane) are conserved (Keller and Simons, 1997; Barlow and Miller, 2013). This conservation of mechanism and organization allows for the study of exocytosis in less complex organisms such as the budding yeast *S. cerevisiae*. In the late exocytic pathway of *S. cerevisiae*, Golgi-derived vesicles are transported from the mother cell to sites of polarized growth by myosin motors (Govindan *et al.*, 1995). These vesicles are then tethered to the plasma membrane by the exocyst complex (Guo *et al.*, 1999), followed by the assembly of vesicle-associated (v)-SNAREs (Snc1p or Snc2p) and plasma membrane-associated t-SNAREs (Sso1p or Sso2p and Sec9p) into *trans*-SNARE complexes (Protopopov *et al.*, 1993; Rossi *et al.*, 1997). These *trans*-SNARE complexes subsequently scaffold fusion triggers (Wickner & Rizo, 2017) that promote vesicle fusion with the plasma membrane allowing for the release of vesicle cargo into the extracellular environment.

In addition to proteins, lipids contribute to exocytosis as well. In particular the phosphatidylinositol phosphates (PIPs) have emerged as particularly important for successful exocytosis. For instance, Sec3p and Exo70p, the two plasma membrane-associated exocyst complex subunits, are localized to specific regions of the plasma membrane based on their interaction with plasma membrane phosphatidylinositol-4,5-

bisphosphate (PI4,5P) (He *et al.*, 2007; Zhang *et al.*, 2008). This interaction is key because the assembly of the vesicle-associated exocyst subunits (Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and Exo84p) with a plasma membrane-associated subunit is required for vesicle docking at the plasma membrane (Boyd *et al.*, 2004). An additional role for a PIP in exocytosis centers on phosphatidylinositol-4-phosphate (PI4P). When post-Golgi vesicles form, they are enriched with PI4P and marked by the Rab Ypt32p (Ortiz *et al.*, 2002; Strahl and Thorner, 2007). However, successful exocytosis requires the replacement of Ypt32p with the Rab Sec4p (Mizuno-Yamasaki *et al.*, 2011), for which PI4P must be removed from the vesicle membrane (Mizuno-Yamasaki *et al.*, 2011; Rossi and Brennwald, 2011). A subsequent study suggested that Osh4p, an Oxysterol Binding Protein (OSBP) homologue in *S. cerevisiae*, facilitates the removal of vesicular PI4P to promote Sec4p loading onto vesicles (Ling *et al.*, 2014). This observation provided an important example of an OSBP binding a lipid ligand to facilitate an important cellular event, in this case the maturation of exocytic vesicles from a docking incompetent form to a docking competent form.

OSBPs are a large protein family conserved from yeast to humans, with twelve members in humans and seven in budding yeast. Members of this protein family bind one or more lipids, including phosphatidylinositol-4-phosphate (PI4P) (Im *et al.*, 2005; de St. Jean *et al.*, 2011; Maeda *et al.*, 2013; Mesmin *et al.*, 2013; Tong *et al.*, 2013; Chung *et al.*, 2015). While many OSBPs bind two different lipid species, the binding pocket can only accommodate one lipid at a time because the binding sites for each lipid species within the lipid-binding pocket are mutually exclusive (de St. Jean *et al.*, 2011). In the budding yeast *S. cerevisiae* the seven OSBP homologues comprise the Osh protein family, the absence of which results in a loss of proper cell polarization (Kozminski *et al.*, 2006) and growth (Beh *et al.*, 2001). Any one family member is sufficient to support cell viability (Beh *et al.*, 2001) and in the absence of the other family members Osh4p alone is sufficient to maintain proper cell polarization (Kozminski *et al.*, 2006). Within the Osh protein family, as with the mammalian OSBP family, there is a variety of lipid binding activities. All seven Osh family proteins bind or are modeled structurally to bind PI4P. Osh6p and Osh7p can also bind phosphatidylserine, whereas Osh1p, Osh2p, Osh4p and Osh5p also bind sterol (Im *et*

al., 2005; Maeda *et al.*, 2013; Tong *et al.*, 2013). However, it has not been shown what essential role lipid binding by the Osh protein family fulfills in yeast (Beh *et al.*, 2001). It has been proposed that sterol transfer is the essential role filled by the Osh protein family, however considering that a subset of the Osh protein family does not bind sterol this is unlikely (Im *et al.*, 2005; Maeda *et al.*, 2013; Tong *et al.*, 2013), leaving in doubt what, if any, essential role lipid binding by Osh proteins fulfills.

Previous studies with *S. cerevisiae* showed that Osh proteins are required for efficient polarized exocytosis, a key cellular process, but important questions remained (Kozminski *et al.*, 2006; Alfaro *et al.*, 2011). Those studies did not determine what specific step in the late exocytic pathway requires Osh proteins and whether lipid binding by Osh proteins is a requirement for Osh protein function in exocytosis. Here we show that a specific pathway of polarized exocytosis requires Osh protein family activity, in a lipid-dependent manner, and that Osh4p solely can supply this activity. Further, we show that Osh protein activity is required for efficient vesicle docking at the plasma membrane and that lipid binding by Osh proteins is a requirement for this function. We describe specific *in vivo* roles for lipid binding by an OSBP and propose a novel two-step mechanism for Osh-dependent regulation of polarized exocytosis.

Methods and Materials

Culture Media and Growth Conditions

S. cerevisiae strains were grown in synthetic media at 25°C unless otherwise stated (Sherman *et al.*, 1986). When temperature-sensitive alleles were used, 25°C was the permissive temperature and 37°C was the restrictive temperature. When using strains with genes under the control of *pMET25* promoter, methionine was added to 100 mg/L to repress transcription (Mumberg *et al.*, 1994; Alfaro *et al.*, 2011).

Strains

All *S. cerevisiae* strains used in this study are described in Supplemental Table 2.2. To generate strains with N-terminal 6xHA-tagged *SNC2*, we used the protocol of Gauss *et al.*, 2005. In brief, using primers oKK355 and 356 and pOM12 as a template, we produced by PCR an integration cassette consisting of *K. lactis URA3* flanked by loxP recombination sites and a 6xHA tag. This cassette was transformed into strains KKY279, 280, 533, and 802, generating strains KKY1289, 1290, 1288, and 1287, respectively. PCR with primers oKK357 and 358 confirmed the integration. Induction of Cre recombinase on another plasmid with 2% galactose removed the loxP-flanked *K.l. URA3* marker, producing 6xHA-*SNC2* (Gauss *et al.*, 2005). Successful recombination was confirmed by PCR with primers oKK357 and 358 as well as by immunoblotting, using an anti-HA antibody (#901501, Biolegend, San Diego, CA). For all experiments, a minimum of two independent clones or transformants were analyzed.

Plasmids

All plasmids and oligonucleotides used in this study are described in Supplemental Tables 2.3 and 2.4, respectively.

Plasmids generated specifically for this study were made as follows. pRS316-*osh4*^{Y97F} (pKK1990) was made by subcloning the SacI/KpnI fragment from pCB662 (Im *et al.*, 2005) into pRS316.

pRS316-*osh4*^{Y97F+H143A/H144A} (pKK1988) was made by PCR-mediated site-directed mutagenesis (#210518-5, Agilent, Santa Clara, CA) using primers oKK295 and 296, to incorporate the Y97F mutation into pRS316-*osh4*^{H143A/H144A}, which was validated by DNA sequencing using primer oKK193.

All pRS316-*OSH4-YFP* (pKK 2089, 2092, 2093, 2094) plasmids were made as follows. Using primers oKK367 and oKK369, *OSH4-YFP* was amplified by PCR from plasmid pCB866 (pKK1965). The PCR product was cloned into the SpeI and XhoI sites of pRS316-*pMET25* (pKK2005), forming *pMET25-osh4*^{Y97F}-*YFP* (pKK2089). Following this, the *pMET25* promoter was removed by SacI/EcoRI digest and replaced with a SacI/EcoRI fragment from plasmids containing an *OSH4* promoter and an *osh4* allele of interest (pKK1921, 1950, and 1988).

All pRS414-*OSH4-RFP* (pKK2109, 2110, 2111, 2112, 2113) plasmids were made as follows. First, using primers oKK367 and oKK322, *OSH4-RFP* was amplified by PCR from genomic DNA isolated from KKY1240. The PCR product was cloned into the SpeI and XhoI sites of pRS316-*pMET25* (pKK2005), forming *pMET25-OSH4-RFP* (pKK2107). To construct pRS316-*pMET25* (pKK2005), pKK1990 was digested with BamHI/XhoI to remove *osh4*^{Y97F} followed by blunt-end ligation after treatment with Klenow fragment. The *pMET25-OSH4-RFP* SacI/KpnI fragment was the subcloned into pRS414 forming *pMET25-OSH4-RFP* (pKK2108). Following this, the *pMET25* promoter was removed by SacI/EcoRI digest and replaced with a SacI/EcoRI fragment from plasmids containing an *OSH4* promoter and an *osh4* allele of interest (pKK1921, 1950, and 1988).

To construct pRS316-*SUC2* (pKK2012), a *SUC2* fragment extending extending 125 bp upstream and 447 bp downstream of the start of the coding sequence was amplified by PCR,

using *S. cerevisiae* genomic DNA as a template and primers oKK319 and oKK320. This fragment was cloned into the SacI/XhoI sites of pRS316.

Invertase Assay

This assay was performed according to Dighe and Kozminski (2008) with the following modification. After washout of synthetic medium containing 5% glucose, cells were grown in synthetic medium containing 0.1% glucose for 4h at 25°C or 37°C prior to analysis. For the purpose of analyzing the kinetics of invertase exocytosis at early time points after the de-repression of invertase, cells were grown in minimal medium containing 0.1% glucose at the times indicated, after washout of minimal medium containing 5% glucose, beginning 30 min post shift from 25°C or 37°C.

Bgl2p Accumulation Assay

The Bgl2p accumulation assay was performed according to Kozminski *et al.* (2006). ~3.0 O.D._{600nm} units of cells were washed with 10 mM NaN₃/KF and dewalled with zymolyase (Seikagaku No. 120488 Tokyo, Japan). Cells were then gently pelleted in a microcentrifuge at 5,000xg for 10 min before being resuspended in SDS loading buffer (50 mM Tris-Cl pH 6.8, 100 mM β-mercaptoethanol, 2% SDS (w/v), 10% glycerol (v/v), 0.1% (w/v) bromophenol blue). Samples were then boiled for 5 min and clarified by centrifugation for 1 min at 18,000xg prior to analysis by SDS-PAGE and immunoblotting. To increase the efficiency of sample processing, Bgl2p levels were compared to the level of b-tubulin, which served as a loading control, after total Bgl2p levels were determined to be constant among the isogenic strains analyzed.

Lucifer Yellow Assay

Per Beh *et al.* (2004), cells were grown to ~0.1 O.D._{600nm}, and pelleted at 8,000 rpm in a 1.5 mL microfuge tube, followed by resuspension in 40 μL synthetic medium, in an aerated tube to prevent Lucifer Yellow (L-0144, Sigma, St. Louis, MO) quenching by CO₂. The

cells were then grown at the 25°C or 37°C for 1 h with agitation. Following incubation, 10 µL of 40 mg/mL Lucifer Yellow were added and the cells were grown for another 2 h at the 25°C or 37°C temperature, after which 1 mL of 50 mM phosphate buffer (pH 7.5) with 10 mM NaN₃/NaF was added. The cells were pelleted, and then washed thrice in the same buffer. Lucifer Yellow-treated cells were observed using a fluorescence microscope with a FITC filter (Beh and Rine, 2004).

SNARE Pulldown Assay

To assay the formation of assembled SNARE complexes we used the protocol of Grote, *et al.* (2001). 30 O.D._{600nm} units of cells were harvested and immediately treated with ice cold 10X TAF (100mM Tris-Cl 7.5, 100mM NaN₃, 100 mM NaF) and left on ice for 10 min. Cells were then harvested by centrifugation in a clinical centrifuge for 5 min at 2,500 rpm at 4°C and then washed with TAF and centrifuged as before. These cells were then resuspended in 1mL IP Buffer (50 mM HEPES pH 7.4, 150 mM KCl, 1 mM EDTA, 1mM DTT, and 0.5% (v/v) NP-40) and vortexed at 4°C with 425-600 µm diameter glass beads (G9268, Sigma, St. Louis, MO) thrice for 4 min each with a 1 min pause on ice between beatings. Lysates were clarified by centrifugation in a microfuge for 20 min at 14,000 rpm at 4°C. The protein concentration was measured by spectrophotometry at 280 nm. Samples were then normalized to a total protein concentration of 4 mg/mL with IP Buffer. Lysates were then precleared with unbound protein A-conjugated agarose (Pierce 20333, ThermoFisher Scientific, Waltham MA) for 1 h 4°C with gentle rocking. Following this, Protein A-agarose blocked with 1 mg/mL BSA (Fraction V, A3059, Sigma, St. Louis, MO) and prebound to anti-HA monoclonal antibody (MMS-101p, Convance, Princeton, NJ) was added to the pre-cleared lysates and rocked gently overnight at 4°C. The anti-HA bound Protein A beads were collected by centrifugation (1000xg for 30s at 4°C) and washed five times with ice cold IP buffer without DTT, then resuspended in SNARE pulldown SDS-PAGE Sample Buffer (60 mM Tris-CL pH 6.8, 100 mM DTT, 2% (w/v) SDS, 100 mg/mL sucrose, 0.05% (w/v) bromophenol blue; Carr *et al.*, 1999). Samples were then boiled for 15 min and clarified by centrifugation for 1 min at 18,000xg prior to analysis by SDS-PAGE.

Vesicle Isolation

As per Alfaro *et al.* (2011), 34 O.D.₆₀₀ units of cells were lysed and loaded onto a Nycodenz gradient (18% to 30%, containing 0.8 M D-sorbitol) and centrifuged in a Beckman SW-41 rotor at 30,000 rpm for 18 h at 4°C. 500 µL fractions were collected and diluted 1:10 in Tris-EDTA (pH 7.5) followed by centrifugation for 1 h at 100,000xg in a Beckman TLA 100.2 rotor at 4°C prior to resuspension in 4X SDS (125 mM Tris-Cl pH 6.8, 1.43 M β-mercaptoethanol, 4% (w/v) SDS, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue) loading buffer. Samples were then heated at 65°C for 10 and clarified by centrifugation for 1 min at 18,000xg prior to analysis by SDS-PAGE. Fraction density was determined by refractometry.

Plasma Membrane Isolation

To isolate plasma membrane, the protocol of Alfaro *et al.* (2011) for vesicle isolation was used with the following modifications. The sample was loaded on top of a discontinuous sucrose gradient (1.10 M, 1.65 M, and 2.25 M sucrose, with 0.8 M D-sorbitol) and centrifuged in a Beckman SW-41 rotor at 80,000xg for 14 h at 4°C. Successive layers of 3.5 mL of 2.25, 1.65 and 1.1 M sucrose in Tris-EDTA (pH 7.5) Buffer containing 0.8 M D-sorbitol formed the gradient (Panaretou and Piper, 2006). Fractions were taken from the 2.25/1.65 M interface and the 1.65/1.1 M interface, diluted 1:4 with Tris-EDTA (pH 7.5) and pelleted for 40 min at 30,000xg in a Beckman TLA100.2 rotor prior to resuspension in 4x SDS-PAGE (125 mM Tris-Cl pH 6.8, 1.43 M β-mercaptoethanol, 4% (w/v) SDS, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue) loading buffer. Samples were then heated at 65°C for 10 min and clarified by centrifugation for 1 min at 18,000xg prior to analysis by SDS-PAGE. To determine total membrane, sucrose fractions were diluted four fold with 25 mM Tris-Cl (pH 7.5), after which 200 µg/mL FM4-64 (#T13320, Molecular Probes, Eugene, OR) diluted in DMSO was added to 18 µg/mL. After 1 h of gentle shaking at room temperature, fluorescence was measured using a PTI 814 photomultiplier detection system (515nm/650nm).

Electron Microscopy

Samples were prepared, imaged, and processed for thin-section electron microscopy per Dighe and Kozminski (2008). Images were processed using Image J (NIH) to uniformly adjust brightness and contrast. Vesicle diameters were measured using Image J (NIH).

Immunofluorescence Microscopy

Sec4p was detected using the protocol of Orlando *et al.* (2011) with the following modification. After the initial fixation in 4.4% (v/v) formaldehyde (F79-500, ThermoFisher, Waltham, MA), the cells were fixed for an additional hour in 5 mL of buffered fixative (PBS, 2% glucose, 20 mM EGTA, 3.7% (v/v) formaldehyde (F79, ThermoFisher, Waltham, MA)). Next the cells were probed with anti-Sec4p monoclonal antibody (Kind gift from P. Brennwald (University of North Carolina); Rossi and Brennwald, 2011) diluted 1:100 in PBS with 1 mg/mL BSA and then with FITC-conjugated anti-mouse IgG (#715-095-150, Jackson ImmunoLabs, West Grove, PA) diluted 1:100 in PBS with 1 mg/mL BSA. Mounting medium consisted of PBS, 90% (v/v) glycerol, and 1 mg/mL p-phenylenediamine (P-6001, Sigma, St. Louis, MO). Images were processed using Image J (NIH); brightness and contrast adjustments were uniformly applied.

Fluorescence Microscopy

Single channel microscopy was performed on a Zeiss Axioplan 2 microscope with a 100 X (Plan-Fluor, N.A. 1.45) objective with a Zeiss AxioCam MRM camera (Zeiss, Oberkochen, Germany). Dual channel microscopy for colocalization was performed on a Zeiss Axiovert S.100 microscope with a 100X (Plan-Apochromat, N.A. 1.4) objective with a Hamamatsu EM-CCD (C9100 13) ImageM camera (Hamamatsu Photonics, Japan). Bleed through between channels was not detected under the conditions used. Images were processed using Image J (NIH); brightness and contrast adjustments were uniformly applied.

Preparation of Whole Cell Extracts

To prepare whole cell extracts for analysis by SDS-PAGE, 5 O.D._{600nm} units of cells were harvested by centrifugation at 2,500 rpm for 5 min then washed twice with deionized water. 100 μ L of 425-600 μ m diameter glass beads were added to each sample followed by 50 μ L of 2X SDS Sample Buffer (125 mM Tris-Cl pH 6.8, 1.43 M β -mercaptoethanol, 2% (w/v) SDS, 20% (v/v) glycerol, 0.15% (w/v) bromophenol blue). Samples were boiled for three min and then immediately vortexed for 1 min. An additional 100 μ L of 2X SDS Sample Buffer were added to each sample, and the samples were vortexed briefly again. Samples were then boiled for 5 min and clarified by centrifugation for 1 min at 18,000xg prior to analysis by SDS-PAGE.

Immunoblotting

Proteins samples were separated using standard SDS-PAGE on 15% gels. The protein samples were then transferred to 0.2 μ m nitrocellulose membranes (#162-0112, Biorad, Hercules, CA). Following this the membranes were blocked with 5% (w/v) powdered milk solution, probed with primary antibody, and then probed with near-infrared fluorescent anti-rabbit secondary antibody (#92632211, Licor, Lincoln, NE) or anti-mouse secondary antibody (#926-32210, Licor, Lincoln, NE). The probed membranes were scanned on an Odyssey[®] Infrared Imaging System (Licor, Lincoln, NE) and band intensity was measured using Image Studio[™] (#9202-500, Licor, Lincoln, NE).

Primary antibodies used are anti-Pma1p (sc-57978, Santa Cruz Biotech, Dallas, TX), anti-Kex2p (ab34772, Abcam, Cambridge, UK), anti-Dpm1p (A-6429, ThermoFisher Scientific, Waltham, MA), anti-Pho8p (ab113688, Abcam, Cambridge, UK), anti-Sec4p and anti-Sso1/2p antibodies (kind gifts of P. Brennwald, University of North Carolina), anti- β -tubulin antibody (kind gift of A. Frankfurter, University of Virginia), anti-Bgl2p, anti-HA (901502, BioLegend, San Diego, CA), and anti-Osh4p (kind gift of C. Beh, Simon Fraser University).

Results

Lipid-Dependent Osh4p Activity Is Required in a Specific Exocytic Pathway

At the beginning of this study, we examined two defined exocytic pathways in *S. cerevisiae* – the Bgl2p-marked pathway that supports polarized cell growth, most notably at the bud tip and neck (Harsay and Bretscher 1995; Adamo *et al.*, 2001) and the invertase (Suc2p)-marked pathway that supports non-polarized cell growth (Harsay and Bretscher 1995; Adamo *et al.*, 2001). Specifically, we asked with quantitative assays whether Osh4p functions in one or both of these pathways and whether its activity requires the binding of a specific lipid. We found that Osh4p is required in one exocytic pathway, the Bgl2p-marked pathway, and that its activity in this pathway is lipid dependent.

