The Distribution and Function of PI4P During Osh-dependent Polarized Secretion in *Saccharomyces cerevisiae*

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ABSTRACT

Polarized secretion is an essential cellular process in which vesicles release exocytic cargo at one pole of the cell after trafficking from the *trans*-Golgi to the PM. Many studies in the budding yeast *S. cerevisiae* have identified conserved proteins required for vesicle maturation, a process that converts a PM docking incompetent vesicle to a PM docking competent state. However, the role of lipids in vesicle maturation is less well studied. In the current model for polarized secretion, the lipid transfer protein Osh4p is posited to mediate a loss of vesicle-associated PI4P that drives vesicle maturation. Currently, it is unclear whether other Osh proteins can substitute for Osh4p to support polarized secretion. Due to a lack of direct data, it is also unclear whether vesicle-associated PI4P decreases in an Osh-dependent manner. In this dissertation, I demonstrate Osh6p can independently support polarized secretion in a lipid-dependent manner. I also show that deletion of *OSH4* does not alter vesicle-associated PI4P levels, though loss of any individual member of the *OSH* family or complete loss of *OSH* family function alters the intracellular distribution of PI4P. I propose a new model for polarized secretion in which the Rab GTPases Ypt32p and Sec4p remain associated with a secretory vesicle during trafficking, independent of PI4P levels and Osh4p. In addition, I highlight that despite sharing a yet unknown function, Osh protein family members play unique roles in establishing and/or maintaining intracellular lipid distribution. Together, these findings provide a strong framework to guide future high spatial and temporal resolution studies of lipids and ORPs in yeast and mammalian cells to elucidate the function of proteins in polarized secretion.

DEDICATION

This dissertation is dedicated to everyone that helped me complete graduate school and shaped me into the person I am today.

To Keith, for all he taught me, for his insight, for his kindness, for every email addressed to the "denizens of the Koz Lab," for being a "clucking hen" when I was ill, for replying to good news with "Yeehaw!" and for never losing faith in my work.

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TABLE OF CONTENTS

Abstract I

Dedication II

Table of Contents III

Abbreviations VI

Figure List VIII

Table List IX

Supplemental Figure List X

Supplemental Table List XI

Chapter 1: Introduction 1

Polarized Secretion in *S. cerevisiae* 1

Establishing Polarity 1

Vesicle Formation at the trans-Golgi 3

Vesicle Trafficking and Maturation 6

Exocyst-mediated Tethering 11

SNARE-mediated Docking 14

Oxysterol Binding Protein Homologues 15

Osh Protein Structure and Homology 16

Role of Osh4p in Secretion 19

Current Model of Polarized Secretion and Remaining Questions 20

Chapter 2: Osh6p Can Independently Support Polarized Secretion In a Lipid-dependent Manner 23

Abstract 24

Introduction 25

Materials and Methods 27

Yeast Strains and Growth Conditions 28

Bgl2p Accumulation Assay 28

Results 29

Osh6p Can Substitute for Osh4p to Independently Support Polarized Secretion 29

Discussion 33

Chapter 3: Osh-dependent and -independent Regulation of PI4P Levels During Polarized Cell Growth in *Saccharomyces cerevisiae* 35

Abstract 36

Introduction 37

Materials and Methods 40

Yeast Strains and Growth Conditions 40

Design and Construction of Lipid Probes 44

Bgl2p Accumulation Assay 47

Fluorescence Microscopy 47

Vesicle Trafficking and Quantification 48

Results 49

Lipid-probe P4M is Specific to PI4P in S. cerevisiae 49

P4M Expression Does Not Significantly Hinder Vesicle Trafficking or Cellular Function 54

Post-Golgi Ypt32p-marked Vesicles Do Not Lose PI4P While Trafficking from the TGN to PM 59

Members of the Osh Protein Family Play Unique Roles in Establishing or Maintaining PI4P Distribution 65

Discussion 71

Do Vesicle-associated PI4P Levels Change as Vesicles Traffic? 73

What Is the Role of Osh4p in Polarized Secretion? 74

Acknowledgements 78

Chapter 4: Discussion and Future Directions 86

Summary.....86

Can Osh6p Substitute for Osh4p to Independently Support Polarized Secretion? 88

How Do All Osh Proteins Independently Support Polarized Secretion? 87

Do Vesicle-associated PI4P Levels Change as Vesicles Traffic? 90

Is Phosphorylation of Sec2p Biologically Relevant?.....91

What Is the Role of Osh4p in Polarized Secretion?..... 92

Do Exocytic Vesicles Lose Vesicle-associated PI4P at the trans-Golgi? 92

Does Osh4p Mediate a Non-specific Lipid Exchange at the PM?.....94

Concluding Remarks 97

References.....98

ABBREVIATIONS

ARF	ADP ribosylation factor
ER	Endoplasmic reticulum
GEF	Guanine nucleotide exchange factor
ORD	OSBP-related ligand-binding domain
ORP	OSBP-related protein
OSBP	Oxysterol binding protein
OSH	OSBP protein homologue
PC	Phosphatidylcholine
PH	Pleckstrin homology [domain]
PI	Phosphatidylinositol
PI4P	Phosphatidylinositol 4-phosphate
PI(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
PM	Plasma membrane
PS	Phosphatidylserine
SNARE	Soluble N-ethylmaleimide-sensitive factor adaptor protein receptor
TGN	*trans*-Golgi network