Consistent with that suggested in the literature (Beh and Rine, 2004), we found no role for Osh4p in the invertase-marked exocytic pathway (Fig. 2.1A). When the exocytosis of invertase was assayed, neither cells containing wild-type *OSH4* nor a temperature-sensitive allele (*osh4-1^{ts}*) displayed a difference in invertase exocytosis when shifted from 25°C to 37°C for 4 h (Fig. 2.1A), the time period normally used to examine *osh4-1^{ts}* at non-permissive temperatures (Beh and Rine, 2004; Kozmisnki *et al.*, 2006; Alfaro *et al.*, 2011). Functional redundancy, which is known among members of the Osh protein family (Beh *et al.*, 2001), did not mask an exocytosis defect in the mutant strain because the cells, which lack chromosomal copies of all seven *OSH* family genes (*oshΔ*) are entirely dependent upon plasmid-borne *OSH4* or *osh4-1^{ts}* for Osh protein activity. An exocytosis defect was also not masked by changes in cell physiology or invertase expression over time. At earlier time points, within 90 min post-temperature shift (t=60 min after invertase de-repression (glucose washout)), both strains exhibited nearly identical levels of invertase exocytosis (Fig. 2.1B). In contrast to these two strains, the temperature-sensitive mutant *sec6-4^{ts}*, which encodes a defective subunit of the exocyst complex and which is known to be defective in invertase exocytosis at 37°C (Novick *et al.*, 1980; TerBush and Novick, 1995), exhibited markedly different kinetics under the same conditions (Figs. 2.1A and B). Therefore, the exocytic pathway marked by invertase does not appear to utilize Osh4p,

Figure 2.1

Figure 2.1 Osh4p activity promotes polarized Bgl2p-marked exocytosis. (A) Ratio of external to total invertase activity in *S. cerevisiae oshΔ* cells containing a *CEN* plasmid-borne copy of wild-type *OSH4* or a temperature-sensitive *osh4* allele (*osh4-I^{ts}*) and *SUC2*. *sec6-4^{ts}* cells with a known invertase exocytosis defect served as a positive control. Shown is the average of five independent experiments in which log-phase cells were cultured at 25°C or 37°C for 4h, following growth at 25°C. (B) Average of two independent experiments in which external and total invertase activity were measured for the same *S. cerevisiae* strains as in (A). Cells were grown at 25°C and then shifted to 37°C, with aliquots taken at the indicated time points post temperature shift and glucose washout (invertase de-repression). (C) Average of two independent experiments in which internal levels of Bgl2p were measured, by immunoblotting, in log-phase *S. cerevisiae oshΔ* strains containing *CEN* plasmid-borne *OSH4* or a temperature-sensitive allele (*osh4-I^{ts}*). Cells were grown at 25°C and then shifted to 37°C, with aliquots taken at the indicated time points. Data were standardized to time 0 for each strain respectively. (D) Relevant genotype of isogenic *S. cerevisiae* used for analysis of Osh protein function in (E and F). Cells lack all seven endogenous *OSH* family genes (*oshΔ*) but contain two *CEN* plasmids; the first contains a temperature-sensitive *osh4-I^{ts}* allele and the second an *OSH4* allele of interest, wild-type *OSH4*, or no insert (vector). At 37°C, cells are entirely dependent on the second plasmid to supply Osh protein function. (E) Fold change in the amount of internal Bgl2p in log-phase cells as described in (D) at 25°C or after shift to 37°C for 90 min, relative to time 0 at 25°C, as measured by immunoblotting. Tubulin served as a loading control. Total Bgl2p levels were constant across these strains. Shown is the average of two independent experiments. Same as in (E), except the first plasmid in these *oshΔ* cells contained wild-type *OSH4*. Shown is the average of three independent experiments in which cells, grown overnight in the presence of methionine, were assayed before and 8h after methionine washout. (Error bars in (A, B, C, E, F) indicate SEM. Data were analyzed with a two-tailed Student's t-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.)

though our results do not preclude a requirement for other Osh protein family members in this pathway.

In contrast to the exocytosis of invertase, we found, consistent with previous report (Kozminski et al., 2006), that the Osh protein family is necessary for Bgl2p-marked exocytosis. We show for the first time that an *oshΔ* [*osh4-I^{ts}*] strain accumulates Bgl2p internally 45-60 min after shift from 25°C to 37°C. In contrast, the same strain containing a wild-type copy of *OSH4* on a plasmid (Fig. 2.1D), rather than *osh4-I^{ts}*, exhibited no appreciable accumulation of Bgl2p post temperature shift. Deletion of *OSH4* alone (all other *OSH* family genes present) produced a negligible (<5%) block in Bgl2p-marked exocytosis. These results indicate that Osh4p is sufficient to support Bgl2p-marked exocytosis in the absence of all other Osh family proteins but is not necessary in the presence of all other Osh family proteins.

Observing that Bgl2p-marked exocytosis requires Osh protein activity led us to ask whether lipid binding by an Osh protein is required as well. To answer this question, we took advantage of the aforementioned observation that Osh4p is sufficient to support Bgl2p-marked exocytosis. We introduced into the *oshΔ* [*osh4-I^{ts}*] strain a second plasmid that contains wild-type *OSH4* or one of several *osh4* alleles, which express at wild-type levels and confer specific defects in lipid binding (Table 2.1), thereby generating a panel of strains that are dependent upon the activity of Osh4p expressed from this second plasmid when grown at 37°C (Fig. 2.1D). Within this panel of isogenic strains, differing only in the identity of the *OSH* allele on the second plasmid, we found that strains with *osh4* alleles that confer a defect either in PI4P binding (H143A/H144A; de St. Jean *et al.*, 2011) or in both sterol and PI4P binding (Y97F + H143A/H144A; Im *et al.*, 2005; de St Jean *et al.*, 2011) accumulated Bgl2p internally upon temperature shift from 25°C to 37°C (Fig. 2.1E). In contrast, a strain containing wild-type *OSH4* did not accumulate Bgl2p at either temperature. A similar result was obtained when we assayed a strain carrying *osh4^{Y97F}* (Fig. 2.1F), which encodes an amino acid substitution that blocks sterol binding (Im *et al.*, 2005). Because the *osh4^{Y97F}* allele is dominant lethal (in the absence of the H143A/H144A mutation), its expression had to be regulated with a methionine-repressible promoter,

Table 2.1 Lipid binding capacity of Osh4p mutants used in this study

	PI4P Binding	Sterol Binding
Osh4p	Yes ^a	Yes ^b
osh4p ^{Y97F}	Yes ^c	No ^b
osh4p ^{H143/144A}	No ^a	Yes ^b
osh4p ^{Y97F+H143/144A}	No ^c	No ^c

a (de St. Jean *et al.*, 2012); **b** Im *et al.*, 2011; **c** Inferred from structure or aforementioned studies.

requiring it to be assayed independently of the other strains. Taken together, these results indicate that PI4P and sterol must bind Osh4p for it to function in the Bgl2p-marked exocytic pathway.

Earlier studies indicated Osh4p has a role in the late exocytic pathway (Kozminski *et al.*, 2006; Alfaro *et al.*, 2011). To exclude the possibility that lipid binding by Osh4p is required in an early exocytic pathway (i.e., pre-Golgi), we examined thin sections of the strains described above by electron microscopy. Specifically, we looked to see whether cells of these strains, which showed defects in Bgl2p-marked exocytosis, accumulated vesicles of ~50 nm diameter, the signature size of pre-Golgi transport vesicles (Kaiser and Schekman, 1990), under conditions that made these strains dependent upon a mutant Osh4p with a specific lipid binding defect. We found that cells dependent upon PI4P-binding defective Osh4p (H143A/H144A) or an Osh4p defective in binding both PI4P and sterol (Y97F + H143A/H144A) accumulated vesicles (Fig. 2.2A), similar to a strain in which no functional Osh proteins are available (vector alone; Fig. 2.2A and Alfaro *et al.*, 2011; Ling *et al.*, 2014.) The vesicles were ~80nm in diameter, the signature diameter of post-Golgi exocytic vesicles, rather than ~ 50nm, the signature diameter of Golgi and pre-Golgi transport vesicles (Suppl. Fig. 2.1A; Kaiser and Schekman, 1990). Some vesicles appeared empty while others appeared filled with electron dense material, even within the same cell (Figure 2.2: Column 2, row 4 and Column 4, rows 2 and 3). This variation in vesicle hollowness appeared due to variations in staining across a section and did not correlate with any experimental variable. In contrast, vesicle accumulation was observed in only one-third of the cells of the strain that carries a wild-type copy of *OSH4*. Normally wild-type cells do not show any notable accumulation of vesicles (Novick, Field, and Schekman, 1980) but here wild-type *OSH4* is present in an *oshΔ* background that, under these assay conditions, produces a weak vesicle accumulation phenotype. The only cells with an Osh4p-lipid binding defect that did not accumulate vesicles were of strains expressing dominant lethal *osh4*^{Y97F}, which confers a sterol-binding defect (Fig. 2.2B). This was an unexpected observation because these cells, under the same experimental conditions, displayed a defect in Bgl2p-marked exocytosis (Fig. 2.1F). This may be explained by the

Figure 2.2

A

B

Figure 2. Exocytic vesicles accumulate in cells dependent on lipid binding deficient Osh4p. (A) Electron micrographs of thin sections showing vesicle accumulation in log-phase *osh4* cells with *CEN* plasmid-borne *osh4-1^{ts}* and a second *CEN* plasmid containing wild-type *OSH4*, an *osh4* allele of interest, or no insert (vector), 90 min after shift from 25°C to 37°C. Note accumulation of vesicles in cells with *osh4* alleles that confer lipid-binding defects. Scale bar is 1 micron in 5,000x column and 0.5 micron in 20,000x column. (B) Same as (A), except the second plasmid in the cells contained an *osh4* allele under the control of a methionine-repressible *MET25* promoter. Prior to embedding, cells were grown for 8h at 25°C in the absence of methionine to de-repress the *MET25* promoter. Image was processed with an unsharp mask. (Please see the digital files, if the micrographs appear low resolution when printed.)

observed accumulation of other membranous structures in these cells, with Bgl2p perhaps accumulating in those structures.

Taken together, these data strongly suggest that neither PI4P nor sterol binding by Osh4p is required in a pre-Golgi step of exocytosis. Rather, PI4P-Osh4p binding appears essential for one or more events in the Bgl2p-marked exocytic pathway post-Golgi.

Vesicle Docking at the Plasma Membrane Requires PI4P Binding by Osh4p

After establishing that polarized, Bgl2p-marked exocytosis requires lipid binding to Osh4p, we asked where this regulation occurs. A previous study showed that exocytic vesicles can transit from the mother cell into the bud, in the absence of Osh4p function (Alfaro *et al.*, 2011), making it unlikely that any lipid regulation of Osh4p affects the motility or direction of vesicle trafficking significantly. In addition, our data show a post-Golgi vesicle accumulation defect when PI4P-Osh4p binding is impaired (see Fig. 2.2A). Therefore, a more likely point of regulation exists when exocytic vesicles dock with the plasma membrane, at the end of their journey from mother cell to bud. As reported in an earlier study (Alfaro *et al.*, 2011), the time an exocytic vesicle dwells subjacent to the plasma membrane at the bud tip is greater in the absence of Osh protein family activity than in cells containing wild-type Osh4p as the sole representative of the Osh protein family, suggesting that Osh4p is necessary for the efficient docking of exocytic vesicles with the plasma membrane.

To determine whether Osh proteins are necessary for the docking of exocytic vesicles at the plasma membrane, we assayed, in an *oshΔ* strain background, for assembled SNARE complexes, in the presence or absence of *OSH4*. When an exocytic vesicle docks at the plasma membrane, v-SNAREs tightly bind plasma membrane-associated t-SNAREs to form an assembled *trans*-SNARE complex (Grote, Carr, and Novick 2000). Because specific SNAREs are associated with specific membranes (Hong, 2005), we used the formation of HA-Snc2p-Sso1/2p complexes as a read-out for exocytic vesicle docking with the plasma membrane. Snc2p is an exocytic v-SNARE that binds directly to either plasma

membrane-associated t-SNAREs Sso1p or Sso2p (Rossi *et al.*, 1997). From whole cell lysates of *S. cerevisiae* expressing 6xHA epitope-tagged Snc2p (HA-Snc2p), under the control of the endogenous *SNC2* promoter, we pulled-down HA-Snc2p and then assayed the ratio of Sso1/2p to HA-Snc2p by immunoblotting. In the absence of the HA tag or primary antibody, little Sso1/2p was detected on immunoblots, indicating that the pull-down of Sso1/2p depends upon HA-Snc2p (not shown). When we shifted cells that lack functional Osh proteins at 37°C (*oshΔ* [*osh4-I^{ts}*][*vector*]), from 25°C to 37°C for 90 min, we noted a trend toward less vesicle-plasma membrane SNARE complex formation (HA-Snc2p:Sso1/2p; Fig. 2.3; Suppl Fig. 2.5), with a decrease on average of ~35% ($p=0.081$; $n=3$) relative to control cells that contained a wild-type copy of *OSH4* (*oshΔ* [*osh4-I^{ts}*][*OSH4*]). Although this trend suggested that Osh proteins are necessary for efficient vesicle-plasma membrane docking, we anticipated a more significant effect considering the robust exocytosis defect observed when Osh activity is absent (Figs. 2.1 and 2.2).

Therefore, we considered the possibility that a defect in endocytosis alters the amount of target SNAREs and other proteins required for docking on the plasma membrane of mutant cells relative to control, diminishing the relative difference in the amount of assembled SNARE complexes detected between strains.

To determine whether an endocytosis defect exists in any of our *osh* mutants, we assayed microscopically for the vacuolar accumulation of Lucifer Yellow, a fluorescent marker for fluid phase endocytosis (Dulic *et al.*, 1991). With cells that are wild-type for endocytosis, Lucifer Yellow accumulates in one or more large, round, vacuolar compartments shortly after the addition of Lucifer Yellow to the culture medium (Dulic *et al.*, 1991). We saw the same with cells expressing a wild-type copy of *OSH4* in the absence of any other wild-type *OSH* family genes (*oshΔ* [*osh4-I^{ts}*][*OSH4*]) or when, at 25°C, the second plasmid in the *oshΔ* [*osh4-I^{ts}*] strain background was an empty vector (Fig. 2.4), consistent with previous report (Beh and Rine, 2004). This accumulation pattern was rarely observed when cells containing the empty vector were assayed after shifting to 37°C (Fig. 2.4). When fluorescence accumulation was observed in these cells post-temperature shift, compartments containing Lucifer Yellow had irregular shapes and sizes. Similar results were observed for the strain dependent upon a PI4P-binding defective Osh4p (H143A/H144A) or an Osh4p defective in binding both PI4P and sterol (Y97F +

Figure 2.3

Figure 2.3 Osh4p promotes SNARE complex assembly at the plasma membrane.

Ratio of the amount of t-SNARE (Sso1/2p) associated with the amount of v-SNARE (HA-Snc2p), as determined by immunoblotting, after HA-Snc2p was pulled down from clarified lysates of log-phase *S. cerevisiae osh4* cells containing a *CEN* plasmid with a temperature-sensitive *osh4-I^{ts}* allele and a second *CEN* plasmid containing wild-type OSH4 or no insert (vector), when grown at 25°C or after shift from 25°C to 37°C, for 90 min. Shown are the averages of three independent experiments (n=3 for each) Data normalized to time 0, indicated by the dashed line. Error bars indicate SEM. Data were analyzed with a paired one-tailed Student's t-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 2.4

Figure 2.4 Lipid binding by Osh4p is required for fluid phase endocytosis.

Fluorescence micrographs that show Lucifer Yellow uptake in *S. cerevisiae osh4Δ* strains containing a *CEN* plasmid-borne temperature-sensitive *osh4* allele (*osh4-1^{ts}*) and a second plasmid containing wild-type *OSH4*, an *osh4* allele of interest, or no insert (vector), after growth at 25°C or 37°C. See *Materials & Methods* for experimental details. Scale bar is 5 microns.

H143A/H144A), though in the latter case defects in compartment morphology were noted even prior to temperature shift. These results suggest that the inability of Osh4p to bind a specific lipid such as PI4P, in the absence of all other Osh proteins, is sufficient to inhibit endocytosis.

To account for the potential impact of endocytosis on the availability of docking sites on the plasma membrane and to challenge our initial conclusion that Osh proteins are required for efficient vesicle docking, we assayed docking by a different method (Fig. 2.5A). In this method, the read-out for docking is the association of an exocytic vesicle-associated protein (e.g., Sec4p, HA-Snc2p) with plasma membrane. We isolated a plasma membrane fraction and determined the ratio of the amount of a vesicle-associated protein present to the total amount of plasma membrane, calculated as the ratio of Sso1 and 2 proteins to total membrane, which corrects for changes in t-SNARE density on the plasma membrane due to endocytosis defects in the *osh* mutants (Fig. 2.5A and Supp. Table 2.1). The plasma membrane fraction showed a >30-fold enrichment of the plasma membrane marker Pma1p relative to a whole cell extract and contained barely detectable cross-contamination by other membrane compartments, as determined by quantitative immunoblots for a panel of known membrane compartment markers (Fig. 2.5B). In a test of this method (Fig. 2.5C), we assayed a known vesicle-plasma membrane docking mutant (*sec6-4^{ts}*; Grote, Carr, and Novick, 2001) and a known vesicle-plasma membrane fusion mutant (*sec1-1^{ts}*; Grote, Carr, and Novick, 2001), along with an isogenic control strain. We found that, as expected, the vesicle-plasma membrane fusion mutant showed a significant association of Sec4p, a vesicle marker, with the plasma membrane. In contrast neither the vesicle docking mutant nor the isogenic control strain exhibited this effect, validating that this method can differentiate defects in vesicle docking with the plasma membrane from defects in vesicle-plasma membrane fusion.

When we applied the “plasma membrane isolation” method to our panel of *osh* mutant strains, we found that the ratio of t-SNAREs (Sso1/2p) to total plasma membrane, on average, varied among strains (Suppl. Figs. 2.2A, B) and, in some cases, in the same strain when cultured at different temperatures, possibly due to allelic variations in the rate of

Figure 2.5

Figure 2.5 Osh protein activity and lipid binding by Osh4p in particular is required for efficient vesicle docking at the plasma membrane. (A) Schema of method to isolate plasma membrane from clarified whole cell extracts (total cellular protein), using a sucrose step gradient, and downstream steps to analyze the plasma membrane fraction. (B) Qualitative and quantitative immunoblot analyses of plasma membrane isolate (from (A)) homogeneity using antibodies against known membrane component markers. PMI, plasma membrane isolate; TCP, total cellular protein. (C) Average of four independent experiments in which the plasma membrane was isolated by step gradient fractionation, as in (A), and tested for association with exocytic vesicle Rab Sec4p. Shown is the ratio of the amount of Sec4p to the amount of plasma membrane (t-SNARE Sso1 and 2p/total membrane). Plasma membrane was isolated from log-phase *S. cerevisiae* cells with a genomic mutation conferring either a vesicle docking (*sec6-4^{ts}*) or a vesicle fusion (*sec1-1^{ts}*) defect at restrictive (37°C) temperature and an isogenic control, at the indicated times after shift of the cultures from 25°C to 37°C. Data were standardized to time zero. (D) Ratio of the amount of v-SNARE (HA-Snc2p) associated with isolated plasma membrane (Sso1 and 2p/total lipid, which corrected for changes in t-SNARE density on the plasma membrane due to endocytosis defects in *osh* mutants; see Fig. 2.4 and Supp. Fig. 2.2). Amounts of SNAREs were determined by immunoblotting and plasma membrane by fluorimetric measurement of membrane bound FM4-64 (see Fig. 2.5A). Plasma membrane was isolated from clarified lysates of log-phase *S. cerevisiae oshΔ* cells with a *CEN* plasmid-borne temperature-sensitive *osh4* allele (*osh4-1^{ts}*) and a second *CEN* plasmid containing wild-type *OSH4*, or no insert (vector) grown for 90 min at 25°C or after shift from 25°C to 37°C. Shown is the average of four independent experiments. (Data are standardized to time 0. Data were analyzed with a two-tailed Student's t-test. Error bars indicate SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.)

endocytosis (Fig. 2.4). This result therefore necessitated a normalizing correction for t-SNARE levels among strains, when calculating the ratio of v-SNARE to plasma membrane, as described above. With the plasma membrane isolation method, we found that cells lacking functional Osh proteins were unable to support the association of vesicles with the plasma membrane to the same extent as cells with a functional Osh family protein (Fig. 2.5D). Using the same assay, we found that mutants encoding PI4P-binding deficient Osh4p did not rescue this defect and had less vesicle marker present on the plasma membrane relative to wild-type cells (Fig. 2.5D). These results indicate that Osh protein activity and PI4P binding by Osh4p in particular, when Osh4p is the only Osh family member present, is required for vesicle docking at the plasma membrane.

We also found a third line of evidence supporting a role for Osh proteins in vesicle docking, vesicle cluster formation (Fig. 2.6). It is known that Sec4p-positive vesicles accumulate and cluster when there is increased Sec4p activity (Salminen and Novick, 1989; Rossi and Brennwald, 2011). We observed this clustering phenotype, by thin section electron microscopy, within populations of cells that lack functional Osh proteins (*oshΔ* [*osh4-I^{ts}*] [vector] at 37°C; ~23%, n=40; Fig. 2.6). The clustered vesicles had a mean diameter approximately that of exocytic vesicles, though less than that of unclustered vesicles (75 vs. 88 nm; Supp. Fig. 2.3). The significance of this difference is unknown, though it may represent greater difficulty in ascertaining the boundaries of clustered structures. Indirect immunofluorescence microscopy, which has been used previously to detect these vesicles clusters (Rossi and Brennwald, 2011), confirmed the presence of Sec4p-positive vesicle clusters in cells lacking Osh protein function (Suppl. Fig. 2.4). This phenotype was partially ameliorated by the presence of a plasmid containing wild-type (*OSH4*) or PI4P-binding defective *osh4* (*osh4^{H143A/H144A}*). These observations are consistent with the idea that a deficiency in PI4P-binding by Osh4p relieves cluster formation by partially blocking the loading of Sec4p onto exocytic vesicles, leading instead to the accumulation of unclustered vesicles (Ling *et al.*, 2014).

Figure 2.6

Figure 2.6 *S. cerevisiae* lacking functional Osh proteins contain clusters of vesicles.

(A) Electron micrographs that show vesicle clusters in thin sections of log-phase *osh1* cells with a *CEN* plasmid-borne temperature-sensitive *osh4* allele (*osh4-I^{ts}*) and a second *CEN* plasmid containing wild-type *OSH4* or no insert (vector) grown for 90 min after shift from 25°C to 37°C. Scale bar is 1 micron in 5,000x column and 0.5 micron in 20,000x column. Image was processed with an unsharp mask. (Please see the digital files, if the micrographs appear low resolution when printed.)

Lipid Binding by Osh4p Regulates Its Association with Different Membranes

To determine how lipid binding to Osh4p regulates polarized exocytosis and in particular the docking of exocytic vesicles to the plasma membrane, we investigated whether lipid binding by Osh4p regulates the association of Osh4p with specific late exocytic pathway organelles. It has been reported that PI4P binding by Osh4p is required to maintain Osh4p at sites of polarized exocytosis (Ling *et al.*, 2014). While it is known that Osh4p localizes to the plasma membrane (Alfaro *et al.*, 2011; Ling *et al.*, 2014) and exocytic vesicles (Alfaro *et al.*, 2011; Ling *et al.*, 2014), it is not known whether specific lipid ligands that bind Osh4p are required for the localization of Osh4p to these membranes. To address this question, we examined by fluorescence microscopy and cell fractionation *oshΔ* cells that contain a wild-type or mutant allele of *OSH4* on a plasmid.

We found that the ability of Osh4p to bind specific lipids affected its ability to associate with exocytic vesicles *in vivo* (Fig. 2.7). As shown by fluorescence microscopy in Figures 2.7A and B and as expected from earlier observations (Alfaro *et al.*, 2011; Ling *et al.*, 2014), wild-type Osh4p-RFP co-localized with puncta of GFP-Sec4p, a marker of exocytic vesicles. This localization pattern was also observed with Osh4p^{Y97F}-RFP, indicating that the association of Osh4p with exocytic vesicles does not require sterol binding. In contrast, PI4P-binding deficient Osh4p^{H143A/H144A}-RFP co-localized less frequently with GFP-Sec4p puncta than wild-type *OSH4*, often adopting instead a diffuse distribution in the cytoplasm. These data suggested that the binding of PI4P by Osh4p promotes the association of Osh4p with exocytic vesicles. Intriguingly, *osh4p*^{Y97F+H143A/H144A}-RFP, which is modeled to be lipid free, associated with exocytic vesicles as frequently as wild-type *OSH4*. Thus, taken together, these results indicate that although lipid binding is not strictly necessary for the association of Osh4p with exocytic vesicles, the competitive binding of PI4P and sterol likely regulates the association of Osh4p with exocytic vesicles.

To independently validate the live cell imaging data, we analyzed Osh4p-exocytic vesicle association by cell fractionation (Fig. 2.7C). We found that Osh4p co-fractionated with the exocytic vesicle markers Sec4p and Bgl2p on buoyant density gradients of membrane

Figure 2.7

Figure 2.7 Lipid binding directs, but is not required for Osh4p association with exocytic vesicles. (A) RFP-Osh4p and GFP-Sec4p visualized by fluorescent microscopy in log-phase *osh4Δ* cells carrying a *CEN* plasmid containing a *RFP*-tagged *OSH4* allele of interest and a second *CEN* plasmid containing *GFP-SEC4* grown at 25°C. Scale bar is 5 microns. Inset is 2x magnification of the main image. Composite is an overlay of Osh4p-RFP signal and GFP-Sec4p signal; white indicates co-localization. Vesicle puncta are defined as GFP-Sec4p positive puncta that are no more than five pixels along one axis and no more than seven pixels along the other, to account for movement of the vesicle during image capture. The micrograph in the first column, third row, was digitally processed to enhance the Osh4p-RFP signal, relative to the other Osh4p-RFP micrographs, to emphasize that Osh4p-RFP was expressed in this cell but more diffusely localized. (B) Quantification of data in (A). Shown is the average of three independent experiments showing the percent co-localization of different Osh4p-RFP alleles of interest with GFP-Sec4p. (C) Immunoblots of 100,000xg pellets of lysate fractions of log-phase *osh4Δ* cells on a continuous 18-34% Nycodenz gradient with sorbitol. Cells contained a *CEN* plasmid with wild-type *OSH4* or the indicated mutant *osh4* allele and were grown at 25°C. Gray brackets indicate the low-density (1.129 g/mL to 1.148 g/mL) vesicle peak, where the exocytic vesicle markers Sec4p and Bgl2p accumulate. (D) Quantification of data in (C). Shown is the average of two independent experiments in which the ratio of the amount of Osh4p to the amount of Sec4p was calculated for the peak Sec4p fraction (Fraction 9 for the top two panels and Fraction 10 for the bottom panel.) (E) Same as in (C), but performed 8 h after methionine washout to de-repress *MET25* promoter. (F) Same as in (D), with Fraction 9 measured in all cases. (For all relevant panels, error bars indicate SEM. (Data were analyzed with a two-tailed Student's t-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.)

preparations made from *osh1* cells containing either plasmid-borne *OSH4* or an allele of *OSH4* that confers a defect in lipid binding. What varied among these gradients, in the fractions containing the peak amount of Sec4p and Bgl2p, was the ratio of Osh4p to Sec4p. The average ratio of Osh4p^{H143A/H144A}, which is defective in PI4P binding, to Sec4p was approximately 50% less than the average ratio of wild-type Osh4p to Sec4p. In contrast, the ratio of Osh4p^{Y97F+H143A/H144A}, which binds neither PI4P nor sterol, or Osh4p^{Y97F}, which is defective in sterol binding, to Sec4p approximated that of wild-type Osh4p (Fig. 2.7 CF). These data are consistent with our *in vivo* observations and suggest that the association of Osh4p with Sec4p-marked exocytic vesicles is an intrinsic property of Osh4p that is only regulated by lipid binding rather than dependent on it.

We also analyzed Osh4p association with the plasma membrane by gradient fractionation (Fig. 2.8 and Suppl. Fig. 2.5). We found that a portion of a wild-type Osh4p pool co-fractionated with plasma membrane (~1% of total Osh4p). In addition we found that PI4P binding deficient Osh4p^{H143A/H144A} accumulated significantly on plasma membrane (~ 6.5 fold relative to wild-type), consistent with the fact that this Osh4p can still bind sterol, a lipid enriched in the plasma membrane (van Meer, Voelker, and Feigenson, 2008). In contrast sterol binding deficient Osh4p^{Y97F} co-fractionated with the plasma membrane at roughly wild-type levels (Fig. 2.8B and Suppl. Fig. 2.5). These observations are inconsistent with the idea that Osh proteins associate with membranes solely based on lipid binding capacity.

We also asked whether lipid binding by Osh4p is required for its localization at sites of polarized exocytosis, in contrast to co-localization with vesicles traversing a cell as shown in Figure 2.7. To this end we looked for localization of wild-type or lipid-binding defective Osh4p-YFP to sites of polarized exocytosis marked with RFP-Sec4p, i.e., small buds (Fig. 2.9A) and mother-bud necks (Fig. 2.9B)(Sheu *et al.*, 2000; Roumanie *et al.*, 2005; Zajac *et al.*, 2005). As expected we found wild-type Osh4p present at these sites, co-localizing with RFP-Sec4p (Fig. 2.9A, B; Alfaro *et al.*, 2011). We also expected lipid-binding defective Osh proteins to localize to these sites with the same allele-specific pattern observed in Figure 2.7. This was the case for Osh4p^{Y97F+H143A/H144A}. Contrary to prediction however,

Figure 2.8 Lipid binding by Osh4p regulates, but is not required for, plasma membrane association. (A) Ratio of the amount of plasma membrane-associated Osh4p to plasma membrane (Sso1 and 2p/total lipid) isolated from clarified lysate of log-phase *S. cerevisiae* cultures, containing an *osh4Δ* strain with a *CEN* plasmid containing wild-type Osh4p or a mutant Osh4p that has a specific lipid binding defect. See Fig. 2.5 legend for methodology. Shown is the average of three independent experiments. (B) Same as (A), except that the expression of the mutant Osh4p was regulated by a methionine-repressible *MET25* promoter. Methionine was washed out of the culture medium 8 h prior to fractionation. (For both panels, error bars indicate SEM. Data were analyzed with a two-tailed Student's t-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.)

Figure 2.9

Figure 2.9 Sterol binding by Osh4p is required for localization to sites of polarized cell growth. (A) YFP-Osh4p and RFP-Sec4p visualized by fluorescent microscopy in small budded log-phase *osh4Δ* *S. cerevisiae* carrying a *CEN* plasmid containing an *YFP* tagged *OSH4* allele of interest and a 2μ plasmid containing *pADHI-RFP-SEC4*, grown at 25°C. Scale bar is 5 microns. Inset is 2X magnification of the main image. Composite is an overlay of Osh4p-YFP signal and RFP-Sec4p signal; yellow indicates co-localization. The micrographs in the first column, second row, in both (A) and (B), were processed to enhance the Osh4p-YFP signal, relative to the other Osh4p-YFP micrographs, to emphasize that Osh4p-YFP was expressed in this cell but more diffusely localized. (B) Same as in A, except examining large-budded cells. (C) Quantification of data in (A and B). Shown is the average of three independent experiments that reported the percentage of cells in which wild-type or mutant Osh4p-YFP co-localized with RFP-Sec4p at the bud tip or bud neck. Error bars indicate SEM. (Data were analyzed with a two-tailed Student's t-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

we found a different allele-specific pattern for two of the Osh mutants. We found PI4P-binding deficient Osh4p^{H143A/H144A} present at sites of polarized exocytosis as often as wild-type Osh4p, indicating that PI4P binding does not promote Osh4p localization at sites of polarized exocytosis (Fig. 2.9). As for the other mutant, we found that sterol binding-deficient Osh4p^{Y97F} did not localize to these sites as often as wild type Osh4p-YFP (Fig. 2.9), consistent with a role for sterol binding by Osh4p at the plasma membrane just prior to vesicle docking.

Discussion

In this study we found that polarized exocytosis depends upon the binding of specific lipid ligands to an Osh protein. In addition, we determined that Osh proteins function in the process of exocytic vesicle docking at the plasma membrane, making this study the first to demonstrate an *in vivo* regulatory role for lipid binding by an OSBP in an essential cellular process and to establish a role for an OSBP in vesicle docking at a target membrane. From our data, we propose below a two-step model for how an Osh protein mediates the maturation and subsequent docking of an exocytic vesicle at the plasma membrane.

Osh4p served as our model for Osh/OSBP function in polarized exocytosis. Although significant evidence points to Osh4p having a role in this cellular process (Kozminski *et al.*, 2006; Alfaro *et al.*, 2011), functional redundancy within the Osh protein family (Beh *et al.*, 2001; Ling *et al.*, 2014; this study) necessitated the use of an *oshΔ* background for some of our assays. Thus, although we can conclude from these assays that an Osh protein has a role in a given cellular process, in a lipid dependent-manner, we can only state in the context of these assays that Osh4p is sufficient, rather than necessary.

Because of the strains *in extant* at the beginning of our study, and the ability to generate new strains limited by the number of usable selectable markers, we were unable to test directly whether other Osh family members could also support the cellular processes examined. If Osh6p, for example, which binds PI4P and phosphatidylserine (Maeda *et al.*, 2013), can substitute in these processes for Osh4p, which binds PI4P and sterol (Im *et al.*, 2005; de St. Jean *et al.*, 2012), in the absence of other Osh proteins, this would suggest that Osh-dependent regulation is driven by lipid exchange rather than lipid identity. We anticipate that newly developed gene editing techniques will allow, for future studies of a given cellular process, the generation of strains amenable to answering questions of necessity and sufficiency for each Osh family member.

An additional caveat we recognize in our study is that the *osh4-1^{ts}* allele may only be defective in a subset of functions. That is, *osh4-1^{ts}* may not be truly null at non-permissive temperatures. This possibility is significant when wild-type *OSH4* and *osh4-1^{ts}* display no

phenotypic difference in an *oshΔ* background at a non-permissive temperature, for example when the exocytosis of invertase was assayed. In these cases, we can only state, strictly speaking, that a role for Osh4p in a given cellular process was not found, rather than excluding a role for Osh4p in the process. Even with this caveat, *osh4-1^{ts}* has proven in this study and others to be a valuable tool for providing insight into Osh protein function (Beh and Rine, 2004; Im *et al.*, 2005; Kozminski *et al.*, 2006; Alfaro *et al.*, 2011; Georgiev *et al.*, 2011; Stefan *et al.*, 2011).

A two-step model for Osh protein-dependent regulation of polarized exocytosis

Based on our results and data in the literature we propose a two-step model of Osh protein activity in polarized exocytosis (Figure 2.10). When vesicles bud from the *trans*-Golgi network they are enriched with PI4P and are marked by the Rab Ypt32p (Ortiz *et al.*, 2002; Strahl and Thorner, 2007). Before these vesicles can dock and fuse with the plasma membrane, it appears that they must undergo a maturation process that involves a change of molecular identity.

In the first step of the model (Fig. 2.10), an Osh protein, in this case Osh4p, is required for PI4P removal from the vesicle membrane to facilitate the loading of Sec4p onto the vesicle and produce docking competent vesicles, as proposed by Novick and colleagues (Ling *et al.*, 2014). Our data provides support for and fulfills predictions of this model. Firstly, a lipid-free Osh4p associates with exocytic vesicles (Fig. 2.7), as one would predict for an Osh protein primed to extract lipids from a membrane. This is also consistent with a PI4P requirement for Osh4p-endomembrane association (Mousley *et al.*, 2012). Secondly, in accord with prediction, PI4P-binding deficient Osh4p is not sufficient to support polarized exocytosis (Figs. 2.1E and 2.2A). Thirdly, expression of a PI4P-binding deficient Osh4p protein ameliorates the clustering of docking competent (Sec4p-positive) vesicles (Fig. 2.6), which is expected if mutant Osh4p does not remodel the lipid composition of vesicles and the number of docking competent vesicles decreases. All these observations are consistent with the first step of our model in which Osh4p promotes vesicle maturation, as posited by Novick and colleagues (Ling *et al.*, 2014). However, it is important to note that vesicle

Figure 2.10. Two step model for Osh protein function in polarized exocytosis. In the first step of the model, Osh proteins are required for vesicle maturation by removing PI4P from the vesicle membrane to facilitate the loading of Sec4p onto the vesicle, thereby producing a docking competent vesicle. In the second step of the model, at sites of polarized cell growth, Osh-bound PI4P exchanges for sterol in the plasma membrane to promote an efficient transition from an initial docking state, presumably exocyst-dependent, to one that is mediated by the formation of trans-SNARE complexes. After SNARE-mediated vesicle fusion with the plasma membrane, Osh4p is recycled from the plasma membrane.

maturation, including the loading of Sec4p onto vesicles, occurs to some degree in the absence of Osh protein activity (Alfaro *et al.*, 2011). This observation suggests a later, second, Osh-dependent step in polarized exocytosis.

The existence of a second step is inferred from a series of observations. First, Alfaro *et al.* (2011) showed that in the absence of Osh protein family activity, Sec4p positive puncta accumulate in the cell, indicating that GFP-Sec4p loaded onto vesicles. Further that same study showed that in the absence of Osh protein activity vesicles transited into the bud, indicating that the myosin Myo2p was properly attached to the vesicle, and that Sec5p-GFP puncta arrived at the plasma membrane, indicating that the exocyst is assembled on the vesicle. Neither event would have occurred without Sec4p bound to the vesicle (Guo *et al.*, 1999; Santiago-Tirado *et al.*, 2011). Furthermore, we show in this study that *osh4-1^{ts}* at restrictive temperature promotes vesicle cluster formation, an event that is dependent on Sec4p loading onto a vesicle (Fig. 2.6). These observations collectively indicate that the proposed role of Osh4p in the removal of vesicular PI4P to facilitate Sec4p loading onto a vesicle is complete in the absence of functional Osh proteins.

For the second step of the model we propose that Osh4p-bound PI4P must be exchanged for plasma membrane sterol for vesicles to efficiently transition from a state of exocyst-mediated tethering to a state of SNARE-mediated docking in order for vesicle-plasma membrane fusion to proceed. Existence of these two states was proposed by Merz and colleagues (Lo *et al.*, 2011). At the start of the second step in the model, a docking competent Sec4p positive vesicle already exists, having transited to the site of polarized exocytosis as it matured. When the vesicle with PI4P-bound Osh4p associates with the plasma membrane, in essence forming a membrane contact site, albeit transient, Osh4p exchanges its bound PI4P for plasma membrane sterol (Fig. 2.10). Examples of OSBP-mediated PI4P-sterol exchange at membrane contact sites have been found in mammalian cells (Mesmin *et al.*, 2013; Chung *et al.*, 2015). We posit that lipid exchange occurs after the exocyst tethers a vesicle to the plasma membrane, though further study is needed to validate this idea. This terminal lipid exchange event serves as a spatial signal to indicate that the vesicle is at a proper distance to dock at the target membrane, one highly enriched

in sterol. Thus, an Osh protein in this capacity would serve as a spatial regulator of *trans*-SNARE complex formation.

A less direct interpretation of available data, though a formal possibility, is that an Osh protein is regulating *cis*-SNARE complex disassembly. We consider this a less likely possibility because it was observed previously that an Osh deficiency increases the dwell time of exocytic vesicles subjacent to the plasma membrane (Alfaro *et al.*, 2011). If SNARE complex disassembly was Osh-dependent, we would predict wild-type and *osh4-1^{ts}* cells to have equivalent vesicle dwell times post-temperature shift, at least until the available pool unassembled SNAREs is exhausted. Moreover, our data do not point to a membrane fusion defect. Such a defect would be expected if SNAREs became limiting. Further studies will be required to discern the relationship of Osh4p or other Oshs to SNAREs and SNARE-associated proteins.

The existence of a terminal lipid exchange event is support by a number of observations from this and previous studies. Firstly, Drin and colleagues showed that Osh4p can in fact exchange PI4P and sterol between membranes (de St. Jean *et al.*, 2012). Secondly and as noted earlier, vesicle dwell time at the plasma membrane increases in the absence of Osh family activity, suggesting Osh proteins may need to function at the plasma membrane for efficient exocytosis (Alfaro *et al.*, 2011). Thirdly, this study has shown that the absence of Osh protein activity does lead to a defect in vesicle docking, which places the observed exocytosis defect at the plasma membrane in proximity to where the lipid exchange event would occur, but temporally after Sec4p loads onto vesicles (Fig. 2.5). Fourth, we observed that sterol binding deficient *osh4p^{Y97F}* did not accumulate at sites of exocytosis to the same extent as sterol binding competent Osh4p (Fig. 2.9). This observation suggests that sterol binding by Osh4p is required for the interaction of Osh4p with the plasma membrane at sites of exocytosis, consistent with a model in which lipid exchange by Osh4p at the plasma membrane needs to occur for successful vesicle docking to occur.

Other lipids enriched at the plasma membrane could be involved in facilitating vesicle docking at sites of polarized exocytosis as well. PS-PI4P exchange by Osh6p or Osh7p

could serve the same role as sterol-PI4P exchange by Osh4p because PS is enriched at sites of polarized exocytosis in *S. cerevisiae* just as sterol is enriched at sites polarized exocytosis in *S. pombe* (Tiedje, *et al*, 2007; Fairn *et al.*, 2011; Makushok *et al.*, 2016). These observations imply a significant role for lipids as mediators of polarized exocytosis.

If vesicle associated Osh4p must exchange its bound PI4P for plasma membrane sterol why doesn't the sterol binding deficient Osh4p^{Y97F} lead to vesicle accumulation? This apparent inconsistency can be explained by the nature of the *osh4*^{Y97F} mutation. Because *osh4*^{Y97F} is a dominant allele that is hyperactive, it may be interfering with an unknown upstream process that leads to a block in vesicle formation (Alfaro *et al.*, 2011). This idea is consistent with the absence of vesicle accumulation in cells expressing *osh4*^{Y97F} (Fig. 2.2B).

One essential function the Osh proteins share is support of polarized exocytosis, a function that is only lost upon removal of all functional Osh proteins (Kozminski *et al.*, 2006). Therefore it can be inferred that all seven Osh proteins must contribute to polarized exocytosis to some extent and that, even if Osh4p is not the primary exocytic Osh protein, Osh4p is clearly sufficient to fill the role and serve as a model for Osh protein function in polarized exocytosis. As polarized exocytosis is a conserved essential function and the OSBPs are a conserved protein family we anticipate that other studies will identify a role for OSBPs in exocytosis in other cell types.