TRAPP Transport protein particle

WT Wild-type

FIGURE LIST

Figure 1.1 Polarized and non-polarized cell growth in *S. cerevisiae* 2

Figure 1.2 Proteins involved in vesicle formation at the *trans*-Golgi 6

Figure 1.3 PI4P-dependent maturation of polarized secretory vesicles 10

Figure 1.4 Structural model of the exocyst complex.....13

Figure 1.5 Oxysterol binding protein homologue structure 18

Figure 1.6 Existing model of polarized secretion in *S. cerevisiae* 22

Figure 2.1 Osh6p promotes polarized secretion in a lipid-dependent manner.....31

Figure 3.1 P4M localizes to PI4P-enriched membranes in *S. cerevisiae* 52

Figure 3.2 mCherry2b-P4M localizes to GFP-Ypt32p-marked regions as well as GFP-Sec4p-marked regions and does not inhibit polarized secretion...57

Figure 3.3 GFP-Ypt32p- and GFP-Sec4p-marked vesicles can be tracked *in vivo* 61

Figure 3.4 mCherry2b-P4M intensity on GFP-Ypt32p-marked vesicles does not change significantly over time 63

Figure 3.5 Loss of individual Osh family proteins results in altered PI4P distribution 68

Figure 3.6 Lethality from loss of Osh protein family function in *osh*Δ [*osh4-1*^{ts}] cells is not directly caused by PI4P re-distribution 70

Figure 3.7 Model of secretory vesicle maturation post-Golgi.....72

Figure 4.1 New model of polarized secretion in *S. cerevisiae* 87

TABLE LIST

Table 1.1 SNAREs involved in polarized secretion in *S. cerevisiae* 14

Table 2.1 *S. cerevisiae* strains used in Chapter 2.....28

Table 3.1 *S. cerevisiae* strains used in Chapter 3.....41

Table 3.2 Plasmids used in Chapter 3 43

SUPPLEMENTAL FIGURE LIST

Supplemental Figure 3.1 Expression of Sec7p-mCherry2bx6 with Clover-P4M or Clover-D4H does not significantly affect cell growth...79

Supplemental Figure 3.2 Diagram of gBlocks used in Chapter 3 80

Supplemental Figure 3.3 Trajectory of GFP-Ypt32p-marked vesicles.....81

Supplemental Figure 3.4 Trajectory of GFP-Sec4p-marked vesicles 82

SUPPLEMENTAL TABLE LIST

Supplemental Table 3.1 Oligonucleotides used in Chapter 3 83

Supplemental Table 3.2 gBlocks used in Chapter 3 84

CHAPTER 1

Introduction

Polarized Secretion in *S. cerevisiae*

Polarized secretion is an essential process that involves the exocytic release of cargo from vesicles at one pole of the cell after they traffic from the TGN to the PM. Notable examples include but are not limited to the release of zymogen from the apical surface of pancreatic acinar cells and the release of neurotransmitters from axon terminals (reviewed by Low *et al.*, 2010; Morris, 2020). In the budding yeast *Saccharomyces cerevisiae*, polarized secretion is essential for polarized cell growth, daughter cell formation, and cell division. Because many of the key events and molecules involved in polarized secretion are evolutionarily conserved (reviewed by Zeng *et al.*, 2017), *S. cerevisiae* is a well-characterized experimental model organism with smaller protein families than mammalian cells that allows us to elucidate the often complex relationship between the lipids and proteins that regulate polarized secretion (reviewed by Feyder *et al.*, 2015).

Establishing polarity

In *S. cerevisiae*, cells undergo polarized and non-polarized or isotropic growth, processes with distinct vesicle populations (Figure 1.1; Harsay and Bretscher, 1995). Vesicles involved in polarized cell growth are marked by the cargo protein Bgl2p whereas vesicles involved in non-polarized cell growth are marked by the cargo protein Suc2p, otherwise known as invertase (Harsay and Bretscher, 1995). Polarized secretion drives bud

emergence and bud growth. Once the bud has reached a certain volume, it undergoes non-polarized isotropic growth before re-establishing polarity to deliver vesicles from the mother cell and bud to the bud neck.

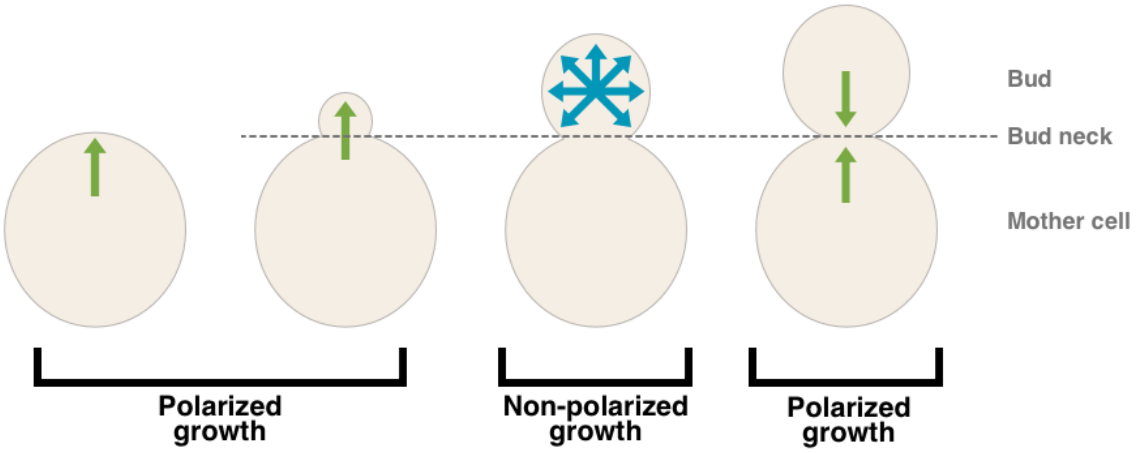


Figure 1.1: Polarized and non-polarized cell growth in *S. cerevisiae*. Proper yeast cell proliferation relies on both polarized and non-polarized cell growth.