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Supplemental Tables

Supplemental Table 2.1 Representative measurements of immunoblot band intensity from a SNARE assembly assay (Figure 2.3).

		<i>oshΔ HA-SNC2 [osh4-1^{ts}]</i>				:Second Plasmid
		<u>[<i>OSH4</i>]</u>		<u>[<i>Vector</i>]</u>		
Time at 37°C (Min)		0	75	0	75	
Clone 1	Signal Intensity (AU) Anti-Sso1/2p (R) 1:1000	268000	185000	93500	102000	
	Signal Intensity (AU) Anti-HA (M) 1:2000	214000	130000	66900	79300	
	Sso1/2p:Ha-Snc2p Ratio	1.252	1.423	1.397	1.286	
	t=0 set to 1	1	1.136	1	0.920	
Clone 2	Signal Intensity (AU) Anti-Sso1/2p (R) 1:1000	90400	101000	69200	168000	
	Signal Intensity (AU) Anti-HA (M) 1:2000	226000	207000	31200	163000	
	Sso1/2p:Ha-Snc2p Ratio	0.895	1.091	2.217	1.030	
	t=0 set to 1	1	1.219	1	0.464	
Clone 3	Signal Intensity (AU) Anti-Sso1/2p (R) 1:1000	11400	8780	103000	45800	
	Signal Intensity (AU) Anti-HA (M) 1:2000	219000	239000	239000	175000	
	Sso1/2p:Ha-Snc2p Ratio	0.052	0.036	0.430	0.261	
	t=0 set to 1	1	0.705	1	0.6072	
Average of t=0 set to 1		1	1.020	1	0.664	

Supplemental Table 2.2 *S. cerevisiae* strains used in this study

Strain	Relevant Genotype	Source	Alias
SEY6210	<i>MAT a ura3-52 his3Δ200 lys2-801am leu2-3,112 trp1Δ901 suc2Δ9</i>	Robinson <i>et al.</i> , 1988	KKY278
HAB821	SEY6210 <i>kes1/osh4Δ::HIS3</i>	Jiang <i>et al.</i> , 1994	KKY501
CBY791	SEY6210 <i>osh1Δ::kanMX4 osh3Δ::LYS2 osh4Δ::HIS3 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3</i>	C. Beh ^a	KKY1302
CBY803	SEY6210 <i>TRP1::P^{MET3}-OSH2 osh1Δ::kanMX4 osh2Δ::kanMX4 osh3Δ::LYS2 osh4Δ::HIS3 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3</i>	Beh <i>et al.</i> , 2001	KKY700
CBY924	SEY6210 <i>osh1Δ::kanMX4 osh2Δ::kanMX4 osh3Δ::LYS2 osh4Δ::HIS3 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3</i> [pCB254]	Beh and Rine, 2001	KKY279
CBY926	SEY6210 <i>osh1Δ::kanMX4 osh2Δ::kanMX4 osh3Δ::LYS2 osh4Δ::HIS3 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3</i> [pCB255]	Beh and Rine, 2001	KKY280
NY3	<i>MAT a sec1-1 ura3-52</i>	Novick, Field, and Sheckman, 1980	KKY531
NY13	<i>MAT a ura3-53</i>	Novick, Field, and Sheckman, 1980	KKY802
NY17	<i>MAT a sec6-4 ura3-52</i>	Novick, Field, and Sheckman, 1980	KKY533
KKY1240	SEY6210 <i>OSH4-RFP::HIS3</i>	This Study	
KKY1287	NY13 <i>6xHA-SNC2</i>	This Study	
KKY1288	NY17 <i>6xHA-SNC2</i>	This Study	
KKY1289	CBY924 <i>6xHA-SNC2</i>	This Study	
KKY1290	CBY926 <i>6xHA-SNC2</i>	This Study	

^a a Kind gift of C. Beh (Simon Fraser University, Burnaby, BC)

Supplemental Table 2.3 Plasmids used in this study

Plasmid	Relevant Genotype	Source
pAD54-RFP-SEC4	pAD54 (<i>pADH1-RFP-SEC4</i>)	Aronov <i>et al.</i> , 2007 ^a
pCB231	pRS316 (<i>OSH4</i>)	Beh and Rine, 2004
pCB254	pRS414 (<i>osh4-I^{ts}</i>)	Beh and Rine, 2001
pCB255	pRS414 (<i>OSH4</i>)	Beh and Rine, 2001
pCB662	pRS426 (<i>pMET25-osh4^{Y97F}</i>)	Im <i>et al.</i> , 2005
pCB866	<i>2μ URA3 MX-OSH4-YFP::HIS3-MX</i>	C. Beh ^b
pKK1950	pRS316 (<i>osh4^{H143A/H144A}</i>)	This Study
pKK1988	pRS316 (<i>osh4^{Y97F+H143A/H144A}</i>)	This Study
pKK1990	pRS316 (<i>pMET25-osh4^{Y97F}</i>)	This Study
pKK2005	pRS316 (<i>pMET25</i>)	This Study
pKK2012	pRS316 (<i>SUC2</i>)	This Study
pKK2089	pRS316 (<i>pMET25-osh4^{Y97F}-YFP</i>)	This Study
pKK2092	pRS316 (<i>osh4^{Y97F+H143A/H144A}-YFP</i>)	This Study
pKK2093	pRS316 (<i>osh4^{H143A/H144A}-YFP</i>)	This Study
pKK2094	pRS316 (<i>OSH4-YFP</i>)	This Study
pKK2107	pRS316 (<i>pMET25-OSH4-RFP</i>)	This Study
pKK2108	pRS414 (<i>pMET25-OSH4-RFP</i>)	This Study
pKK2109	pRS414 (<i>OSH4-RFP</i>)	This Study
pKK2111	pRS414 (<i>pMET25-osh4^{Y97F}-RFP</i>)	This Study
pKK2112	pRS414 (<i>osh4^{H143A/H144A}-RFP</i>)	This Study
pKK2113	pRS414 (<i>osh4^{Y97F+H143A/H144A}-RFP</i>)	This Study
pOM12	<i>6XHA K.1 URA3 AmpR</i>	Gauss <i>et al.</i> , 2005
pRC2098	pRS316 (<i>GFP-SEC4</i>)	Calero <i>et al.</i> , 2003 ^c
pRS316	<i>CEN URA3</i>	Sikorski and Hieter, 1989
pRS414	<i>CEN TRP1</i>	Sikorski and Hieter, 1989

a. Kind gift of J. Gerst (Weizmann Institute of Science, Rehovot, Israel)

b. Kind gift of C. Beh (Simon Fraser University, Burnaby, BC)

c. Kind gift of R. Collins (Cornell University, Ithaca, NY)

Supplemental Table 2.4 Oligonucleotides used in this study

Name	Sequence
oKK193	GTCCTTGCTATCTTTAGAG
oKK295	TTAAAATCTCAATTCTGTTCTCG
oKK296	CGAGAACAGAATTGAGATTTTAA
oKK319	ATGCGAGCTCGATATGAATTATTCTTCAAAACATTC (SacI underlined)
oKK320	ATGCCTCGAGCCCTCACTACTTCTTTTTGAGAAC (XhoI underlined)
oKK322	ATGCCTCGAGTTAGGCGCCGGTGGAGTGG (XhoI underlined)
oKK355	CATACATTCGAAACACTTCCAAATACAAAATAA GAACGCGCAACGATGTGCAGGTCGACAACCCTTAAT
oKK356	ACTCTCCTCTGGAGGCACATATGGATCGTATGGC ACTGATGACGAGCGGCCGCATAGGCCACT
oKK357	CAGTGAATAGTATCTGTAAGTC
oKK358	CTCTCATTATTCCCACCGTGTC
oKK367	ATGC <u>ACTAGT</u> ATGTCTCAATACGCAAGCTCATC (SpeI underlined)
oKK369	ATGCCTCGAGTTACTTGTACAGCTCGTCCATG (XhoI underlined)

Supplemental Figures

Supplemental Figure 2.1 Diameters of vesicles in thin section electron micrographs of *S. cerevisiae*. Vesicle diameters were measured in *oshΔ* cells containing two *CEN* plasmids, the first of which contained *osh4-I^{ts}* and the second no insert (vector), *osh4*^{H143A/H144A}, or *osh4*^{Y97F+H143A/H144A}. Vesicle diameters were measured with Image J. Vesicles in clustered with other vesicles were not scored.

A **B**

Supplemental Figure 2.2. Amount of Sso1 and 2p on the plasma membrane varies with *OSH4* allele expressed . (A) Shown is the average ratio, from four independent experiments, of the amount of t-SNAREs Sso1 and 2p, as measured by immunoblotting, to total membrane, as determined by FM4-64 binding, in a plasma membrane fraction. Plasma membrane was isolated by step gradient fractionation (see Supplemental Figure 2.5A) from clarified lysate of log-phase *S. cerevisiae oshΔ* cells containing a *CEN* plasmid-borne temperature-sensitive *osh4* allele (*osh4-1^{ts}*) and a second plasmid containing an *osh4* allele of interest, at the times indicated after shift of the cultures from 25°C to 37°C. (B) Same as in (A), except results are the average of three experiments in which a plasma membrane fraction was isolated from an *oshΔ* strain with a *CEN* plasmid-borne wild-type *OSH4* and a second plasmid containing an *osh4* allele of interest regulated by a methionine-repressible *MET25* promoter. Cells were grown in the presence or absence of methionine for 8 h at 25°C prior to fractionation. Data are standardized to time 0. (For both panels, error bars indicate SEM. Data were analyzed with a two-tailed Student's t-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.)

Supplemental Figure 2.3. Diameters of vesicles in vesicle clusters, observed in thin section electron micrographs of *S. cerevisiae*. Vesicle diameters were measured in *osh4* cells containing two *CEN* plasmids, the first of which contained *osh4-1^{ts}* and the second no insert (vector), *osh4^{H143A/H144A}*, or *osh4^{Y97F+H143A/H144A}*. Vesicles were scored as either randomly distributed within the cell (Non-Clustered Vesicles) or as found in clusters of 5 or more vesicles abutting each other (Clustered Vesicles). See Figure 2.6 for example of clustered vesicles.

Supplemental Figure 2.4 Sec4p positive structures accumulate in cells with vesicle clusters. Sec4p visualized by indirect immunofluorescence in log-phase *oshΔ S. cerevisiae* with a *CEN* plasmid-borne temperature-sensitive *osh4* allele (*osh4-1^{ts}*) and a second plasmid containing an *osh4* allele of interest, grown at 25°C or shifted from 25°C to 37°C for 90 min. Arrows (see [Vector] 37°C) indicate irregularly shaped accumulations of Sec4p indicative of the vesicle clusters observed by EM (Fig. 2.6). Scale bar is 5 microns. Inset is a 2x magnification of the main image.

Supplemental Figure 2.5 Lipid binding by Osh4p regulates, but is not required for, plasma membrane association. (A) Percent of total Osh4p associated with the plasma membrane. Plasma membrane was isolated from clarified lysate of log-phase *S. cerevisiae* cultures, containing an *osh4Δ* strain with a *CEN* plasmid containing wild-type Osh4p or a mutant Osh4p that has a specific lipid-binding defect. Whole cell extracts were made from the same strains and used to calculate total Osh4p. Shown is the average of four independent experiments. (B) Same as (A), except that the expression of the mutant Osh4p was regulated by a methionine-repressible *MET25* promoter. Methionine was washed out of the culture medium 8 h prior to fractionation. For both panels, error bars indicate SEM. (Data were analyzed with a one-tailed Student's t-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.)

Chapter 3: Functional Analysis of the ALPS Domain in *S. cerevisiae* OSBP homologue 4 (Osh4p)

The following publication was used as the basis for Chapter 3. The figures and tables have been renumbered to maintain sequence with other figures and tables in this dissertation.

Smindak RJ, Kozminski KG (2017). Members of the OSBP Homologue (Osh) Protein Family in *S. cerevisiae* Require Specific Lipids to Regulate Polarized Exocytosis. *PloS One*. (In preperation)

Author Contributions:

Keith Kozminski, discussion of data and editing of manuscript

Abstract

A wide range of cellular events require polarized exocytosis including, but not limited to, the formation of polarized epithelia in the intestine and the formation of new daughter cells in yeast. In the budding yeast, *S. cerevisiae*, the oxysterol binding protein homologue (Osh) family is required for polarized exocytosis. In the absence of other Osh family members, Osh4p is sufficient for polarized exocytosis, though in a manner that requires lipid binding. While known that the Osh4p ALPS domain influences sterol binding and the localization of Osh4p to the Golgi what, if any role, this protein domain has in Osh-dependent polarized exocytosis is unclear. In this study we analyzed the function of the ALPS domain of Osh4p in polarized exocytosis. We show that the Osh4p ALPS domain is essential for Osh4p function in polarized exocytosis. We also show that the Osh4p ALPS domain inhibits Osh4p localization to exocytic vesicles and sites of polarized exocytosis on the plasma membrane. We propose the Osh4p ALPS domain inhibits Osh4p-mediated lipid extraction and deposition from cellular membranes, through which it inhibits Osh4p vesicle and plasma membrane association.

Introduction

Oxysterol binding proteins (OSBP) are a family of lipid binding proteins conserved from yeast to humans (Beh *et al.*, 2012). Structurally OSBPs consist of a lipid binding β -barrel closed at one end and open at the other with a lid that can cover the open end of the β -barrel (Im *et al.*, 2005). The lid itself consists of the N-terminus of the protein to the beginning of the β -barrel and can be either short, enough to cover the open end of the β -barrel, or long with a portion that can cover the open end of the β -barrel while also containing other protein domains such as the phosphatidylinositol 4,5-bisphosphate binding PH domain (Beh *et al.*, 2012). There are seven OSBP homologues in yeast, known collectively as Osh proteins and they are required for the essential process of polarized exocytosis (Beh *et al.*, 2012; Kozminski *et al.*, 2006). One of the yeast OSBPs, Osh4p, localizes to exocytic vesicles, from which it likely exerts its essential activity in support of polarized exocytosis (Im *et al.*, 2005; Beh *et al.*, 2001; Alfaro *et al.*, 2011; de St. Jean *et al.*, 2011). A later study showed that lipid ligand binding by Osh4p is required for polarized exocytosis when cells are dependent on Osh4p for Osh protein activity (Smindak *et al.*, 2017). In the case of Osh4p, the lid over the β -barrel is an ALPS domain, and the functional role of the lid in Osh4p-dependent polarized exocytosis has not been explored (Smindak *et al.*, 2017). ALPS domains are amphipathic peptide α -helices that associate with highly curved membranes because of the lipid packing defects caused by high membrane curvature (Drin *et al.*, 2007; Bigay *et al.*, 2005). ALPS domains form into an amphipathic α -helix upon interacting with the membrane surface and insert the hydrophobic r -groups of their amino acids into gaps between membrane lipids (Bigay *et al.*, 2005). Notably, *in vivo*, this secondary structure leads to the localization of ALPS domain-containing proteins to the Golgi apparatus due to lipid packing defects in the membrane of that organelle (Parnis *et al.*, 2006). Because of the role of ALPS domains in membrane association, we hypothesized that the Osh4p ALPS domain is essential for Osh4p function in polarized exocytosis and that the Osh4p ALPS domain would contribute to the localization of Osh4p to exocytic vesicles.

We found that the Osh4p ALPS domain is required for Osh4p function in polarized exocytosis, however it is not required for vesicle association, despite the curvature of that

organelle. We observed increased Osh4p vesicle and plasma membrane association when Osh4p did not have an ALPS domain. This observation, in conjunction with previously conducted sterol and PI4P binding studies with Osh4p missing its ALPS domain has led us to propose that the Osh4p ALPS domain inhibits lipid binding to provide temporal control of Osh4p function in polarized exocytosis.

Methods and Materials

Strains, Culture Media and Growth Conditions

All *S. cerevisiae* strains used in this study are described in Table 3.1. *S. cerevisiae* strains were grown in minimal media at 25°C unless otherwise stated (Sherman *et al.*, 1986). For all experiments, at least two independent clones or transformants were analyzed.

Table 3.1 *S. cerevisiae* strains used in this study

Strain	Relevant Genotype	Source	Alias
SEY6210	<i>MAT α ura3-52 his3Δ200 lys2-801am leu2-3,112 trp1Δ901 suc2Δ9</i>	Robinson <i>et al.</i> , 1988	KKY278
CBY926	SEY6210 <i>osh1Δ::kanMX4 osh2Δ::kanMX4 osh3Δ::LYS2 osh4Δ::HIS3 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3</i> [pCB255]	Beh <i>et al.</i> , 2001	KKY280
HAB821	SEY6210 <i>kes1/osh4Δ::HIS3</i>	Jiang <i>et al.</i> , 1994	KKY501
KKY1240	SEY6210 <i>OSH4-RFP::HIS3</i>	Smindak <i>et al.</i> , 2017	
KKY1290	CBY926 <i>6xHA-SNC2</i>	Smindak <i>et al.</i> , 2017	

Plasmids

All plasmids and oligonucleotides used in this study are described in Tables 3.2 and 3.3, respectively.

To construct *pRS316-osh4^{Δ29}* (pKK 2090), the *SacI*/*EcoRI* fragment of *pRS316-OSH4* (pCB231) was removed and replaced by a *SacI*/*EcoRI* fragment of synthesized *OSH4* (IDT, Coralville, IA) missing the first 29 codons. The *EcoRI* site is 729 bp 3' of the endogenous translational start site of the wild type *OSH4* coding sequence and the *SacI* site is 608 bp 5' of the endogenous translational start site

The *pRS316-osh4^{Δ29}-YFP* (pKK2091) plasmid was made as follows. Using primers oKK367 and oKK369, *OSH4-YFP* was amplified by PCR from plasmid pCB866. The PCR product was cloned into the *SpeI* and *XhoI* sites of *pRS316-pMET25* (pKK2005), forming *pRS316-pMET25-OSH4-YFP* (pKK2082). Following this, the *pMET25* promoter was removed by *SacI*/*EcoRI* digest and replaced with a *SacI*/*EcoRI* fragment from a synthesized piece of DNA containing *pOSH4-osh4* without the first 29 codons of *OSH4* (IDT, Coralville, IA).

The *pRS414-osh4^{Δ29}-RFP* (pKK2110) plasmid was made as follows. First, using primers oKK367 and oKK322, *OSH4-RFP* was amplified by PCR from KKY1240. The PCR product was cloned into the *SpeI* and *XhoI* sites of *pRS316-pMET25* (pKK2005), forming *pRS316-pMET25-OSH4-RFP* (pKK2107). The *pRS316-pMET25-OSH4-RFP* *SacI*/*KpnI* fragment was the subcloned into *pRS414* forming *pRS414-pMET25-OSH4-RFP* (pKK2108). Following this, the *pMET25* promoter was removed by *SacI*/*EcoRI* digest and replaced with a *SacI*/*EcoRI* fragment a synthesized piece of DNA containing *pOSH4-osh4* without the first 29 codons of *osh4* (IDT, Coralville, IA).

Table 3.2. Plasmids used in this study

Plasmid	Relavent Genotype	SOURCE
pAD54-RFP-SEC4	pAD54 (<i>pADH1-RFP-SEC4</i>)	Aronov <i>et al.</i> , 2007 ^a
pCB231	pRS316 (<i>OSH4</i>)	Beh <i>et al.</i> , 2004
pCB254	pRS414 (<i>osh4-1</i>)	Beh <i>et al.</i> , 2004
pCB255	pRS414 (<i>OSH4</i>)	Beh <i>et al.</i> , 2004
pCB866	<i>2μ URA3 MX-OSH4-YFP::HIS3-MX</i>	C. Beh ^b
pKK2005	pRS316 (<i>pMET25</i>)	Smindak <i>et al.</i> , 2017
pKK2082	pRS316 (<i>pMET25-OSH4-YFP</i>)	Smindak <i>et al.</i> , 2017
pKK2090	pRS316 (<i>osh4^{Δ29}</i>)	This Study
pKK2091	pRS316 (<i>osh4^{Δ29}-YFP</i>)	This Study
pKK2094	pRS316 (<i>OSH4-YFP</i>)	Smindak <i>et al.</i> , 2017
pKK2107	pRS316 (<i>pMET25-OSH4-RFP</i>)	Smindak <i>et al.</i> , 2017
pKK2108	pRS414 (<i>pMET25-OSH4-RFP</i>)	Smindak <i>et al.</i> , 2017
pKK2109	pRS414 (<i>OSH4-RFP</i>)	Smindak <i>et al.</i> , 2017
pKK2110	pRS414 (<i>osh4^{Δ29}-RFP</i>)	This Study
pOM12	<i>6XHA K.l URA3 AmpR</i>	Guass <i>et al.</i> , 2005
pRC2098	pRS316 (<i>GFP-SEC4</i>)	Calero <i>et al.</i> , 2003 ^c
pRS316	<i>CEN URA3</i>	Sikorski and Hieter, 1989
pRS414	<i>CEN TRP1</i>	Sikorski and Hieter, 1989

a Kind gift of J. Gerst (Weizmann Institute of Science, Rehovot, Israel)

b Kind gift of C. Beh (Simon Fraser University, Burnaby, BC)

c Kind gift of R. Collins (Cornell University, Ithaca, NY)

Table 3.3. Oligos used in this study

Name	Sequence
oKK193	GTCCTTGCTATCTTTAGAG
oKK322	ATGCCTCGAGTTAGGCGCCGGTGGAGTGG (XhoI underlined)
oKK355	CATACATTCGAAACACTTCCAAATACAAAATAA GAACGCGCAACGATGTGCAGGTCGACAACCCTTAAT
oKK356	ACTCTCCTCTGGAGGCACATATGGATCGTATGGC ACTGATGACGAGCGGCCGCATAGGCCACT
oKK357	CAGTGAATAGTATCTGTAAGTC
oKK358	CTCTCATTATTCCCACCGTGTC
oKK367	ATGC <u>ACTAGT</u> ATGTCTCAATACGCAAGCTCATC (SpeI underlined)
oKK369	ATGCCTCGAGTTACTTGTACAGCTCGTCCATG (XhoI underlined)

Bgl2p Accumulation Assay

To assay cells for the internal accumulation of Bgl2p, we used the protocol of (Smindak *et al.*, 2017).

Whole Cell Extracts and Cell Fractionation

Performed as per (Alfaro *et al.*, 2011; Smindak *et al.*, 2017).

Fluorescence Microscopy

Dual channel microscopy for colocalization studies was performed on a Zeiss Axiovert S.100 microscope with a 100X (Plan-Apochromat, N.A. 1.4) objective with a Hamamatsu EM-CCD (C9100 13) ImageM camera (Hamamatsu Photonics, Japan). Bleed through between channels was not detected under the conditions used. Images were processed using Image J (NIH) to adjust brightness and contrast uniformly.

Growth Assays

0.025 OD₆₀₀ units of cells grow at 25°C were resuspended in 50 µL of minimal medium in a 48 well titer plate. Cultures were then serially diluted 1:10, 1:100 and 1:1000 prior to plating. In all cases, cells were grown for 5 d on minimal medium at indicated temperature prior to analysis.

Immunoblots

Immunoblots were performed as in (Smindak *et al.*, 2017) with the following modification. When analyzing osh4p^{Δ29}, Biorad Any kDtm 12 lane gels (BioRad, 4569035, Hercules, Ca) were used.

Results and Discussion

The ALPS Domain Is Necessary for Osh4p Function in Polarized Exocytosis

A previous study showed that an *osh4* allele that encodes an Osh4p protein lacking the first 29 amino acids (*osh4p*^{Δ29}), the Osh4p ALPS domain, was sufficient to support cell growth, when expressed on a high copy number plasmid (Im *et al.*, 2005). We wanted to know if, when expressed on a low copy (*CEN*) plasmid, closer to physiological levels, if *osh4*^{Δ29} was sufficient, for cell growth and viability (Supp. Fig 3.1). For this assay, we compared the growth of cells with all seven *OSH* family genes deleted (*oshΔ*) but containing a low copy (*CEN*) plasmid carrying the temperature-sensitive *osh4-1^{ts}* allele, and a second *CEN* plasmid containing wild-type *OSH4*, *osh4*^{Δ29}, or an empty vector. At 37°C these cells are dependent on the second plasmid for viability. The *oshΔ* background was required because it is known that functional redundancy exists among members of the *OSH* gene family (Beh *et al.*, 2001). Cells in which the second plasmid contained wild-type *OSH4* grew at 37°C (Fig. 3.1A), similar to all three strains at 25°C. In contrast, we found that when cells were entirely dependent on *osh4*^{Δ29} no growth occurred, indicating that the Osh4p ALPS domain is in fact required for Osh4p function, when protein levels are more physiological than in previous reports (Fig. 3.1 A) (Im *et al.*, 2005).

Previous studies established that Osh protein activity is required for polarized exocytosis, as is lipid binding by Osh4p, when cells are dependent on Osh4p (Alfaro *et al.*, 2011; Smindak *et al.*, 2017). Because of the latter observation, we asked whether Osh4p also requires its ALPS domain to support polarized exocytosis (Kozminski *et al.*, 2006; Smindak *et al.*, 2017). To this end, we assayed for the exocytosis of Bgl2p, which is a marker of vesicles mediating polarized exocytosis. We found that cells reliant on *osh4*^{Δ29} accumulated Bgl2p internally (Fig. 3.1B), similar to cells without functional Osh proteins (Kozminski *et al.*, 2006; Smindak *et al.*, 2017). This internal accumulation of Bgl2p observed in cells dependent on *osh4*^{Δ29} indicates that the Osh4p ALPS domain is required for polarized exocytosis. Having established that the ALPS domain is necessary for Osh4p to support polarized exocytosis, we next asked if the Osh4p ALPS domain is required for

Figure 3.1: The ALPS domain of Osh4p is required for Osh4p function, including its role in vesicle docking at the plasma membrane. **A)** *S. cerevisiae* dependent on Osh4p lacking its ALPS domain (*osh4p*^{Δ29}) do not grow. Equivalent dilutions of *S. cerevisiae* cultures were grown for 5 d on minimal medium at either 25°C or 37°C. At 37°C, cells are dependent upon the indicated allele for Osh function. **B)** Fold change of internal Bgl2p levels in log-phase cells at 25°C or after shift to 37°C for 90 min, relative to time 0 at 25°C, as measured by immunoblotting. Strains are identical except for the allele on the second plasmid. Tubulin served as a loading control. Shown is the average of 4 or 7 independent experiments for cells carrying an empty vector or *osh4*^{Δ29}, respectively. **C)** Ratio of the amount of v-SNARE (HA-Snc2p) associated with isolated plasma membrane (Sso1 and 2p / total membrane). Amounts of SNAREs were determined by immunoblotting and total membrane by fluorometric measurement of membrane bound FM4-64. Plasma membrane was isolated from clarified lysates of log-phase *S. cerevisiae oshΔ* cells with a *CEN* plasmid-borne temperature-sensitive *osh4* allele (*osh4-I^{ts}*) and a second *CEN* plasmid containing wild-type *OSH4*, or no insert (vector), after growth for 90 min at 25°C or after shift from 25°C to 37°C. Shown is the average of 4 or 3 independent experiments for cells carrying an empty vector or *osh4*^{Δ29} respectively. (Data were analyzed with a one-tailed Student's t-test in **B** and a two tailed Student's t-test in **C**. Error bars indicate SEM. * $p \leq 0.05$, ** $p \leq 0.01$)

Osh4p function in exocytic vesicle docking at the plasma membrane (Smindak *et al.*, 2017). To assay vesicle docking at the plasma membrane we utilized a previously established assay (Smindak *et al.*, 2017) that measures the amount of v-SNARE (HA-Snc2p) associated with the plasma membrane. We found that *osh4*^{Δ29} did not support the association of HA-Snc2p with the plasma membrane, observing a significant decrease in plasma membrane-associated HA-Snc2p when only *osh4*^{Δ29} was available, relative to a strain with wild-type *OSH4* (Fig. 3.1C). This observation suggests that the Osh4p ALPS domain is required for Osh4p to support exocytic vesicle docking at the plasma membrane.

Finally, using Bgl2p exocytosis competent *osh4Δ* cells, we asked whether the Osh4p ALPS domain is required for Osh4p localization to sites of polarized exocytosis (Smindak *et al.*, 2017). We looked for colocalization of YFP-tagged Osh4p and *osh4p*^{Δ29} with RFP-Sec4p at sites of polarized exocytosis in yeast, that is the bud tip and neck (Fig. 3.2). We found that *osh4p*^{Δ29}-YFP colocalized with RFP-Sec4p at sites of polarized exocytosis in a greater percentage of cells than wild type Osh4p-YFP (Fig. 3.2). In addition we observed Osh4p-YFP staining in and near the bud that did not colocalize with RFP-Sec4p (Fig. 3.2A and B). This may be due to a population of Sec4p-negative but Osh4p-positive vesicles near the site of polarized growth or Osh4p-YFP localization to another, Sec4p-negative, organelle near the site of polarized growth. Further analysis will be required to validate either of these hypotheses. This observation suggests an inhibitory role for the Osh4p ALPS domain in localization to regions of polarized exocytosis in yeast.

The ALPS Domain Negatively Regulates the Association of Osh4p with Exocytic Vesicles and the Plasma Membrane

The above data suggest that the ALPS domain is a key requirement for Osh4p to exercise a role in polarized exocytosis. To our surprise however, the ALPS domain is not required for the association of Osh4p with exocytic vesicles in Bgl2p exocytosis competent *osh4Δ* cells (Figs. 3.3A, B). We found that a similar percentage of *osh4p*^{Δ29}-RFP puncta colocalized with Sec4p-GFP puncta as wild-type Osh4p-RFP, indicating that the ALPS domain does not contribute to Osh4p-RFP-exocytic vesicle association. We independently

A

B

C

Figure 3.2 The Osh4p ALPS domain is not required for localization to sites of polarized cell growth. **A)** YFP-Osh4p and RFP-Sec4p visualized by fluorescence microscopy in log phase *osh4Δ* cells carrying a *CEN* plasmid containing a YFP-tagged *OSH4* allele of interest and an additional 2μ plasmid containing *RFP-SEC4* grown at 25°C. Scale bar is 5 microns. Inset is a 2x magnification of the main image. Composite is an overlay of Osh4p-YFP signal and RFP-Sec4p signal, yellow indicates colocalization. **B)** Same as in A, except examining large-budded cells **C)** Quantification of data in (**A and B**), Average of three independent experiments in which wild-type and mutant Osh4p-YFP were colocalized with RFP-Sec4p, after which the percent colocalization of RFP-Sec4p at the bud tip and neck with Osh4p-YFP was calculated for each replicate and averaged. For Osh4p-YFP 82, 74, and 68 were counted and for *osh4p*^{Δ29} 62, 63, and 43 were counted Data were analyzed with a two tailed Student's t-test. Error bars indicate SEM. * $p \leq 0.05$

Figure 3.3

A

B

C

D

Figure 3.3: The ALPS domain of Osh4p does not contribute to vesicle or plasma membrane localization **A)** RFP-Osh4p and GFP-Sec4p visualized by fluorescent microscopy in log phase *osh4Δ* cells carrying a *CEN* plasmid containing an *RFP*-tagged *OSH4* allele of interest and another *CEN* plasmid containing *GFP-SEC4* grown at 25°C. Scale bar is 5 microns. Inset is a 2x magnification of the main image. Vesicle puncta are defined as GFP-Sec4p positive puncta that are no more than 5 pixels along one axis, and no more than seven pixels along the other, to account for vesicle movement during the exposure. **B)** Quantification of data in **(A)**. Average of four for Osh4p-RFP and three for *osh4p*^{Δ29} independent experiments in which different Osh4p-RFP alleles of interest were colocalized with GFP-Sec4p, after which the percent of colocalization of GFP-Sec4p puncta with Osh4p-RFP puncta was calculated for each replicate and then averaged. For Osh4p-RFP 120, 87, 79, and 241 puncta, over 93, 83, 47, and 140 cells were counted and for *osh4p*^{Δ29} 57, 103, and 191 puncta over 37, 61, and 108 cells were counted **C)** Average of two independent cell fractionations for which the ratio of Osh4p or *osh4p*^{Δ29} to Sec4p was calculated in the peak Sec4p buoyant density gradient fraction in an *osh4Δ* strain **D)** Average of three independent experiments in which the plasma membrane was isolated by gradient fractionation and tested for association of Osh4p, expressed from a *CEN* plasmid, after which the ratio of the amount Osh4p to the amount of plasma membrane (Sso1 and 2p/total membrane) was calculated. Plasma membrane was isolated from clarified lysate of log-phase *S. cerevisiae* cultures, containing an *osh4Δ* strain with a *CEN* plasmid containing wild type or mutant *OSH4* (Data were analyzed with a one-tailed Student's t-test in **C** and a two tailed Student's t-test in **B**, **D**, and **F**. Error bars indicate SEM. * $p \leq 0.05$, ** $p \leq 0.01$)

confirmed this result by fractionating cells expressing either wild-type Osh4p or osh4p^{Δ29}. We found more osh4p^{Δ29} present in buoyant density gradient fractions containing Sec4p and Bgl2p-marked exocytic vesicles than wild-type Osh4p, even after normalizing for Osh4p and osh4p^{Δ29} expression levels (Fig. 3.3C; Supp. Fig. 3.1). Collectively these data indicate that the ALPS domain of Osh4p is not required for association of Osh4p with exocytic vesicles, and that in fact the ALPS domain may inhibit the association of Osh4p with exocytic vesicles.

Next we tested for the ability of osh4p^{Δ29} to associate with the plasma membrane using cell fractionation in the same strains. We found that osh4p^{Δ29} is present at greater levels in the plasma membrane fraction than wild-type Osh4p (Fig. 3.3D), suggesting an inhibitory role for the Osh4p ALPS domain in plasma membrane association.

The ALPS Domain Is a Protein Domain that Temporally Regulates Osh4p Activity

We propose that the ALPS domain of Osh4p, and further the “lid” over the lipid-binding domain of other OSBPs, negatively regulates lipid binding and extraction from membranes by Osh4p to provide temporal regulation to Osh4p function (Im *et al.*, 2005; von Filseck *et al.*, 2015). Several lines of evidence presented here and previously in the literature support this claim.

The first line of evidence supporting this hypothesis comes from a 2005 study that showed that in a low stringency condition, i.e. no detergent added, osh4p^{Δ29} binds cholesterol at higher affinity than wild-type Osh4p (Im *et al.*, 2005). This observation suggested that the ALPS domain inhibits sterol binding to some extent, perhaps by occluding the lipid-binding pocket. Additionally, and paradoxically, that same study showed that in a higher stringency condition, i.e. in the presence of non-ionic detergent, osh4p^{Δ29} bound cholesterol with less affinity than wild-type Osh4p (Im *et al.*, 2005). The release of sterol into the cytosol, a hydrophilic environment, would be unlikely, but would have been more likely in the high stringency conditions used *in vitro* (Im *et al.*, 2005). It should be noted that it is possible that detergent could enter the lipid binding pocket and inhibit lipid binding by doing so, thus repeating this experiment in the presence of a lipid Osh4p cannot bind may provide a more accurate assessment of the Osh4p ALPS domain in

lipid binding. Thus, it appears osh4p^{Δ29} is able to load and unload sterols at greater rates than wild-type Osh4p (Im *et al.*, 2005). However, the on- and off-rates of sterol and PI4P binding to Osh4p has not been calculated and should be to confirm this hypothesis. A later 2015 study built upon these results and showed that osh4p^{Δ29} can only extract sterol from membranes at ~25% of wild type capacity (von Filseck *et al.*, 2015). Moreover, osh4p^{Δ29} possesses greatly reduced PI4P extraction capacity relative to wild-type and almost no ability to transfer lipids between membranes, supporting the view that the Osh4p ALPS domain is required for extraction of lipids from membranes (von Filseck *et al.*, 2015).

Second, the observations made in this study are consistent with those made by Im *et al.* and von Filseck *et al.* in (Im *et al.*, 2005; von Filseck *et al.*, 2015) and support the hypothesis that the “lid” negatively regulates lipid binding. We show, both by gradient fractionation and analysis of fluorescent images, that osh4p^{Δ29} is present on exocytic vesicles and the plasma membrane to a greater extent and in greater amounts than wild-type Osh4p (Fig. 3.2 and Fig. 3.3A, B, C, D). Increased osh4p^{Δ29} association with the vesicle or plasma membrane is consistent with the reported role for the Osh4p ALPS domain in inhibiting lipid deposition but promoting lipid extraction (von Filseck *et al.*, 2015). It would be expected that osh4p^{Δ29} would be enriched at locations where it could deposit its bound lipid because upon deposition osh4p^{Δ29} would not be able to efficiently extract the reciprocal lipid, and therefore osh4p^{Δ29} would remain on that membrane until lipid extraction occurs (Im *et al.*, 2005; von Filseck *et al.*, 2015). Further we show osh4p^{Δ29}-RFP is present at sites of polarized exocytosis at greater than wild-type levels, again consistent with a model where Osh4p requires its ALPS domain to inhibit lipid deposition but promote lipid extraction at membranes enriched with the lipids it binds (Fig. 3.3; von Filseck *et al.*, 2015).

Temporal regulation of Osh4p function in polarized exocytosis is likely necessary to coordinate the two proposed points of Osh protein function in post-Golgi polarized exocytosis (Ling *et al.*, 2014; Smindak *et al.*, 2017). First, Golgi exit, the budding of vesicles at the *trans*-Golgi, is dependent on the Rab Ypt32p, or its paralog Ypt31p, but ultimately Ypt32p needs to be replaced with the terminal post-Golgi exocytic vesicle Rab Sec4p for vesicle docking at the plasma membrane (Benli *et al.*, 1996; Jedd *et al.*, 1997; Mizuno-Yamasaki *et al.*, 2010). Because vesicular membrane PI4P removal by Osh4p is

required for the replacement of Ypt32p with Sec4p, the kinetics of Osh4p PI4P removal must be regulated temporally to ensure that the exchange of Ypt32p for Sec4p does not occur prematurely (Ling *et al.*, 2014). First, due to the dependence of Golgi exit on Ypt32p, we hypothesize that the premature removal of Ypt32p would cause an exocytic block in the late Golgi, however this has not been demonstrated (Benli *et al.*, 1996). Second, when a docking competent, Sec4p positive vesicle is at the plasma membrane we postulate that Osh4p without its ALPS domain will exchange its bound vesicle-derived PI4P for plasma membrane sterol too quickly, prior to exocyst complex based vesicle tethering, and therefore prevent vesicle tethering, ultimately leading to the cytosolic accumulation of docking competent vesicles (Smindak *et al.*, 2017). Thus, accelerated lipid exchange could be sufficient to cause the exocytic block observed in cells dependent on osh4p^{Δ29}.

Because all known OSBs possess a lid of some form, even if that lid is not an ALPS domain, we hypothesize that the role of the OSBP lid in inhibiting lipid extraction and deposition is conserved throughout the OSBP protein family (Im *et al.*, 2005).

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Supplemental Figures

A)

B)

Supplemental Figure 3.1 Expression of *osh4p*^{Δ29} is approximately equal to the expression of wild-type Osh4p at restrictive temperature. A) Immunoblot of *S. cerevisiae* whole cell extracts of *osh4Δ* cells containing wild-type *OSH4* or *osh4*^{Δ29} on a *CEN* plasmid grown at 25°C, showing wild-type full length Osh4p and truncated *osh4p*^{Δ29}. B) Average of two experiments for wild-type Osh4p and three for *osh4p*^{Δ29} in which whole cell extracts of *osh4Δ* cells containing either wild-type *OSH4* or *osh4*^{Δ29} on a *CEN* plasmid were analyzed by SDS-PAGE followed by immunoblotting for total Osh4p or *osh4p*^{Δ29} and β-tubulin (loading control) from which the ratio of Osh4p to β-tubulin was calculated. Error bars indicate SEM. ** $p \leq 0.01$

Chapter 4: Discussion and Future Directions

Oxysterol binding proteins (OSBPs) have been implicated in several important cellular processes in eukaryotes. These include transfer of lipids between membranes, regulation of signaling complexes, viral particle maturation, and polarized exocytosis. Prior to this study, it was not known if polarized exocytosis required lipid binding by Osh proteins or whether lipid transfer and polarized exocytosis were mutually exclusive activities. Further, the specific role of OSBPs in polarized exocytosis was not known. In this section, I will first reiterate the key observations from both chapter two and three, then I will discuss caveats of my studies, followed by open questions, and then finally I will propose a series of future directions.

Lipid-Dependent Regulation of Exocytosis in *S. cerevisiae* by OSBP Homologue (Osh) 4

Using the yeast, *S. cerevisiae*, as a model, I addressed the questions of whether lipid binding by an Osh protein is required to support polarized exocytosis and what function in polarized exocytosis Osh protein activity fulfills. I showed that lipid binding by the yeast OSBP Osh4p is required for polarized exocytosis. I also found that during polarized exocytosis, Osh4p supports vesicle docking at the plasma membrane in at least two ways.

First, consistent with a previous study, I provide support for the hypothesis that Osh4p removes PI4P from the exocytic vesicle membrane to facilitate the loading of the post-Golgi Rab Sec4p onto the vesicle membrane, which makes a vesicle competent for docking at the PM (Ling *et al.*, 2014). Second, my data support a role for Osh4p in another process after and distinct from promoting vesicle maturation. I found that in the absence of Osh protein activity, Sec4p-positive, docking competent vesicles cluster in the cytoplasm. This suggests that after Sec4p loading, Osh4p must execute another activity. My data suggest that Osh4p supports vesicle docking at the PM. I show that Osh protein activity supports SNARE assembly at the plasma membrane, albeit not with strong statistical significance. I also show that Osh protein activity is required for the association of vesicle SNAREs with the plasma membrane, also consistent with a role in

vesicle docking (Fig. 2.5). Based on observations from my study and others I have developed a two-step model for Osh protein function in polarized exocytosis at the PM.