Sites of polarized growth are established by Cdc42p, a highly conserved, small, monomeric GTPase (Adams *et al.*, 1990; Johnson and Pringle, 1990). Cells lacking functional Cdc42p are large, unbudded, and multinucleated, indicating a failure in polarized cell growth (Adams *et al.*, 1990). Cdc42p regulates both the actin cytoskeleton upon which vesicles are trafficked and the recruitment of vesicle docking machinery known as the exocyst complex, which will be discussed later in this chapter (reviewed by Chiou *et al.*, 2017). Cdc42p interacts with formins, proteins that facilitate the assembly of actin filaments, which are bundled into actin cables upon which polarized vesicles are trafficked to sites of polarized growth. Cdc42p and the GTPase Rho1p recruit the formin

Bni1p to the bud cortex while a downstream effector of Cdc42p recruits the formin Bnr1p to the bud neck (Evangelista *et al.*, 1997; Buttery *et al.*, 2007).

Vesicle formation at the trans-Golgi

Secretory vesicles form at the *trans*-Golgi and traffic to designated sites of polarized secretion on the PM. In mammals, the Golgi is made of polarized stacks of cisternae, which proteins traverse through inter-cisternal connections or intra-cisternal transport (Beznoussenko *et al.*, 2014). In yeast, Golgi are discrete structures that mature from *cis* to *trans* (Preuss *et al.*, 1992; Losev *et al.*, 2006; Matsuura-Tokita *et al.*, 2006). Because the idea of compartmentalization inherently contradicts the concept of Golgi maturation, some studies refer to the Golgi by kinetic stages where "early Golgi" and "late Golgi," also referred to as the TGN, are distinguished based on what trafficking pathways are active (reviewed by Pantazopoulou and Glick, 2019). In addition, more recent studies posit that yeast possess a minimal endomembrane system that lacks both an early and a recycling endosome, whose functions are instead performed the TGN, adding a new layer of difficulty when defining the Golgi as *cis* or *trans* (Day *et al.*, 2018; Casler *et al.*, 2019). Herein, "*trans*-Golgi" will refer to the Golgi structure capable of forming polarized secretory vesicles and "TGN" will refer to the site on the Golgi where vesicle formation occurs (Brigance *et al.*, 2017).

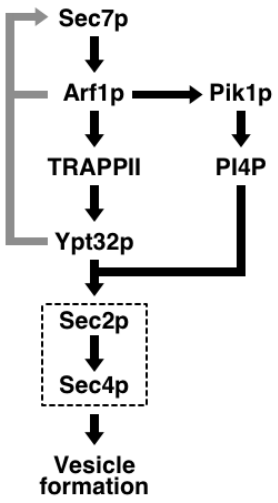
Maturation of the *trans*-Golgi and vesicle formation require the sequential recruitment and activation of GTPases as well as changes in Golgi lipid composition (Figure 1.2). A key protein in Golgi maturation and a commonly used *trans*-Golgi marker is Sec7p, a GEF for Arf proteins (Novick *et al.*, 1980; Sata *et al.*, 1998). In both mammalian

and yeast cells, Arf GTPases are key regulators of intracellular vesicle transport (reviewed by Adarska *et al.*, 2021). In yeast, Sec7p recruits Arf1p, which regulates Sec7p by positive feedback and continues *trans*-Golgi maturation via the recruitment of a kinase that alters *trans*-Golgi PI4P levels (Figure 1.2, gray arrows; Richardson *et al.*, 2012). The TGN is enriched in PI4P relative to other membranes including the ER and nucleus (Zinser *et al.*, 1991; Levine and Munro, 2002; Klemm *et al.*, 2009). TGN PI4P levels are maintained by the essential phosphatidylinositol 4-kinase, Pik1p, which contains a binding site for the Ca^{2+}-binding protein Frq1, a frequenin homologue (Flanagan *et al.*, 1993; Pongs *et al.,* 1993; Hendricks *et al.*, 1999; Walch-Solimena and Novick, 1999). The Pik1p-Frq1p complex is recruited to the *trans*-Golgi by directly binding Arf1p, indicating *trans*-Golgi PI4P levels likely increase just prior to vesicle formation (Strahl *et al.*, 2005; Highland and Fromme, 2021). Due to the challenges of quantifying lipid changes *in vivo*, we do not know the exact amount of PI4P that must be present at the *trans*-Golgi for vesicle formation to occur, or whether PI4P is uniformly distributed across the *trans*-Golgi.

Another protein required for vesicle formation at the *trans*-Golgi is the Rab GTPase Ypt32p (Benli *et al.*, 1996; Jedd *et al.*, 1997). Like Arf1p, Ypt32p is proposed to stimulate Sec7p activity (Figure 1.2, gray arrows; McDonald and Fromme, 2014). However, it is unclear how Ypt32p is recruited to the *trans*-Golgi. One hypothesis, which is presented in Figure 1.2, is that Arf1p recruits the Ypt32p GEF, the TRAPPII complex, which then recruits and activates Ypt32p (Jones *et al.*, 2000; Thomas and Fromme, 2016; Thomas *et al.*, 2018; Mi *et al.*, 2022). While there is long standing debate regarding TRAPPII and its specificity for Ypt32p, this hypothesis is supported by several studies. Sec7p does not directly interact with TRAPPII, but Sec7p activity is required for TRAPPII localization to

the *trans*-Golgi (Thomas and Fromme, 2016). This suggests a Sec7p effector such as Arf1p recruits TRAPPII to the *trans*-Golgi. Consistent with this, in cells lacking Arf1p, TRAPPII localizes to the cytoplasm, further implicating Arf1p in TRAPPII recruitment (Chen *et al.*, 2011; Thomas and Fromme, 2016). While more studies are needed in *S. cerevisiae* to definitively confirm Arf1p recruits TRAPPII and TRAPPII recruits Ypt32p, this pathway appears to be conserved across eukaryotes. In the filamentous fungi *Aspergillus nidulans*, TRAPPII localizes to the *trans*-Golgi just prior to Rab11, the homologue of Ypt32p (Pinar and Peñalva, 2020). TRAPPII is also required for the localization of Rab11 in male meiotic *Drosophila* cells (Robinnet *et al.*, 2009). Together, these studies provide justification for the pathway presented in Figure 1.2.

Ypt32p is required for exit from the *trans*-Golgi and is present on vesicles, though that is not true for all vesicle-associated proteins recruited to the *trans*-Golgi prior to vesicle formation (Jedd *et al.*, 1997). For example, the Rab GEF Sec2p, which is not required for exit from the TGN, is recruited to the *trans*-Golgi where it binds Ypt32p and PI4P generated by Pik1p (Ortiz *et al.*, 2002; Mizuno-Yamasaki *et al.*, 2010; Stalder *et al.*, 2013). For many years, Sec2p was modeled to recruit the Rab GTPase Sec4p to secretory vesicles during trafficking, though recent evidence suggests Sec4p is recruited to the *trans*-Golgi immediately preceding vesicle formation (Figure 1.2, dotted box; Gingras *et al.*, 2022). While Sec2p and Sec4p are not required for vesicle formation, they are required for vesicle maturation during trafficking, a process which is described below. Despite knowing many of the proteins required for vesicle formation at the *trans*-Golgi, the machinery responsible for vesicle formation and cargo segregation have yet to be identified.



GEFs		
Protein	**Brief description**	**Homologue**
Sec7p	A GEF for Arf1p. *trans*-Golgi marker.	Arfgef1/BIG1 (Morinaga *et al.*, 1996)
TRAPPII	A complex of proteins that functions as a GEF for Ypt32p.	TRAPPII (Cox *et al.*, 2007)
Sec2p	A GEF for Sec4p. Binds PI4P and Ypt32p at the *trans*-Golgi. Not required for vesicle formation.	Rabin8/Rab3IP (Hattula *et al.*, 2002)
GTPases		
Protein	**Brief description**	**Homologue**
Arf1p	A Ras GTPase. Recruits proteins to further *trans*-Golgi maturation.	Arf1 (Kahn *et al.*, 1991)
Ypt32p	A Rab GTPase. Involved in the recruitment of Sec2p.	Rab11 (Benli *et al.*, 1996; Jedd *et al.*, 1997)
Sec4p	A Rab GTPase. Not required for vesicle formation.	Rab8 (Chavrier *et al.*, 1990; Huber *et al.*, 1993)
Kinases		
Protein	**Brief description**	**Homologue**
Pik1p	Phosphorylates PI to generate PI4P at the *trans*-Golgi.	PI4Kβ (Meyers and Cantley, 1997)

Figure 1.2: Proteins involved in vesicle formation at the *trans*-Golgi. A model of the Sec7p-dependent pathway that is required for vesicle formation at the *trans*-Golgi. Sec2p and Sec4p, though not required for vesicle formation (indicated by the dotted box), are recruited to the *trans*-Golgi prior to vesicle formation. Gray arrows indicate positive feedback.

Vesicle trafficking and maturation

As secretory vesicles traffic from the *trans*-Golgi to the PM, they undergo a process known as maturation to generate a PM docking competent vesicle. Vesicle maturation requires a regulated Rab cascade, in which an activated Rab protein recruits the GEF necessary to activate the next Rab protein in the sequence. Though recent data suggest the Rab GTPase Sec4p is recruited to the *trans*-Golgi and is present on newly formed vesicles (Gingras *et al.*, 2022), the model of polarized secretion that served as the basis for this dissertation predated such data (Ling *et al.*, 2014; Smindak *et al.*, 2017). Therefore, I will

discuss and identify weaknesses of the existing model of vesicle maturation in which Sec4p is recruited to immature vesicles.

The Rab GTPase Ypt32p and Rab GEF Sec2p are recruited to the *trans*-Golgi and are present on newly formed vesicles (Figure 1.3A; Jedd *et al.*, 1997; Ortiz *et al.*, 2002). The Rab GTPase Sec4p is then recruited to the vesicle where an active site on the N-terminus of Sec2p facilitates the Sec4p GDP-to-GTP exchange (1.3B; Walch-Solimena *et al.*, 1997; Dong *et al.*, 2007). In yeast, polarized secretory vesicles are trafficked along actin cables by Myo2p, a type V myosin motor that binds Ypt32p and Sec4p through its C-terminal globular tail domain (Johnston *et al.*, 1991; Schott *et al.*, 1999; Lipatova *et al.*, 2008). Ypt32p recruits Myo2p to the vesicle, but vesicle movement is driven by the Myo2p-Sec4p interaction (Figure 1.3A, B; Schott *et al.*, 1999).