In my model, described in Chapter 2 but in brief here, Osh4p first removes PI4P from the vesicle membrane to facilitate Sec4p loading onto the vesicle membrane as proposed by Novick and colleagues (Ling *et al.*, 2014). This could be due to Osh4p-mediated extraction of PI4P from the vesicle membrane upon sterol deposition, consistent with *in vitro* analysis of Osh4p lipid exchange events (de St. Jean *et al.*, 2011; Ling *et al.*, 2014). Second, as the vesicle comes into close proximity with the plasma membrane, PI4P-bound Osh4p on the vesicle exchanges PI4P for plasma membrane sterol which promotes trans-SNARE assembly, by relieving an as yet unknown inhibition on trans-SNARE assembly.

Alternatively, vesicle docking at the plasma membrane may be independent of sterol binding by Osh4p. In this case, Osh4p would extract PI4P from the vesicle membrane and concurrently deposit its bound sterol. Upon arrival at the plasma membrane, Osh4p may then interact with a protein enriched at the site of polarity such as Cdc42p, thereby allowing vesicle docking. Only after vesicle docking would Osh4p then deposit its bound PI4P and extract a sterol molecule prior to recycling to a new vesicle to repeat the process (de St. Jean *et al.*, 2011; Ling *et al.*, 2014). This model would fulfill the predictions of the vesicle maturation model but would suggest that sterol binding and transfer by Osh proteins is chiefly required for vesicle maturation and further enriching the vesicle membrane with sterol (Klemm *et al.*, 2009; Ling *et al.*, 2014). Approaches to differentiate between these two models are discussed in future directions.

Both models are based on observations from my studies and others, however there are questions that need to be answered to validate either of these models (Mizuno-Yamasaki *et al.*, 2010; de St. Jean *et al.*, 2011; Ling *et al.*, 2014; von Filseck *et al.*, 2015)

Figure 4.1

Figure 4.1 Two step model for Osh protein function in polarized exocytosis. In the first step of the model, Osh proteins are required for vesicle maturation by removing PI4P from the vesicle membrane to facilitate the loading of Sec4p onto the vesicle, thereby producing a docking competent vesicle. In the second step of the model, at sites of polarized cell growth, Osh-bound PI4P exchanges for sterol in the plasma membrane to promote an efficient transition from an initial docking state, presumably exocyst-dependent, to one that is mediated by the formation of trans-SNARE complexes. After SNARE-mediated vesicle fusion with the plasma membrane, Osh4p is recycled from the plasma membrane.

Functional Analysis of the ALPS Domain in *S. cerevisiae* OSBP homologue 4 (Osh4p)

I also analyzed the requirement of the Osh4p ALPS domain in supporting polarized exocytosis and its contribution to vesicle localization of Osh4p. ALPS domains are amphipathic alpha helices that associate with highly curved membranes due to lipid packing defects associated with such membranes (Bigay *et al.*, 2005; Drin *et al.*, 2007). Previously, the Osh4p ALPS domain was thought to be only partially required for Osh4p function (Im *et al.*, 2005). However, my study revealed that the Osh4p ALPS domain is an essential domain required for polarized exocytosis. I had hypothesized that the Osh4p ALPS domain would confer vesicle localization to Osh4p because of the ability of ALPS domains to bind to highly curved membranes (Bigay *et al.*, 2005; Drin *et al.*, 2007). However that proved not to be the case. In fact, I observed increased vesicle localization by osh4p^{Δ29}, relative to wild-type Osh4p.

This result prompted me to consider how lipid binding and transfer defects caused by a lack of the Osh4p ALPS domain could cause the increase in association of osh4p^{Δ29} to vesicles. Prior to this study, osh4p^{Δ29} had been shown to have lower affinity for sterol and that osh4p^{Δ29} was defective in extracting both sterols and PI4P from membranes (~25% and 57% of wild-type extraction capacity, respectively) (Im *et al.*, 2005; von Filseck *et al.*, 2015). Moreover, osh4p^{Δ29} possesses almost no ability to transfer lipid between membranes, consistent with the view that the Osh4p ALPS domain is required for lipid extraction from the membrane (von Filseck *et al.*, 2015). An interesting possibility is that the ALPS domain contributes to the lipid binding specificity of Osh4p, and that osh4p^{Δ29} may bind lipids more promiscuously than wild-type Osh4p, however this hypothesis will require experimental validation.

My data are consistent with this model but I would also propose that *in vivo* the ability of Osh4p to extract lipids is coupled to release of Osh4p from the membrane to which it is bound. In this way, Osh proteins only leave a membrane when loaded with a required lipid from the source membrane, increasing the efficiency of lipid exchange, because Osh proteins would be trapped on the donor membrane pending lipid loading after lipid deposition. This idea is consistent with a previous study that indicated that the

Osh4p ALPS domain promotes lipid extraction from the membrane (von Filseck *et al.*, 2015).

In the context of exocytosis, I hypothesize that osh4p^{Δ29} is sufficiently PI4P-extraction competent to support Sec4p loading onto exocytic vesicles (von Filseck *et al.*, 2015). Analysis of PI4P extraction ability revealed that the ability of osh4p^{Δ29} to extract PI4P, while ~57% of wild-type Osh4p, was still superior to that of osh4p^{H143A/H144A} (~43% of wild-type) (von Filseck *et al.*, 2015). Whether this level of PI4P extraction is sufficient to remove vesicular PI4P and promote loading of Sec4p onto vesicles at wild type levels is not known. I also hypothesize that osh4p^{Δ29} would not be able to fulfill a sterol binding dependent role in vesicle docking at the plasma membrane because osh4p^{Δ29} can only extract sterol from membranes at ~25% of wild type capacity (von Filseck *et al.*, 2015).

Based on the above, it appears that the Osh4p ALPS domain has an inhibitory role that slows the rate of sterol deposition into a vesicle and, by extension, reciprocal PI4P extraction from a vesicle (de St. Jean *et al.*, 2011). In this way I propose that the ALPS domain could prevent Osh4p from removing vesicular PI4P too quickly, thereby allowing Ypt32p to finish its required role in Golgi exit (Jedd *et al.*, 1997). This idea is consistent with the molecular role for the ALPS domain inhibiting sterol deposition, as suggested in a previous study (von Filseck *et al.*, 2015). This hypothesis will need to be validated by testing for inhibition of vesicle formation by osh4p^{Δ29}.

The observations made here and in other studies in regards to the effect of the Osh4p ALPS domain on lipid binding and exocytosis further highlight the link between lipid binding and exocytosis. Work done by other labs has determined that osh4p^{Δ29} is more sterol binding deficient than PI4P binding deficient, and my *in vivo* results are consistent with these lipid-binding capacities (von Filseck *et al.*, 2015). As stated before, whether or not osh4p^{Δ29} is sufficiently competent to bind PI4P and support Sec4p loading onto vesicles at wild type levels remains an open question as is whether osh4p^{Δ29} inhibits vesicle formation at the TGN (Ling *et al.*, 2014).

Caveats

While I have presented a model consistent with both my observations and those of other labs, there are several caveats that warrant discussion.

A caveat of my results is that I was not able to show statistically significant ($p=0.08$) differences in trans-SNARE assembly (Fig. 2.3). Trans-SNARE assembly assays have been previously used to determine if particular mutants cause a vesicle docking defect (Grote *et al.*, 2000). This experiment could potentially be improved by repeating the SNARE assembly assay with cells treated with N-ethylmaleimide. The SNARE disassembly protein N-ethylmaleimide sensitive fusion protein (NSF), Sec18p in yeast, is sensitive to N-ethylmaleimide, thus in cells treated with N-ethylmaleimide cis-SNARE complexes could accumulate on the plasma membrane (Block *et al.*, 1988). Thus, instead of assaying for the absence of assembled SNARE complexes in strains with vesicle docking defects relative to wild-type strains, one would assay for the accumulation of assembled SNARE complexes in a wild-type strain, relative to a vesicle-docking deficient strain (Grote *et al.*, 2000). This could increase the sensitivity of the SNARE assembly assay.

Another caveat is the assumption of my model that osh4-1p^{ts} is sterol binding deficient and PI4P binding competent at restrictive temperature. This hypothesis has not been tested and is presented as a future direction later in this dissertation.

In addition, demonstration of PI4P deposition coupled to sterol extraction at the plasma membrane will need to be demonstrated to confirm the hypothesis that sterol binding by Osh proteins supports vesicle docking at the plasma membrane. Further, while PI4P-sterol exchange is proposed to relieve some, as yet unknown, inhibition on vesicle docking, the nature of this effect remains unknown. These issues are presented as future directions later in this document.

There are also caveats from earlier studies that need to be addressed in order to validate this model (Mizuno-Yamaskai *et al.*, 2010; de St. Jean *et al.*, 2011; Ling *et al.*, 2014; von Filseck *et al.*, 2015). For instance, clear demonstration of the dependence of Sec4p loading onto vesicles on Osh protein activity will be needed to confirm the role of Osh proteins in vesicle maturation (Mizuno-Yamasaki *et al.*, 2010; Ling *et al.*, 2014).

Further, while data suggestive of a role for Osh proteins in depleting vesicular PI4P levels has been published, more direct demonstration of increased PI4P levels on individual vesicles in the absence of Osh protein activity is needed to have confidence in Osh proteins being the factor required for vesicular PI4P removal (Ling *et al.*, 2014).

Moreover, the mechanism of PI4P depletion in the vesicle membrane is also in question. While I propose that Osh protein based extraction is the mechanism of action, Novick and colleagues suggest that Sac1p metabolizes vesicular PI4P to PI (Foti *et al.*, 2001). This is another point that will require experimental validation.

Open Questions

Are ORPs Dedicated Lipid Transfer Proteins?

A number of studies have suggested that ORPs execute non-vesicular lipid transfer between cellular membranes (Mesmin *et al.*, 2013; Chung *et al.*, 2015). In one model, ORPs, particularly OSBP, tether the ER to the Golgi, forming an ER-Golgi membrane contact site (Mesmin *et al.*, 2013). The OSBP lipid-binding domain binds ER membrane sterol, translocates to the Golgi membrane, and deposits the bound sterol into the Golgi membrane (Mesmin *et al.*, 2013). Finally, OSBP binds a Golgi membrane PI4P molecule, transfers it to the ER membrane, and repeats the process (Mesmin *et al.*, 2013). The transfer of sterol from the ER to the Golgi enriches sterol in the Golgi relative to the ER, which is required for vesicle sorting and formation (Klemm *et al.*, 2009; Mesmin *et al.*, 2013). In this model, sterol is translocated from an area of low concentration, the ER, to an area of higher concentration, the Golgi, which requires the reciprocal movement of Golgi PI4P back to the ER (Mesmin *et al.*, 2013; von Filseck *et al.*, 2015). The energy gained by moving PI4P down its concentration gradient, which is maintained by the action of the PI4P phosphatase Sac1p in the ER, is presumed to power the movement of sterol to the Golgi against its concentration gradient (Mesmin *et al.*, 2013; von Filseck *et al.*, 2015). However, several questions raised from these studies warrant reconsideration of that conclusion, and are discussed below

1. ORPs as Regulators of Membrane Contact Site Formation

The changes in lipid composition observed in specific membranes upon ORP expression or depletion may be due to an additional role for ORPs in regulating membrane contact site formation (Mesmin *et al.*, 2013, Chung *et al.*, 2015). For example, OSBP activity could facilitate lipid transfer between membranes such as the ER and Golgi or ER and PM, by promoting membrane contact site formation between these organelles in addition to transferring the lipids themselves (Mesmin *et al.*, 2013; Chung *et al.*, 2015). The authors of those studies note that OSBP, ORP5 and ORP8 tether membranes together, the ER and Golgi in the case of OSBP and the ER and PM in the case of ORP5 and ORP8 (Mesmin *et al.*, 2013; Chung *et al.*, 2015). OSBP forms Golgi-ER contact sites by binding Golgi PI4P with its PH domain and VAP proteins in the ER with its FFAT domain (Mesmin *et al.*, 2013). ORP5 tethers the PM to the ER dependent on PM PI4P (Chung *et al.*, 2015). In fact PM PI4P is required for ORP5 localization to ER-PM contact sites similar to Osh3p dependence on PI4P to form ER-PM contact sites in yeast (Stefan *et al.*, 2011; Chung *et al.*, 2015). In this context, Osh3p responds to high plasma membrane PI4P levels, which Osh3p binds with its PH domain while concurrently binding to the ER resident proteins Scs2p and Scs22p to form a PM-ER membrane contact site (Stefan *et al.*, 2011).

If ORPs facilitated lipid transfer by modulating membrane contact site formation, another soluble lipid transfer protein such as Sec14p or a lipid transfer tunnel formed by an extended synaptotagmin, could facilitate lipid transfer at an OSBP-dependent membrane contact site (Bankaitis *et al.*, 1990; Schauder *et al.*, 2014; Yu *et al.*, 2016). Although Osh3p, ORP5 and ORP8 facilitate ER-PM contact sites formation and OSBP tethers the ER to the Golgi, it is not clear if all ORPs can tether or facilitate the tethering of membranes (Stefan *et al.*, 2011; Mesmin *et al.*, 2013; Chung *et al.*, 2015).

Alternatively OSBPs could facilitate membrane hemifusion events between apposing membranes, again by supporting membrane contact site formation (Zhao *et al.*, 2016). Hemi- or full fusion between opposing membranes such as at an ER-Golgi contact site could facilitate lipid transfer under the appropriate conditions, however convincing data supporting the formation of a hemi- or full fusion between the ER and Golgi membranes at ER-Golgi contact sites does not exist. Fusion at membrane contact

sites in general has also not been reported. Nevertheless, non-vesicular lipid transfer, via hemifusion, is particularly intriguing in light of the role for Osh4p in vesicle docking in polarized exocytosis. Membrane fusion is presumed to be preceded by hemifusion of the two apposed leaflets, and indeed this has been demonstrated to be the case for vesicle fusion with the plasma membrane in neuroendocrine chromaffin and pancreatic β -cells (Zhao *et al.*, 2016). It was also demonstrated that lipid mixing did occur upon both hemi- and full fusion between the vesicle and plasma membrane and that hemifusion was reversible (Zhao *et al.*, 2016). Thus, hemifusion could support lipid transfer, so long as lipids transfer was from an area of high concentration to one of low concentration (Zhao *et al.*, 2016).

One possible *in vivo* demonstration of hemifusion mediated lipid transfer would be to express GFP-tagged P4M, a bacterial PI4P binding protein, and follow GFP localization after membrane contact site formation (Hammond *et al.*, 2014). GFP-P4M, while it localizes to the plasma membrane and endomembrane system in addition to the Golgi, does not localize to the ER. Therefore movement of GFP-P4M between the ER and Golgi, indicative of PI4P transfer to the ER, should be observable (Hammond *et al.*, 2014). Plasma membrane and endosomal GFP-P4M would be excluded from analysis by marking the ER with Sec63-RFP and the Golgi cisternae with Kex2p, Sed5p, or Tlg1p-BFP (Inadome *et al.*, 2005; Alfaro *et al.*, 2011). Alternatively, one could express a GFP tagged Golgi localized integral membrane protein such as Sed5p or Tlg1p representing early and late Golgi compartments, respectively, and follow their distribution after membrane contact site formation with the ER (Inadome *et al.*, 2005). If GFP-Sed5p or Tlg1p protein diffused into the ER membrane, that would suggest full fusion of the ER and Golgi membranes (Stroupe, 2012).

I do not exclude a role for ORPs in lipid transfer between membranes for the purpose of maintaining lipid homeostasis. Indeed lipid binding and exchange is a central feature of OSBP activity in polarized exocytosis. However, although data consistent with a role for OSBPs in dedicated lipid transfer exist, these same data are also consistent with additional roles for OSBPs in lipid transfer through the regulation of membrane contact sites, as discussed above (Mesmin *et al.*, 2013). Studies to exclude membrane contact

site formation and maintenance by OSBPs as a driving force for lipid transfer will be needed before a primary role for lipid transfer by an OSBP can be confirmed.

2. Technical Considerations

ORP5 and ORP8 based Phosphatidylserine Transfer at ER-PM Contact Sites

Studies suggesting *in vivo* lipid transfer as a main function of the OSBPs have technical shortcomings that warrant consideration. It was found that phosphatidylserine levels at the plasma membrane increased when the OSBP homolog ORP5 was expressed in HeLa cells (Chung *et al.*, 2015). Changes in membrane PS levels in this case suggested that ORP5 may transfer PS to the plasma membrane (Chung *et al.*, 2015.) In that study, the authors noted that this could be an artifact due to overexpression of ORP5 (Chung *et al.*, 2015). The authors addressed this issue by using a recombinant ORP5 gene, which upon treatment with rapamycin is recruited to the plasma membrane (Chung *et al.*, 2015). In this system, rapamycin induces the dimerization of an FK506 binding protein, incorporated into ORP5, with plasma membrane localized FKBP12-rapamycin-binding protein, thus recruiting the FK506 binding protein containing protein to the PM (Chung *et al.*, 2015). PI4P was removed from the PM in a rapamycin-dependent manner in this study, however it is possible that inducing localization of ORP5 to the plasma membrane led to increased ER-PM membrane contacts and concomitant PI4P phosphatase activity, which could account for the PI4P removal observed (Stefan *et al.*, 2011; Chung *et al.*, 2015). In addition, it is not clear whether this method enriches ORP5 at the plasma membrane above physiological levels (Chung *et al.*, 2015). Over-enrichment of ORP5 at the plasma membrane could be equivalent to ectopic ORP5 overexpression, and therefore the removal of PI4P from the plasma membrane could be an artifact (Chung *et al.*, 2015).

This critique does not detract from other results in the Chung *et al.* (2015) study that showed that ORP5 induced PS translocation from the cytoplasm to the PM, a result which strongly suggested that ORP5 can act as a dedicated PS transfer protein (Chung *et al.*, 2015). They also observed an increase in PM PI4P and decrease in PM PS upon ORP5 and ORP8 depletion, again strongly supporting a role for ORP5 and ORP8 in PS

transfer (Chung *et al.*, 2015). The changes in PI4P and PS distribution observed in the absence of ORP5 and ORP8 are consistent with a role for ORP5 and ORP8 in transfer of PS to the plasma membrane at ER-PM contact sites (Chung *et al.*, 2015). Despite the *in vivo* evidence in favor of a role for ORPs in lipid transfer, alternative hypotheses regarding ER-PM contact site formation by ORP5 allowing other mechanisms of lipid transfer should be excluded (Chung *et al.*, 2015). For instance, PS transfer to the PM should be assessed in cells in which other lipid transfer proteins have been depleted. If, for instance, extended synaptotagmins were depleted from the cell and exogenously expressed ORP5 could still enrich PS in the PM to the same extent, that would exclude a role for extended synaptotagmins in PS transfer in this context, and further support a role for ORP5 in PS transfer (Schauder *et al.*, 2014; Chung *et al.*, 2015).

OSBP Based Sterol Transfer at ER-Golgi Contact Sites

In addition to PS transfer at ER-PM membrane contact sites another ORP, OSBP, has been shown to support sterol transfer from the ER to the Golgi, with the caveat that OSBP was expressed above wild-type levels in this study (Mesmin *et al.*, 2013). To address the issue of OSBP overexpression, I would repeat the experiments of Mesmin *et al.*, (2013), with the following modifications. I would deplete endogenous OSBP in HeLa cells by siRNA knockdown or CRISPR and assess the effects of expressing wild-type OSBP or a peptide containing both the PH and FFAT domains of OSBP (Mesmin *et al.*, 2013). It is possible that only complete OSBP will support sterol transfer or that both OSBP and the PH-FFAT construct will support sterol transfer from the ER to the Golgi (Mesmin *et al.*, 2013). Because both of these constructs can tether the ER to the Golgi, a lack of tethering could not be responsible for a change in sterol distribution between the two organelles in this contact (Mesmin *et al.*, 2013). The amount of cholesterol in the ER and Golgi would then be determined in two ways.

First, the ER and Golgi would be isolated and the amount of cholesterol in each organelle would be determined by HPLC (Whitters *et al.*, 1994; Rieder and Emr, 2001; Inadome *et al.*, 2005; Georgiev *et al.*, 2011; Liu *et al.*, 2017). If OSBP is required for ER to Golgi sterol movement, the amount of cholesterol in the Golgi of cells expressing only the PH and FFAT domains of OSBP will be less than in cells expressing

wild-type OSBP. This would support a role for OSBP as a dedicated ER to Golgi sterol transfer protein (Mesmin *et al.*, 2013).

Second, to further test for OSBP dependent lipid transfer, I would stain live cells with a fluorescently labeled cholesterol binding protein, the modified D2 domain of perfringolysin from *C. perfringens* (Liu *et al.*, 2017). The relative amount of cholesterol in a given membrane could then be determined by measuring fluorescence intensity (Liu *et al.*, 2017). If the ratio of Golgi to ER fluorescence decreases when only the PH and FFAT domains of OSBP are expressed that would suggest that OSBP lipid binding activity, not OSBP tethering alone, is required for sterol transfer, further supporting a role for OSBP as a dedicated ER to Golgi sterol transfer protein (Mesmin *et al.*, 2013).