Historically, many studies used Sec4p as a marker for mature or "late" vesicles, though maturation continues after Sec4p activation. Sec15p, an effector of Sec4p and a component of the hetero-octameric exocyst complex, must also be recruited to the vesicle. Myo2p interacts with Sec15p through a separate binding site than Ypt32p and Sec4p, though further studies are required to elucidate the relevance of this interaction (Jin *et al.*, 2011). Unlike on Myo2p, Ypt32p and Sec15p share a mutually exclusive binding site on Sec2p suggesting that dissociation of Ypt32p from the vesicle prior to Sec15p recruitment is required, though there are no direct data supporting Ypt32p dissociation (Figure 1.3C, D; Medkova *et al.*, 2006). The current model of polarized secretion is also unclear regarding when and where this Ypt32p to Sec15p exchange takes place relative to vesicle formation at the *trans*-Golgi. Ypt32p has been observed on vesicles near the PM and we know multiple components of the exocyst complex are recruited to the vesicle prior to

arrival at the PM (Boyd *et al.*, 2004; Rivera-Molina and Novick, 2009; Mizuno-Yamasaki *et al.*, 2010; Gingras *et al.*, 2022). These observations suggest that only a fraction of Ypt32p dissociates prior to Sec15p binding, a nuance that is often overlooked when discussing the current model of polarized secretion. This uncertainty in the timing and location of essential events during vesicle maturation highlights the necessity of high temporal resolution imaging.

The dissociation of Ypt32p and the recruitment of Sec15p is hypothesized to occur after a loss of vesicle-associated PI4P (Figure 1.3C, D; Mizuno-Yamasaki *et al.*, 2010). This is referred to as a PI4P gradient, wherein vesicles have lower PI4P levels at the PM relative to when they formed at the *trans*-Golgi. Specifically, the PI4P gradient is suggested to affect whether Sec2p preferentially binds Ypt32p or Sec15p (Stalder *et al.*, 2013). *In vitro* binding assays demonstrated that phosphorylation of Sec2p increases Sec2p binding to Sec15p and decreases Sec2p binding to Ypt32p (Elkind *et al.*, 2000; Stalder *et al.*, 2013). However, in the same study, Stalder *et al.* (2013) demonstrated that phosphomimetic and nonphosphorylatable Sec2p mutants did not affect cell growth or Sec2p localization in WT cells and provided no evidence of altered Sec15p localization. Despite this, Stalder *et al.* (2013) conducted further *in vitro* experiments under the assumption that Sec2p phosphorylation is relevant *in vivo* and claimed that loss of vesicle-associated PI4P resulted in a conformation change in Sec2p that triggered the Ypt32p to Sec15p exchange, with phosphorylation of Sec2p increasing the strength Sec2p-Sec15p interaction. However, there is no data to support the hypothesis that Sec2p-PI4P interaction affects Sec2p conformation (Stalder *et al.*, 2013). Therefore, although there is strong evidence

demonstrating that phosphorylation of Sec2p affects Sec2p binding to Ypt32p and Sec15p *in vitro*, whether this is an essential step in vesicle maturation is unclear.

In addition to the uncertainty regarding the role of Sec2p-phosphorylation *in vivo*, the data presented in Mizuno-Yamasaki *et al.* (2010), which first proposed the PI4P gradient, are not entirely convincing. This is largely due to a lack of *in vivo* data and the promiscuity of early generation lipid probes (reviewed by Wills *et al.*, 2018 and Hammond *et al.*, 2022). Mizuno-Yamasaki *et al.* (2010) quantified co-localization in micrographs of cells co-expressing Sec2p-3xGFP and mCherry-FAPP1-PH, a lipid binding domain that binds both PI4P and PI(4,5)P_2 and requires Arf1p to localize (Godi *et al.*, 2004; Lenoir *et al.*, 2015). No direct quantification of vesicle-associated PI4P was provided and there was no mention of which puncta were scored. This is an issue because Sec2p localizes to both the *trans*-Golgi and secretory vesicles, but FAPP1-PH localization is restricted to the *trans*-Golgi where Arf1p is located. Therefore, a decrease in co-localization between Sec2p and FAPP1-PH on puncta near sites of polarized cell growth does not necessarily indicate a loss of PI4P on secretory vesicles. The group expands upon their PI4P-dependent model in Ling *et al.* (2014), in which they propose the oxysterol binding protein homologue Osh4p (Kes1p) is responsible for decreasing vesicle-associated PI4P levels by depositing sterol to and extracting PI4P from the vesicle membrane (Figure 1.3C, D). However, once again, there was no direct quantification of vesicle-associated PI4P and the two lipid probes used, Fapp1p-PH and Osh2p-PH, are not specific to PI4P (Roy and Levine, 2004). Loss of vesicle-associated PI4P is central to the current model of polarized secretion in *S. cerevisiae*, yet there are no direct data supporting the existence of a PI4P gradient.

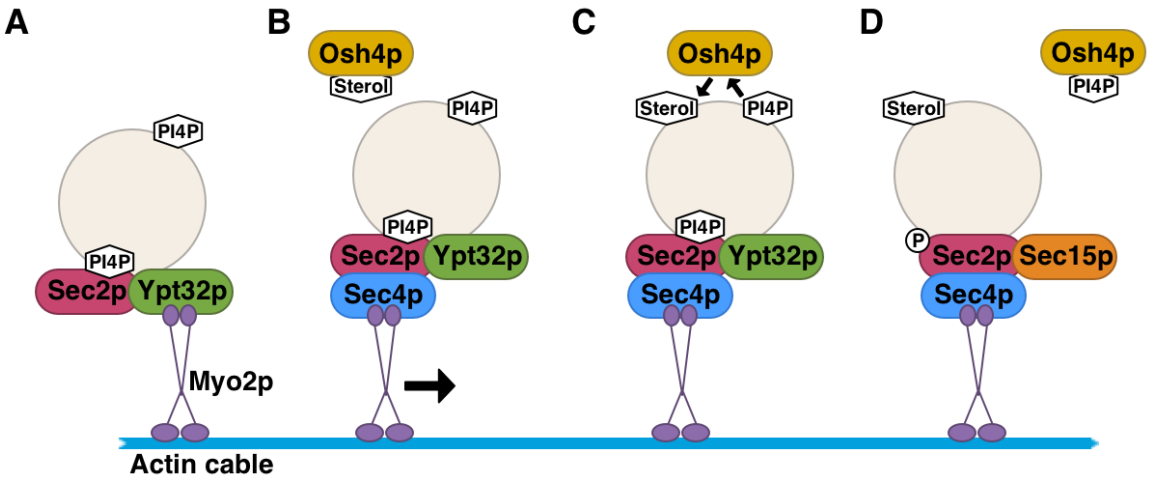


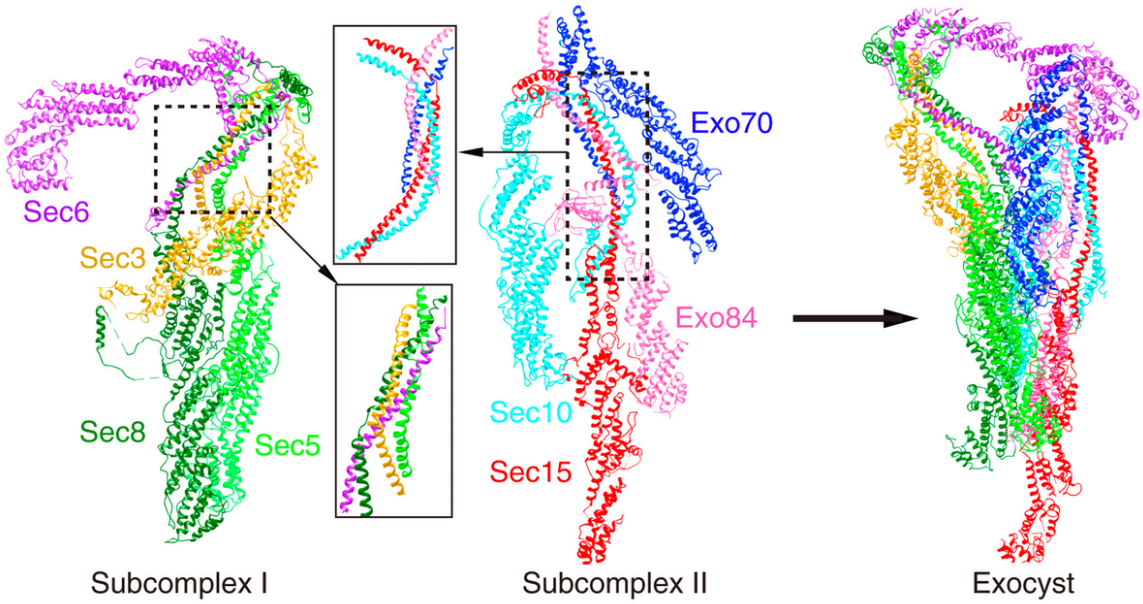
Figure 1.3: PI4P-dependent maturation of polarized secretory vesicles. (A) Sec2p and Ypt32p are present on newly formed vesicles, with Sec2p bound to PI4P inhibiting Sec2p-Sec15p interaction. The motor protein Myo2p is recruited to the vesicle by interacting with Ypt32p, but the vesicle remains immobile. (B) Sec4p is recruited to the vesicle by Sec2p. Myo2p now binds Sec4p and vesicle trafficking begins (indicated by an arrow). (C) Osh4p deposits sterol to the vesicle membrane and binds vesicle-associated PI4P. (D) Osh4p extracts PI4P from the vesicle membrane. Decrease of vesicle-associated PI4P levels triggers the phosphorylation of Sec2p (indicated by P) by an unidentified kinase after which Sec2p no longer binds PI4P (Stalder *et al.*, 2013). Phosphorylation of Sec2p decreases the affinity of Sec2p for Ypt32p and increases affinity for the exocyst complex subunit, Sec15p. Ypt32p dissociates and Sec4p recruits Sec15p, which binds Sec2p at the same site as Ypt32p. Model adapted from Mizuno-Yamasaki *et al.* (2010), Stalder *et al.* (2013), and Ling *et al.* (2014).

Exocyst-mediated tethering

A mature secretory vesicle is capable of tethering and docking at the PM. The definition of tethering and docking can vary among laboratories. Here, tethering refers to the exocyst complex connecting the secretory vesicle to the PM, and docking refers to the formation of *trans*-SNARE complexes. SNAREs are required for vesicle fusion and confer specificity for the target membrane (i.e., the PM), but they do not confer specificity to the sites of polarized growth. Rather, the exocyst complex confers specificity to sites of polarized growth on the PM (reviewed by Lepore *et al.*, 2018).

The exocyst complex is an 845kDa protein complex comprised of eight subunits: Exo70p, Exo84p, Sec3p, Sec5p, Sec6p, Sec7p, Sec10p, and Sec15p (Figure 1.4; TerBush *et al.*, 1996; Guo *et al.*, 1999). In mammalian cells, the exocyst complex is both structurally and functionally similar to the yeast exocyst complex (reviewed by Wu and Guo, 2015). For many years, studies provided structural insight into individual subunits, though advancements in technology have led to the creation of structural models of the entire exocyst complex. Purified intact exocyst complexes from *S. cerevisiae* can, under specific denaturing conditions, be separated into two four-component modules/subcomplexes (Figure 1.4; Heider *et al.*, 2016). One subcomplex is comprised of Sec3p, Sec5p, Sec6p, and Sec8p, and the other is comprised of Exo70p, Exo84p, Sec10p, and Sec15p. These subcomplexes assemble through the formation of four-helix bundles. Cryo-EM and chemical cross-linking studies support the modular/subcomplex model (Mei *et al.*, 2018). As technology continues to advance, further studies will provide insight into the functions of the exocyst complex, including vesicle tethering and SNARE-complex regulation, which is discussed later in this chapter.