3. Is Sterol or PS Transfer by ORPs Essential?

Another shortcoming of the lipid transfer model deserves attention, as discussed in Chapter 1. Osh proteins, powered by the reciprocal transfer of PI4P to the ER, have been proposed to be dedicated lipid transfer proteins, enriching PS and sterols at the plasma membrane and Golgi, respectively (de St. Jean *et al.*, 2011; Mesmin *et al.*, 2013; von Filseck, 2015). If this is the case, broadly applied across organisms, then the observations that all Osh proteins share an essential function in lipid transfer is not consistent with the different lipid binding properties of the Osh family proteins as a whole (Beh and Rine, 2001). For example, cells dependent on Osh6p are viable, despite the fact that Osh6p does not bind sterol (Beh *et al.*, 2001; Im *et al.*, 2005; Maeda *et al.*, 2013). At the same time, cells dependent on Osh4p are viable, even though Osh4p does not bind phosphatidylserine (Beh *et al.*, 2001; Im *et al.*, 2005; Maeda *et al.*, 2013). Therefore, dedicated transfer of sterol or phosphatidylserine by Osh proteins is not an essential function of Osh protein in yeast. It should be noted that Osh proteins could transfer lipids among membranes but this activity may not be essential due to the presence of other protein families able to carry out the same process. For instance, the yeast StART family proteins could carry out sterol transfer in the absence of Osh protein function and extended synaptotagmins could facilitate PS transfer (Georgiev *et al.*, 2011). However, while transfer of PS or sterol is likely not the essential function of the Osh protein family, Osh protein based lipid transfer is not precluded by these observations.

On the other hand, all Osh proteins either bind PI4P or are predicted to do so and therefore a conserved PI4P based function is likely (de St. Jean *et al.*, 2012; Maeda *et al.*, 2013; Tong *et al.*, 2013; von Filseck, *et al.*, 2015). However, dedicated PI4P transfer by Osh proteins is unlikely to be essential because PI4P is made from PI by resident PI4 kinases on the Golgi and plasma membrane, and in the absence of Osh protein function PI4P accumulates on the plasma membrane, contrary to what would be predicted if Osh proteins are required for PI4P enrichment on the PM (Strahl and Thorner, 2007). It should be noted that Osh proteins do contribute to PIP homeostasis by activating the PI4P phosphatase Sac1p and recruiting Sac1p to the plasma membrane to maintain PI4P levels on that organelle (Stefan *et al.*, 2011). Through this mechanism Osh proteins contribute to maintaining organellar PIP identity, by regulating PI4P metabolism rather than by transporting PI4P itself (Stefan *et al.*, 2011). Therefore, it is more likely that PI4P acts to regulate OSBP activity.

Why is Osh Protein Activity Required for Polarized Exocytosis, but Not for Other Exocytic Processes, in Yeast?

I have shown that Osh protein activity is required for polarized exocytosis but not for non-polarized exocytosis (Figs. 2.1 and 2.2; Beh and Rine, 2004; Kozminski *et al.*, 2006). It is noteworthy that the non-polarized exocytic pathway requires a vesicle coat complex, a clathrin coat, for proper vesicle formation at the TGN, whereas the polarized exocytic pathway does not require vesicle coats for vesicle formation (Harsay and Schekman, 2002). Loss of Osh protein activity does not cause accumulation of ER to Golgi, intra-Golgi, or Golgi to ER vesicles, all of which assemble either a COPI or COPII coat (Keller and Simons, 1997; Barlow and Miller, 2013). These observations suggest that Osh proteins might act specifically on coat protein-independent vesicles. The features of coated vesicles that might substitute for Osh protein activity are however unknown.

Another distinguishing feature of the polarized and non-polarized pathways is the lipid composition of the vesicles (Klemm *et al.*, 2009). Vesicles mediating polarized exocytosis have been shown to be enriched in sterol relative to the Golgi membranes from which they arise (Klemm *et al.*, 2009). Due to low sterol levels in other vesicle

population, such as ER to Golgi vesicles, it is also possible that only vesicles enriched in sterol require Osh protein function. It should be noted that the lipid content of vesicles mediating non-polarized exocytosis is less clear, however it is predicted that these vesicles are not as enriched in sterol as vesicles mediating polarized exocytosis because the endosome, which is the destination of these vesicles, has lower sterol content than vesicles mediating polarized exocytosis (Klemm *et al.*, 2009). Thus it would be predicted that if vesicles mediating non-polarized exocytosis had higher sterol content, like vesicles mediating polarized exocytosis, then endosomal sterol content would be higher, however this point requires experimental validation (Klemm *et al.*, 2009). Additionally, whether lipid content differences dictate the requirement of Osh proteins to support polarized but not non-polarized exocytosis is unknown (Klemm *et al.*, 2009).

Additionally, the specific requirement for Osh protein function in polarized exocytosis could be that it facilitates Cdc42p localization at the bud tip of small and medium sized buds (Kozminski *et al.*, 2006; Dighe and Kozminski 2014). Cdc42p requires Osh protein activity to polarize to the bud tip and is posited to support maintenance of Cdc42p polarity at sites of polarized growth (Kozminski *et al.*, 2006). Cdc42p is required for polarized exocytosis and fulfills two roles (Adamo *et al.*, 2000). First Cdc42p is required for the polarized distribution of actin filaments, to allow for Myo2p based trafficking of vesicles to sites of polarized growth (Govindan *et al.*, 1995; Johnson *et al.*, 1999; Zhang *et al.*, 2001). Secondly, Cdc42p is necessary for recruitment of Sec3p and Exo70p to the site of polarized growth on the plasma membrane, which then acts, according to a popular model, as a polarity cue for exocyst complex assembly at the plasma membrane (Finger *et al.*, 1998; Wu *et al.*, 2010). As noted earlier, Osh4p is required for Cdc42p localization at the site of polarized growth, thus Osh4p contributes to both the polarized distribution of actin filaments and for Sec3p and Exo70p stabilization on the plasma membrane both of which contribute to polarized exocytosis (Kozminski *et al.*, 2006). Osh4p does interact with Cdc42p, however where this interaction occurs and whether it is direct or indirect is unknown (Alfaro *et al.*, 2011).

Another possibility is that Osh4p on the vesicle interacts with plasma membrane Cdc42p at the site of polarized growth, providing a form of coincidence detection as vesicles in the polarized pathway would have to then interact not only with the exocyst

but also Cdc42p prior to docking. Further analysis will be required to validate this hypothesis.

How Does Osh4p Associate with Exocytic Vesicles?

Alfaro and colleagues suggested that Osh4p associates with exocytic vesicles by binding the exocyst complex (Alfaro *et al.*, 2011). They demonstrated that disassembly of the exocyst complex led to dissociation of Osh4p from exocytic vesicles (Alfaro *et al.*, 2011). However, technical concerns call this result into question (Alfaro *et al.*, 2011). Alfaro and colleagues used in their study a *sec6-4^{ts}* strain that causes an exocytic block after 15 min and loss of stable exocyst complexes after 30 min (Terbush and Novick, 1995; Grote *et al.*, 2000). Nonetheless, Alfaro and colleagues assayed for Osh4p association with vesicles 90 to 120 mins after the switch to restrictive temperature, considerably longer than the time required for the *sec6-4^{ts}* mutant phenotype to manifest (Terbush and Novick, 1995; Grote *et al.*, 2000; Alfaro *et al.*, 2011). While not excluding the possibility that the exocyst complex is required for Osh4p association with exocytic vesicles, the increased time (90-120 min) at which *sec6-4^{ts}* cells were grown at restrictive temperature opens the possibility that the absence of Osh4p on exocytic vesicles is due to a secondary effect rather than the primary effect of exocyst disassembly (Terbush and Novick, 1995). For instance, membrane fluidity changes induced by prolonged incubation at restrictive temperature could destabilize the association of vesicle associated proteins, after the exocyst complex is no longer assembled (Morano *et al.*, 2012). Osh4p association with exocytic vesicles in the absence of assembled exocyst complexes should be reassessed after an incubation at restrictive temperature of no longer than 30 minutes. This experiment would indicate whether or not Osh4p dissociation from vesicles is temporally linked to the timing of exocyst complex disassembly, however it would not indicate definitively whether Osh4p binds the exocyst subunits on vesicles. Further investigation of other potential Osh4p vesicular binding partners, including transmembrane and peripherally associated proteins, should be considered and is discussed in future directions.

Technical Considerations When Differentiating Vesicle Docking and Fusion Defects

I used two assays to differentiate between vesicle docking and fusion defects. First, I performed a SNARE assembly assay that quantified the amount of plasma membrane t-SNARE Sso1/2p pulled down using antibodies to the v-SNARE HA-Snc2p (Grote *et al.*, 2000). This assay is appropriate if the relative levels of vesicle and plasma membrane SNAREs remain constant on their resident membranes in different strains and under different conditions. However, in cells having defects in endocytosis or those requiring a long (greater than 30 min) temperature shift, changes in plasma membrane t-SNARE levels could influence the outcome of the SNARE assembly assay independent of defects in vesicle docking or fusion (Supp. Fig. 2.2; Grote *et al.*, 2000). Thus, true vesicle docking defects may be overlooked if only the SNARE assembly assay is used. It is possible that addition of NEM to the cells prior to assaying for SNARE assembly may solve this problem.

To assay docking defects in a different, albeit less direct, manner I established the plasma membrane isolation assay to assess the ability of exocytic vesicles to associate with the plasma membrane and normalize for any variance in plasma membrane t-SNARE levels that could mask a vesicle docking or fusion defect (Panaretou and Piper, 2006). A caveat of this technique is that it only assesses vesicle association with the plasma membrane, not SNARE assembly. Thus, the SNARE assembly assay is a more direct assay for analyzing vesicle docking defects than the plasma membrane isolation assay (Grote *et al.*, 2000). I conclude that when assaying for vesicle-docking defects in strains that have variant plasma membrane t-SNARE levels the plasma membrane isolation assay should be used if a significant change in SNARE assembly is not detected (Grote *et al.*, 2001).

Future directions

Is Sterol Binding by Osh4p Required for Polarized Exocytosis?

The data presented herein are consistent with vesicular Osh4p depositing PI4P in the plasma membrane, and concurrently extracting plasma membrane sterol. However, more direct demonstration of this mechanism is needed. An observation in support of our

model is that osh4-1p^{ts} at restrictive temperature leads to formation of Sec4p positive vesicle clusters (Fig. 2.6 and Supp. Fig. 2.4). Vesicle clusters are cytoplasmic accumulations of vesicles that form in a Rab-dependent manner. However, it is possible that vesicles may also cluster in an, as yet unknown, Rab –independent manner as well (Rossi and Brennwald, 2011). Thus, osh4-1p^{ts} at restrictive temperature likely binds PI4P because Sec4p loading onto vesicles is predicted to depend on PI4P removal from the vesicle membrane and Sec4p is observed on vesicles in cells dependent on osh4-1p^{ts} at restrictive temperature (Alfaro *et al.*, 2011; Ling *et al.*, 2014). I hypothesize that osh4-1p^{ts} extracts PI4P from the vesicle membrane, but does not extract sterol from the plasma membrane, and, further, that the inability of osh4-1p^{ts} to bind sterol blocks vesicle docking at the plasma membrane, resulting in the accumulation and clustering of Sec4p-positive, docking competent vesicles (Fig. 2.6). The osh4-1p^{ts} mutation substitutes a glycine at residue 183 to aspartic acid, thus a negative charge is being added to the lipid-binding pocket near, but not in contact with, the hydroxyl group of an Osh4p bound ergosterol which could lessen the affinity of Osh4p for sterols (Fig 1.5). The position and negative charge of G183D, coupled with our other observations, makes a sterol binding defect a likely possibility. To test this hypotheses, I would perform sterol and PI4P binding assays using osh4-1p^{ts} at permissive and restrictive temperatures.

The ability of osh4-1p^{ts} to bind sterol can be tested using a published sterol binding assay (Im *et al.*, 2005), with modifications. Purified, GST tagged, osh4-1p^{ts} and Osh4p would be mixed with the fluorescent ergosterol analog dehydroxyergosterol and incubated at 25°C and 37°C for one hour, after which GST-tagged Osh4p and osh4-1p^{ts} will be pulled out of solution using glutathione-agarose beads. The amount of dehydroxyergosterol associated with the glutathione beads will be quantified by fluorometry, after multiple washes to remove unbound dehydroxyergosterol. A post-incubation sample of glutathione beads will be analyzed by immunoblotting for total bound Osh4p to monitor any protein degradation during incubation at elevated temperature.

I would also test osh4-1p^{ts} for its ability to bind PI4P using PI4P-coated agarose beads (Echelon, P-B004a, Salt Lake City, UT). PI4P-coated beads would be mixed with purified recombinant osh4-1p^{ts} at 25°C and 37°C and incubated for 1 hour. These beads

would then be recovered, washed, and finally the amount of bound osh4-1p^{ts} quantified by immunoblotting.

Analysis of the binding affinity of osh4-1p^{ts} for dehydroxyergosterol and PI4P can be determined using these methods over a range of concentrations. In addition, analysis of dehydroxyergosterol competition with PI4P for the lipid-binding pocket, and vice versa, could be performed by adding free dehydroxyergosterol or PI4P, respectively to the reaction.

Confirming that osh4-1p^{ts} is PI4P binding competent and sterol-binding deficient would fulfill an important prediction of the model of Osh4p function presented herein. First, by showing that a strictly sterol-binding deficient allele does not support polarized exocytosis, sterol binding by Osh4p would be confirmed as essential for polarized exocytosis. Secondly, by showing that osh4-1p^{ts} is PI4P-binding competent, the vesicle clustering phenotype observed will be more closely linked to Osh4p PI4P-binding and therefore Sec4p loading onto exocytic vesicles.

In addition, osh4-1p^{ts} may be an important tool for analyzing the role of sterol binding by Osh4p in cellular processes because osh4p^{Y97F}, while previously thought to be sterol binding deficient, has recently been shown to be more sterol binding competent than previously thought (Im *et al.*, 2005; von filseck *et al.*, 2015). If osh4-1p^{ts} is sterol-binding deficient, the phenotypic differences between cells dependent on osh4-1p^{ts} and those expressing osh4p^{Y97F} may be due to osh4p^{Y97F} not being as sterol-binding deficient as previously thought (Im *et al.*, 2005; von Filseck *et al.*, 2015).

Is Phosphatidylserine Binding By Osh6p or Osh7p Sufficient for Supporting Polarized Exocytosis

My model is that sterol binding by Osh4p at the plasma membrane is required for SNARE-mediated vesicle docking. However, the Osh family does not have uniform lipid binding characteristics. The Osh proteins all bind PI4P, but vary in the other lipid they bind, with Osh1p, Osh2p, Osh4p, and Osh5p binding sterol, Osh6p and Osh7p binding PS, and Osh3p only being known to bind PI4P (Im *et al.*, 2005; Maeda *et al.*, 2013; Tong *et al.*, 2013). Thus, whether PS binding by Osh6p or Osh7p is sufficient to support vesicle docking is unknown.

The ability of Osh6p or Osh7p to support vesicle docking will be addressed by analyzing cells dependent on Osh6p or Osh7p for exocytic defects. First, cells dependent on Osh6p or Osh7p would be tested for the internal accumulation of Bgl2p, a marker of vesicles mediating polarized exocytosis (Harsay and Bretscher 1995; Adamo *et al.*, 2001). If Osh6p or Osh7p is not sufficient to support Bgl2p exocytosis then internal Bgl2p will accumulate in cells dependent on Osh6p and Osh7p. If Bgl2p does accumulate in these cells, they will be analyzed by transmission electron microscopy to confirm that vesicles are accumulating and that Bgl2p is not accumulating in some other cellular compartment. If vesicles are found to accumulate in these cells, that would suggest that PS binding by an Osh protein is not sufficient to support polarized exocytosis.

If post-Golgi exocytic vesicles are found to accumulate in cells dependent on Osh6p or Osh7p then these strains will be tested for vesicle docking defects using the same techniques used in Chapter two of this dissertation, to confirm if Osh6p is not sufficient to support vesicle docking. To increase the sensitivity of the SNARE assembly assay used to assay vesicle docking, the assay would be performed with and without N-ethylmaleimide. The SNARE disassembly protein N-ethylmaleimide Sensitive Fusion protein (NSF), Sec18p in yeast, is sensitive to N-ethylmaleimide. Thus in cells treated with N-ethylmaleimide, cis-SNARE complexes may accumulate on the plasma membrane (Block *et al.*, 1988). Therefore, instead of assaying for the absence of assembled SNARE complexes in vesicle docking deficient strains relative to a wild-type strain, one would assay for the accumulation of assembled SNARE complexes in a wild-type strain, relative to a vesicle-docking mutant (Grote *et al.*, 2000).

On the other hand, if Osh6p or Osh7p is sufficient to support Bgl2p exocytosis that would suggest that PS binding by an Osh protein fulfills the role of sterol binding by other Osh proteins.

What Does Osh4p Sterol Binding at the Plasma Membrane Regulate?

Confirmation that sterol binding by Osh4p is required for Osh4p to support polarized exocytosis would support the model of Osh4p function presented in this dissertation. However it will not reveal the function of sterol binding by Osh4p at the molecular level. I have postulated that sterol binding by Osh4p at the plasma membrane

is required to relieve an inhibition of vesicle docking, as discussed on page 83 of this chapter and expanded on below (Fig. 4.2).

I hypothesize that sterol binding by Osh4p is required for the transition of the vesicle from exocyst-mediated tethering to SNARE-mediated docking (Figure 4.2; Lo *et al.*, 2011). When PI4P is bound, vesicular Osh4p could stabilize the exocyst complex at the plasma membrane and prevent the transition to SNARE-mediated docking (Lo *et al.*, 2011). Upon successful tethering and concomitant proximity to sterol in the plasma membrane, Osh4p would deposit its bound PI4P in the plasma membrane and extract plasma membrane sterol. This could destabilize the assembled exocyst complex and allow for SNARE-mediated vesicle docking. In support of this model, Osh4p has been shown to interact with three of the five vesicular exocyst subunits (Sec5p, Sec6p, and Sec8p) that interact with the plasma membrane subunit Exo70p (Boyd *et al.*, 2004; Kozminski *et al.*, 2006; Alfaro *et al.*, 2011; Hieder *et al.*, 2016). In addition, Osh4p interacts with the only vesicular subunit, Sec5p, reported to interact with the other plasma membrane subunit Sec3p (Kozminski *et al.*, 2006; Hieder *et al.*, 2016). Despite an assembled vesicular exocyst subcomplex not being detectable, it is still possible that unassembled exocyst subunits are carried to the site of polarized growth on exocytic vesicles and that these vesicular subunits would then interact with plasma membrane subunits, such as Sec3p, upon arrival (Boyd *et al.*, 2004; Heider *et al.*, 2016). Thus, Osh4p is well positioned to regulate the transition from vesicle tethering to docking through regulation of the exocyst complex.

To test this model I would analyze the interaction between the vesicular exocyst subunit Sec5p and the plasma membrane exocyst subunit Sec3p by isolating Sec3p and determining the amount of associated Sec5p (Hieder *et al.*, 2016). The interaction between the vesicular subunit Exo84p and the plasma membrane subunit Exo70p would not be suitable due to Exo70p being localized to the vesicle in addition to the plasma membrane (Boyd *et al.*, 2004). If sterol binding by Osh proteins is required for the transition from exocyst-mediated tethering to SNARE mediated docking, then sterol-binding deficient Osh4p should stabilize the interaction between the vesicular and plasma membrane exocyst complex subunits, leading to increased amounts of Sec5p in association with Sec3p. This would suggest that Osh4p inhibits SNARE-mediated

Figure 4.2 Model of Osh4p function in promoting vesicle docking at the plasma membrane. PI4P bound Osh4p on the vesicle stabilizes the exocyst complex after the exocyst tethers the vesicle to the plasma membrane prior to fusion. PI4P bound Osh4p then dissociates from the vesicle and deposits its bound PI4P and extracts a sterol molecule from the plasma membrane. This action serves as a coincidence detector paired with the recognition of plasma membrane PI4,5P₂ and Cdc42p by Exo70p and Sec3p. Sterol binding by Osh4p would prevent Osh4p from reassociating with the exocyst and thereby restabilizing it. Following this, the exocyst disassembles, allowing for trans-SNARE assembly.

vesicle docking by stabilizing the fully assembled exocyst complex at the plasma membrane (Lo *et al.*, 2011).

Alternatively, in the same conditions, one could analyze the persistence of FRET between Sec5p-CFP and Sec3p-YFP (Boyd *et al.*, 2004; Heider *et al.*, 2016). If sterol binding-deficient osh4p inhibits the transition from exocyst mediated vesicle tethering to SNARE-mediated docking then FRET should persist longer when only sterol-binding deficient osh4p is available.

When Does Osh4p Dissociate from the Vesicle

Using a similar assay, but with the addition of Osh4p-RFP, dissociation of Osh4p from the vesicle could be temporally linked to exocyst assembly at the plasma membrane, as discussed above. This would reveal if Osh protein dissociation occurs prior to or after exocyst assembly.

In addition, Osh4p-RFP signal on the vesicle could be temporally linked to FRET between CFP-Snc2p and YFP-Sso1p, CFP and YFP tagged v- and t-SNAREs respectively. In this case, Osh4p-RFP dissociation from the vesicle could be linked to trans-SNARE assembly.

Collectively, these two proposals could establish a time line of exocyst based tethering, trans-SNARE assembly, and Osh4p dissociation from the vesicle, which would provide further insight into Osh4p function at the PM. I hypothesize that Osh4p-RFP would dissociate after exocyst-based tethering, but before trans-SNARE assembly, consistent with a role in stabilizing the exocyst complex. However, this will have to be proven experimentally.