Another model for exocyst complex assembly is based on subunit localization wherein subunits are categorized as vesicle-associated subunits (Exo84p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p) or PM-associated subunits (Exo70p and Sec3p; Boyd *et al.*, 2004). Several lines of evidence support this location-based mode, which is not mutually exclusive with the modular/subcomplex model. First, localization of vesicle-associated subunits is dependent on actin assembly, which means these subunits are likely trafficked to the PM on vesicles (Boyd *et al.*, 2004). Furthermore, as previously discussed, Sec15p interacts with secretory vesicles through Sec4p and Myo2p, while Sec6p is recruited to the vesicle by the v-SNARE Snc2p (Shen *et al.*, 2013). Vesicle-associated subunits are also known to interact with t-SNAREs upon arrival to the PM, which suggests the exocyst complex may assume an active role in docking in addition to providing spatial specificity through tethering. In contrast to vesicle-associated subunits, the PM-associated subunits, Exo70p and Sec3p, localize at sites of polarized secretion independently of the actin cytoskeleton (Finger *et al.*, 1998; Boyd *et al.*, 2004). Rather, localization of Sec3p and Exo70p requires direct interaction with both PM PI(4,5)P_2 and Cdc42p (He *et al.*, 2007; Zhang *et al.*, 2008; Wu *et al.*, 2010). In addition, Sec3p interacts with Rho1p, although Rho1p and Cdc42p compete for Sec3p binding, and Exo70p interacts with Rho3p (Robinson *et al.*, 1999; Zhang *et al.*, 2001). Although these interactions have been identified, the coordination and timing of these interactions between Sec3p, Exo70p, Cdc42p, and Rho GTPases is unclear.



Vesicle-associated		
Sec5p	Sec6p	Sec8p

PM-associated
Sec3p

Vesicle-associated		
Exo84p	Sec10p	Sec15p

PM-associated
Exo70p

Figure 1.4: Structural model of the exocyst complex. A labeled model of the exocyst complex. Sec3p, Sec5p, Sec6p, and Sec8p assemble into a subcomplex through formation of four-helix bundles (left, helices in insets). Exo70p, Exo84p, Sec10p, and Sec15p also assemble into a subcomplex. The two subcomplexes interact to form the hetero-octameric exocyst complex (right). Structural model from Mei and Guo (2018).

SNARE-mediated docking

In eukaryotes, membrane fusion during polarized secretion is mediated by conserved v-SNAREs and t-SNAREs located, respectively, on the vesicle and target membrane (i.e., PM; Table 1.1). Together, v- and t-SNAREs form a *trans*-SNARE complex, which brings the vesicle and PM in close proximity to initiate membrane fusion. In yeast, docking of polarized secretory vesicles is mediated by the v-SNARE Snc1/2p and the t-SNAREs Sso1/2p and Sec9p (Gerst *et al.*, 1992; Brennwald *et al.*, 1994; Rossi *et al.*, 1997). The vesicle-associated exocyst subunit Sec6p is known to interact with the v-SNARE Snc1/2p and the t-SNARE Sec9p (Sivaram *et al.*, 2005; Shen *et al.*, 2013; Dubuke *et al.*, 2015). Sec6p interaction with Snc1/2p is hypothesized to stabilize vesicle binding, while Sec6p interaction with Sec9p is hypothesized to drive *trans*-SNARE complex assembly (Dubuke *et al.*, 2015). The PM-associated exocyst subunit Sec3p binds the t-SNARE Sso1/2p and increases the rate of Sso1/2p and Sec9p formation *in vitro* leading to the hypothesis that binding of Sec3p alters the confirmation of Sso1/2p to interact with Sec9p more readily (Yue *et al.*, 2017). Together, these data suggest the exocyst complex plays an active role in both tethering and docking.

v-SNAREs	Exocyst interactions	Homologue
Snc1/2p	Sec6p	Synaptobrevins (Gerst *et al.* , 1992)

t-SNAREs	Exocyst interactions	Homologue
Sso1/2p	Sec3p	Syntaxins (Aalto *et al.* , 1993)
Sec9p	Sec6p	SNAP-25 (Brennwald *et al.* , 1994)

Table 1.1: SNAREs involved in polarized secretion in *S. cerevisiae*.

Oxysterol Binding Protein Homologues

Lipids are essential cellular components that, as discussed above, have direct impacts on cellular functions such as polarized secretion. Cells establish and maintain lipid distribution through several mechanisms including the vesicle-independent transfer of lipids from one membrane to another. Oxysterol binding protein (OSBP) and oxysterol binding protein related proteins (ORPs) are a family of lipid-binding and -transfer proteins. In humans, there are sixteen ORPs linked to membrane contact sites, where vesicle-independent lipid exchanges occur, as well as to viral replication and cancer progression (reviewed by Arora *et al.*, 2022). For instance, ORP3 modulates cell adhesion and is upregulated in several cancers potentially affecting tumorigenesis by interacting with R-Ras and altering cell migration (Lehto *et al.*, 2008; D'Souza *et al*., 2020), and OSBP is required for hepatitis C virus replication by maintaining the sterol-rich viral replication compartment (Wang *et al.*, 2014).

In yeast, ORPs are called oxysterol binding protein homologues (Osh). There are seven members of the Osh protein family and any one is sufficient for cell proliferation, indicating Osh proteins share a yet unknown essential function (Beh *et al.*, 2001). The study of Osh proteins in *S. cerevisiae* has provided key insights into ORP structure and function. For example, the first known ORP structure was Osh4p (Im *et al.*, 2005) and Osh4p was the first ORP shown to transfer lipids between membranes (de Saint-Jean *et al.*, 2011).