When Does Osh4p Extract Plasma Membrane Sterol

Again using an assay similar to that described in the two previous sections, but in this case with DHE added to the cells, another question related to the role of sterol binding by Osh4p in vesicle docking, when does sterol binding occurs, can be addressed. I hypothesize that after the vesicle is tethered to the plasma membrane by the exocyst complex, Osh4p extracts sterol from the plasma membrane, which then allows the vesicle to dock at the plasma membrane. Further, I hypothesize that when Osh4p is PI4P bound

on the vesicle it stabilizes the exocyst complex, and that it must come off the exocyst, deposit its bound PI4P, and extract a sterol from the plasma membrane. This action could destabilize the exocyst allowing the vesicle to transition from an exocyst-mediated tethered state to a SNARE-mediated docked state. Sterol binding would be required to prevent still PI4P bound Osh4p from returning to the exocyst and restabilizing the complex. Alternatively, PI4P bound Osh4p may prevent assembly of the vesicular exocyst subunits with the plasma membrane exocyst subunits. In this case, PI4P exchange for plasma membrane sterol by Osh4p would be required exocyst-mediated vesicle tethering.

To address this question, I would use TIRF microscopy, combined with FRET, to image vesicles as they approach and dock at the cell cortex, as has been done previously (Alfaro *et al.*, 2011). First, I would test for FRET between Sec5p-CFP and Sec3p-YFP, a vesicular and plasma membrane subunit of the exocyst complex that bind each other upon vesicle tethering (Boyd *et al.*, 2004; Hieder *et al.*, 2016). FRET signal, in this case, would indicate when vesicle tethering begins and, when FRET signal fades, when tethering has ceased, provided Sec5p and Sec3p dissociate upon cessation of tethering. Second, I would utilize the FRET transfer between Osh4p and the fluorescent ergosterol analog DHE (Georgiev *et al.*, 2011; de St. Jean *et al.*, 2011). It should be noted, that FRET between Osh4p and DHE has previously been analyzed using fluorometry rather than microscopy, however there is a two fold difference in measured fluorescence intensity between DHE bound and unbound Osh4p which should be detectable (de St. Jean *et al.*, 2011). Nevertheless, careful optimization will be needed. This assay will necessitate growing the cells under hypoxic conditions for 36 hours prior to analysis so that exogenously added DHE can be incorporated into the plasma membrane (Georgiev *et al.*, 2011). Because DHE is internalized by the cell and will redistribute among internal cellular membranes, it will be important to perform this experiment at various time-points relative to exposing the cells to DHE to determine when an ideal amount of DHE is present in the plasma membrane and internalized signal does not prohibit analysis of FRET between DHE and Osh4p (Georgiev *et al.*, 2011; de St. Jean *et al.*, 2011). I would then test for FRET between Osh4p and DHE. If FRET between Osh4p and DHE occurs prior to cessation of FRET between Sec5p-CFP and Sec3p-YFP, that would be consistent

with sterol binding by Osh4p destabilizing the exocyst complex to allow SNARE-based vesicle tethering at the plasma membrane.

What Does Osh4p Bind on Exocytic Vesicles?

I have confirmed that Osh4p is localized to exocytic vesicles and that lipid-binding by Osh4p regulates Osh4p association with exocytic vesicles (Fig. 2.7). However, the factors involved in the interaction of Osh4p with exocytic vesicles are unknown. To identify vesicular components that bind Osh4p, I propose two strategies: A directed screen to identify proteins in close proximity to Osh4p on the vesicle and an unbiased analysis of vesicles in both the polarized and non-polarized exocytic pathway to determine the protein content of both types of vesicles.

First, I propose to use the Bio-ID system to identify potential Osh4p interacting proteins *in vivo* (Roux *et al.*, 2013). In this system, Osh4p would be fused to a biotin ligase and expressed in wild-type cells. Biotin ligase tagged proteins add biotin to lysines on proteins within 20 to 30 nm of the ligase moiety (Roux *et al.*, 2013). Biotin tagged proteins, putative Osh4p interacting proteins, would then be isolated and identified using mass spectroscopy. This approach will yield candidate Osh4p interacting proteins from which we could identify vesicular proteins. To validate potential candidate Osh4p interactors, I would test for *in vitro* binding between purified recombinant Osh4p and the candidate proteins. It should be noted that non-vesicular proteins, or proteins not known to localize to vesicles, would be considered as well, because these proteins could act as adaptors between Osh4p and the vesicle.

Secondly, I propose a unbiased approach to identify vesicular Osh4p interacting proteins. At least two biochemically distinct subsets of exocytic vesicles exist, heavy (1.165 g/mL) and light (1.14 g/mL), that represent vesicles mediating non-polarized and polarized exocytosis, respectively (Harsay and Bretscher, 1995; Adamo *et al.*, 2001). However, the protein components of these vesicles, including both cargo and exocytic machinery, have not been exhaustively identified. Previously, exocytic vesicles were isolated and analyzed for associated proteins, however the study did not distinguish vesicles mediating polarized and non-polarized exocytosis (Forsmark *et al.*, 2011). I propose to separately isolate and purify the light vesicles of polarized exocytosis and the

dense vesicles of non-polarized exocytosis. I would first isolate light vesicles mediating polarized exocytosis from strains carrying the *exo70-35* mutation that exclusively causes a block in polarized exocytosis while not affecting non-polarized exocytosis, this would provide a relatively pure sample of vesicles mediating polarized exocytosis (He *et al.*, 2007). Secondly, I would isolate vesicles mediating both polarized and non-polarized exocytosis from wild-type cells. In both cases I would first separate the two vesicle populations by density gradient fractionation and then further purify them with Sec4p antibody coupled to protein A conjugated Dynabeads (Dighe and Kozminski, 2006). Lastly, I would submit the purified, Sec4p-positive vesicles of each density for mass spectroscopy analysis to identify vesicle-associated proteins.

Having a definitive list of the protein components of both vesicle classes would be an invaluable tool for researchers who study exocytosis and would provide prospective candidate proteins that designate a vesicle as of the polarized or non-polarized exocytic pathways. Further, this whole vesicle analysis would identify putative Osh4p interacting proteins, which could then be individually tested for interaction with Osh4p. Additionally, separately analyzing the protein content of vesicles mediating polarized and non-polarized exocytosis will shed light on two other important questions. First, upon analysis, I may find other Osh proteins associated with exocytic vesicles. The previous study, analyzing a mixed vesicle sample, did not detect vesicle-associated Osh4p, however later it was found that Osh4p does indeed associate with exocytic vesicles (Alfaro *et al.*, 2011; Forsmark *et al.*, 2011). It is possible, that elevated temperature (37°C) or the effects of the *sec6-4^{ts}* mutation in the strain from which the vesicles were isolated dissociated a subset of vesicle associated proteins, including Osh4p (Forsmark *et al.*, 2011). Therefore more vesicle-associated proteins may be identified when using vesicles isolated from a wild-strain at permissive temperature (25°C), Osh proteins among them (Forsmark *et al.*, 2011). Second, while myself and others have established that Osh protein activity is not required for non-polarized exocytosis, analyzing both populations of vesicles separately could determine whether Osh4p, among other Osh proteins, localizes to only vesicles mediating polarized exocytosis. I hypothesis, that Osh4p will only be found on vesicles mediating polarized exocytosis, due to the specific requirement for Osh protein activity in polarized exocytosis (Kozminski *et al.*, 2006).

However, it is possible that Osh4p localizes to both populations of exocytic vesicles, but is only required for polarized exocytosis.

Is OSBP Activity Required for Exocytosis in Mammalian Cells?

I have shown that Osh protein activity is required for polarized exocytosis in yeast. Due to the conserved nature of this protein family, I predict that OSBP activity is required for polarized exocytosis in other organisms as well. To this end I propose to knockdown or knockout all mammalian OSBP genes, in a suitable polarized cell, using a general OSBP siRNA or CRISPR, respectively, and then assay for exocytic defects (Lipschutz *et al.*, 2003).

If knocking down all ORP genes in mammalian cells is not feasible, sequential knockdown of individual ORP genes could be attempted. Although OSBP has been depleted from cells by siRNA, a post-Golgi exocytic defect caused by its depletion has not been explored (Nishimura *et al.*, 2013). I would take a candidate based approach based on mammalian OSBP domain architecture, starting with ORP4S, as it is most similar to Osh4p, consisting of an ORD with a lid, and then testing other ORPs (Ngo *et al.*, 2010). A loss of exocytosis when OSBP or another ORP is depleted would support my contention that regulation of exocytosis is a conserved essential function of the OSBP family.

Conclusion

Although multiple important cellular functions have been ascribed to the OSBP family, a conserved essential function linked to lipid binding has remained elusive. My research has identified a lipid dependent role for Osh proteins in polarized exocytosis. I have provided data that suggest that the Osh protein family is specifically required for vesicles of the polarized exocytic pathway to dock at the plasma membrane and developed a two-step model of Osh protein function in polarized exocytosis. I provided key data that supports a previous model of Osh protein function in which Osh4p removes vesicular membrane PI4P in order for Sec4p to be loaded onto exocytic vesicles, producing docking competent vesicles. In addition, my work introduces a second step to

the model, in which Osh4p regulates vesicle docking at the plasma membrane. Considering that the OSBP family is conserved from yeast to humans, I expect that a role for OSBPs in regulating polarized exocytosis, through interactions with specific membrane lipids, will be found in other organisms. In addition, I expect future research to reveal OSBP based regulation of contact sites between other organelles and to provide insight into the mechanisms by which Osh proteins regulate the transition from vesicle tethering to vesicle docking at the plasma membrane.

Appendix 1. Osh protein activity is required at all cell cycle stages

S. cerevisiae produce daughter cells by forming a bud. The bud increases in size as the cell progresses through the cell cycle, ultimately culminating in cytokinesis and a new cell. Bud size is associated with different stages of the cell cycle, and bud size can be used as a read out of progression through the cell cycle (Hartwell *et al.*, 1973). In fact, when genes required for progression through the cell cycle are mutated, strains carrying these mutations often accumulate cells of a particular bud size, leading to a change in bud size distribution. Accumulation of cells with a particular size bud can be used to infer if a gene is required to progress past a certain point in the cell cycle (Hartwell *et al.*, 1973).

Alfaro *et al.*, (2011), established that Osh protein activity is required for polarized exocytosis, which facilitates polarized cell growth. Based on this finding I hypothesized that Osh protein activity may be required for cell cycle progression. After incubation at restrictive temperature for four hours I assayed for bud size and found no significant difference in bud size distribution in the absence of Osh protein function or when only lipid binding deficient *osh4p* was present (Table 5). This result suggests that Osh protein activity is required at all cell cycle stages, and that progression through the cell cycle is dependent on Osh protein activity. This result is consistent with the fact that polarized exocytosis occurs at all cell cycle stages, and without polarized exocytosis bud size should not change.

Methods and Materials

To assay for a cell cycle progression block I grew *oshΔ* cells carrying a *CEN* plasmid containing the temperature-sensitive *osh4-I^{ts}* allele, and another *CEN* plasmid carrying an *osh4* allele of interest at permissive (25°C) or restrictive (37°C) for 4 h. These cultures were then analyzed for bud size distribution by light microscopy. Three independent clones were analyzed and the average of the three clones recorded (See Table 5).

Table A.1 Bud size distribution in *oshΔ* cells dependent on the indicated allele for Osh protein function (percent of total cells) (n=3)

A) 25°C

<i>oshΔ</i> (CBY926)+ [Indicated Allele]	Unbudded Cells	Small Budded	Medium Budded	Large Budded	Abnormal Morphology
<i>OSH4</i> (pCB231)	33	28.15	19.05	15.15	4.5
<i>Vector</i> (pRS316)	26.3	30.1	16.2	20.3	7.15
<i>osh4</i>^{H143A/H144A} (pKK1950)	27.75	33.85	17.1	17.85	5.15
<i>osh4</i>^{Y97F+H143A/H144A} (pKK1988)	30.55	28.6	18.05	14.75	5.55

B) 37°C

<i>oshΔ</i> (CBY926)+ [Indicated Allele]	Unbudded Cells	Small Budded	Medium Budded	Large Budded	Abnormal Morphology
<i>OSH4</i> (pCB231)	28	32.25	15.1	17.4	7.25
<i>Vector</i> (pRS316)	30.35	25.9	15.55	21.45	6.75
<i>osh4</i>^{H143A/H144A} (pKK1950)	27.55	29.5	16.9	20.35	5.8
<i>osh4</i>^{Y97F+H143A/H144A} (pKK1988)	30.4	29.25	16.15	17.9	6.25

Appendix 2. *OSH4* and lipid binding deficient *osh4* allele yeast two-hybrid screen

An important observation from Alfaro *et al.*, 2011 was that Osh4p localized to exocytic vesicles. This led to the hypothesis that Osh4p likely operates from the platform of the vesicle to fulfill a role in polarized exocytosis. However, what Osh4p binds to on the vesicle to maintain its vesicular localization was not determined, though pull-downs with TAP tagged Osh4p suggest some possibilities.

To address this question I performed a yeast two-hybrid genomic screen using wild type *OSH4* and three *osh4* mutants that encode lipid-binding deficient osh4p. Unfortunately, I was not able to identify any yeast two-hybrid interactions between wild-type *OSH4* or a mutant *osh4* allele and a gene encoding a vesicle resident protein. Interactions between Osh4p and vesicular proteins may be too transient to detect by yeast two-hybrid interaction. Nevertheless, we did identify a number of genes that interact with wild-type *OSH4* and mutant *osh4* alleles (Table 9). I was able to identify genes encoding proteins that reside on other organelles such as the mitochondria, the late endosome, and the endoplasmic reticulum. This suggests a role for Osh4p at other membrane contact sites, for instance those between the endoplasmic reticulum and mitochondria (Prinz, 2014).

One gene of particular interest that I identified, *VPS21*, encodes a vacuolar Rab (Singer-Kruger *et al.*, 1994). This finding was intriguing because Rabs are key factors in regulating membrane-membrane interactions. Because of its role in regulating membrane interactions, the interaction between *VPS21* and *OSH4* is a high priority for further study. This finding further suggests a greater role for *OSH4* at membrane-membrane contact sites, in this case perhaps the contacts between vacuoles and other organelles. Another interesting interaction is that between *OSH4* and *TOM71*. Tom71p recruits the yeast StART protein Lam6p to the ER-mitochondrial interorganellar contact sites (Elbaz-Alon *et al.*, 2015). Thus, Osh4p may play a role in regulating the localization of Lam6p to the ER-mitochondrial contact site.

Methods and Materials

I used the protocol of Fromont-Racine (2002), to screen for yeast two hybrid interactions. This protocol is carried out over three days. YPD and minimal media used in this study are the same as the media used previously in Chapter 2 of this dissertation (Sherman *et al.*, 1986), except that adenine was added to 200 mg/L and methionine was added to 0.003 mg/L in all cases.

On day one, PJ69-4 α strains carrying a pOBD plasmid with an *osh4* allele of interest were grown overnight in 20 mL of minimal media without tryptophan at 30°C. On day two the 20 mL culture from day one was inoculated into a 150 mL culture of minimal media without tryptophan to an O.D.₆₀₀ of 0.006 and grown overnight at 30°C. On day three, 80 O.D.₆₀₀ units of the PJ69-4 α strain carrying a pOBD plasmid with an *osh4* allele of interest was moved into a new 250 mL baffled flask. This is the bait culture.

At the same time on day three, a vial of PJ69-4a yeast carrying pOAD plasmids with genomic inserts covering the whole yeast genome was thawed on ice. After the vial was thawed, the contents were added to 20 mL of YPD and grown with shaking for 10 min at 30°C. This is the prey culture.

After ten minutes, the prey culture was added to the bait culture and mixed gently by hand. After mixing, the combined culture was harvested by centrifugation in a clinical centrifuge for 3 min at 5,000 rpm at room temperature. The supernatant was removed and the pellet was resuspended in 2 mL of YPD media.

400 μ L aliquots of the combined bait and prey culture was then spread onto YPD plates and incubated for 5 h at 30°C, to allow for the bait and prey strains to mate. After 5 h, the cells were scraped off the plates using a sterile glass scraper and collected in a sterile flask. The plates were then washed with minimal medium without leucine, tryptophan, and histidine and the wash was collected in the same sterile flask. The collected culture was then strained through sterile cheese cloth to remove any agar.

The strained yeast culture was divided into 500 μ L aliquots, and each aliquot was plated onto 150 mm plate containing minimal medium without leucine, tryptophan, and histidine, but with 10 mM 3-amino-1,2,3-triazole (A8056, Sigma, St. Louis, MO) added to prevent the growth of false positives. Plates were then incubated at 30°C for two

weeks. Growth in these conditions indicates a genetic interaction between the bait and prey plasmids, which implies a physical interaction between the proteins they encode.

After two weeks, colonies were collected and grown in minimal medium without leucine and tryptophan. Plasmids were then isolated from these cultures. Using the isolated plasmids as a template, I used oKK342 and 343 to amplify the insert in the pOAD plasmid in each colony. The PCR product was then sequenced using oKK352 and the sequence used to identify the encoded gene using the S288C yeast genome open reading frames as a reference genome.

Because adenine was included in the screening plates, the screen is considered to have been performed under medium stringency conditions rather than high stringency. Medium stringency screening allows for the identification of weaker interactions but also permits more false positives.

Table A.2 *S. cerevisiae* strains used in this study

Strain	Relevant Genotype	Source	Alias
PJ69-4a	MATa <i>trp1-901 leu2-3,112 ura3-52 his3-200 gal4(deleted) gal80(deleted) LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James <i>et al.</i> , 1996 ^a	KKY1243
PJ69-4α	MATα <i>trp1-901 leu2-3,112 ura3-52 his3-200 gal4(deleted) gal80(deleted) LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James <i>et al.</i> , 1996 ^a	KKY1244

^a Kind gift of S. Fields (University of Washington, Seattle, WA)

Table A.3 Plasmids used in this study

Plasmid	Relevant Genotype	Source
pOAD	<i>CEN LEU2 ADH1p-GAL4(AD)</i>	James <i>et al.</i> , 1996 ^a
pOBD2	<i>CEN TRP1 ADH1p-GAL4(DBD)</i>	James <i>et al.</i> , 1996 ^a
pKK1977	pOBD2 (OSH4)	This Study
pKK2036	pOBD2 (<i>osh4</i> ^{Y97F})	This Study
pKK2046	pOBD2 (<i>osh4</i> ^{H143A/H144A})	This Study
pKK2048	pOBD2 (<i>osh4</i> ^{Y97F+H143A/H144A})	This Study

^a Kind gift of S. Fields (University of Washington, Seattle, WA)

Table A.4 Oligonucleotides used in this study

Name	Sequence
oKK261	ACCACCATGGCAAGCTCATCCTCATGG NcoI underlined
oKK262	TCTCTGCAGGTGCAACGGTAACAAGTTG PstI underlined
oKK342	AATTCCAGCTGACCACCATG
oKK343	GATCCCCGGAATTGCCATG
oKK352	GAATCACTACAGGGATGTTTAATAC

Table A.5 Yeast two-hybrid screen results

<i>OSH4</i>	<i>osh4</i> ^{Y97F}	<i>osh4</i> ^{H143A/H144A}	<i>osh4</i> ^{Y97F+H143A/H144A}
<i>ISC10</i> (5) ^C	<i>MET2</i> (1) ^C	<i>EMC2</i> (4) ^{ER}	<i>MTH1</i> (2) ^C
<i>PLB2</i> (4) ^{E/CW}	<i>PET18</i> (1) ^C	<i>YKG9</i> (3) ^{C,N}	<i>HRD1</i> (2) ^{ER}
<i>YER034W</i> (3) ^{C/N}	<i>MTH1</i> (1) ^C	<i>SOR1</i> (2) ^C	<i>FYV6</i> (1) ^N
<i>VPS21</i> (2) ^{LE/M}		<i>ISM1</i> (1) ^M	<i>RRT5</i> (1) ^{PSM}
<i>SDA1</i> (2) ^N		<i>PTR2</i> (1) ^{PM}	<i>MDM30</i> (1) ^M
<i>TUM1</i> (2) ^C		<i>MTH1</i> (1) ^C	<i>RPL22A</i> (1) ^C
<i>TOM71</i> (1) ^M		<i>YNR071C</i> (1) ^C	<i>RPP1A</i> (1) ^C
<i>MTH1</i> (1) ^C		<i>YFR020W</i> (1) ^C	<i>GAT4</i> (1) ^N
<i>MET2</i> (1) ^C			<i>MET2</i> (1) ^C
<i>YKL070W</i> (1) ^M			<i>NMD4</i> (1) ^{C/N}
			<i>ALG14</i> (1) ^{ER}
			<i>YKG9</i> (1) ^{C/N}
			<i>YLR255C</i> (1) ^U
			<i>YMR103C</i> (1) ^U

Number in parenthesis indicates number of time gene identified in screen

^CCytoplasm, ^{ER}Endoplasmic Reticulum, ^NNucleus, ^{LE}Late Endosome, ^MMitochondria,
^{E/CW}Extracellular/Cell wall, ^{PM}Plasma Membrane, ^{PSM}Prospore Membrane, ^UUnknown

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