Osh protein structure and homology

All ORPs and members of the Osh protein family share a conserved C-terminal OSBP-related lipid ligand-binding domain (ORD) that contains the OSBP signature sequence, EQVSHHPP (Figure 1.5A; reviewed by Arora *et al.*, 2022). Within the ORD, lipid binding occurs in the hydrophobic tunnel of an incomplete β-barrel whose opening is covered by a "lid" domain (Figure 1.5B, orange; Im *et al.*, 2005). One method of classifying Osh proteins is based on the presence or absence of additional structural domains: long Osh proteins (Osh1-3p) contain N-terminal domains that allow for additional lipid binding outside the hydrophobic tunnel and/or protein-protein interactions, whereas short Osh proteins (Osh4-7p) lack significant N-terminal domains. For example, Osh1p contains N-terminal ankyrin repeats that direct Osh1p to nucleus-vacuole junctions as well as a PH domain that directs Osh1p to the Golgi (Levine and Munro, 2001; Kvam and Goldfarb, 2004). No other Osh protein is known to localize to nucleus-vacuole junctions including Osh2p, a paralog of Osh1p that also contains ankyrin repeats. This suggests that, despite sharing an essential function, members of the Osh protein family also serve unique functions.

All Osh proteins can bind PI4P and are capable of or suspected to bind another lipid in a mutually exclusive manner (Im *et al.*, 2005). Osh1p, Osh2p, Osh4p, and Osh5p bind sterol, whereas Osh6p and Osh7p bind PS (de Saint-Jean *et al.*, 2011; Maeda *et al.*, 2013). A second lipid ligand of Osh3p has yet to be identified. Sterol interacts with the hydrophobic tunnel within the β-barrel of the ORD domain through direct interactions and water-mediated interactions explaining why Osh4p can bind different sterols, including ergosterol, cholesterol, and 25-hydroxycholesterol among others (Im *et al.*, 2005; de Saint-

Jean *et al.*, 2011). In addition to binding lipids, Osh proteins are also capable of transferring lipids between membranes *in vitro* (de Saint-Jean *et al.*, 2011; von Filseck *et al.*, 2015b). Currently, technology limits our ability to visualize *in vivo* lipid transfer. No study has directly demonstrated lipid transfer, though Osh6p was indirectly shown to transfer PS from the ER to the PM *in vivo* (von Filseck *et al.*, 2015a).

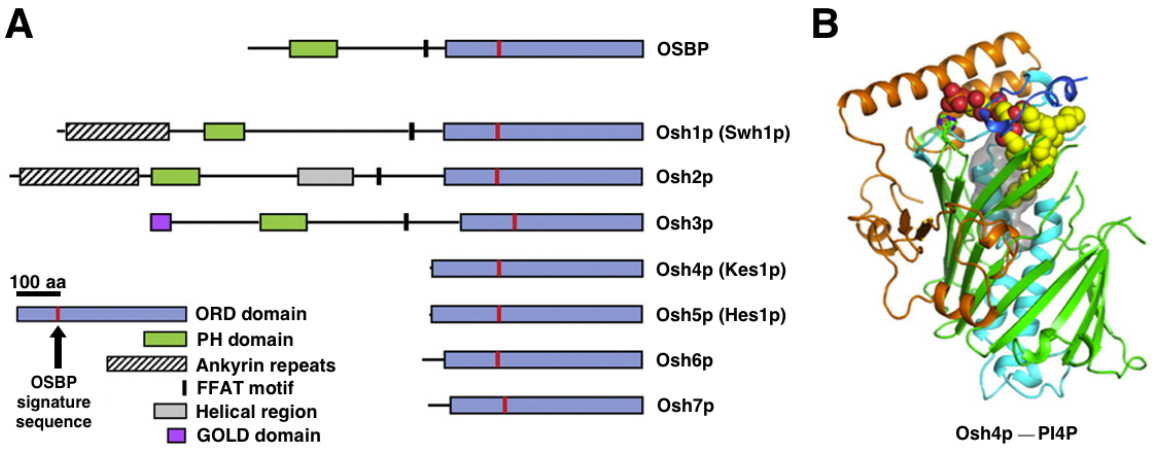


Figure 1.5: Oxysterol binding protein homologue structure. (A) Domain organization of mammalian OSBP and yeast Osh protein family members. The Osh protein family can be broken into two groups: long Osh proteins that contain N-terminal domains that allow for additional lipid binding outside the hydrophobic tunnel and/or protein-protein interactions (Osh1-3p), and short Osh proteins that lack significant N-terminal domains (Osh4-7p). All Osh proteins contain a conserved ORD domain, which contains the OSBP signature sequence, EQVSHHPP. Domains listed are OSBP-related lipid ligand-binding (ORD), pleckstrin homology (PH), and Golgi dynamic (GOLD). (B) Model of Osh4p bound to PI4P. Osh4p has a partial β-barrel with a hydrophobic ligand binding tunnel and a flexible lid to cover the tunnel entrance. PI4P is shown as a space-fill model. Hydrophobic tunnel (transparent, gray), ORD domain (blue), central α-helices (cyan), β-barrel (green), and lid domain (orange). Figure adapted from Tong *et al.* (2016).

Role of Osh4p in secretion

The Osh protein family supports polarized cell growth, with Osh4p localizing to both the TGN and secretory vesicles (Kozminski *et al.*, 2006; Alfaro *et al.*, 2011). However, the function of Osh4p at the TGN is unclear. One hypothesis is Osh4p transfers PI4P down its concentration gradient from the TGN to the ER, where PI4P is hydrolyzed by Sac1p, providing free energy for sterol to be transported from the ER to the TGN against its concentration gradient (de Saint-Jean *et al.*, 2011). This function would be conserved because in metazoan cells, OSBP is required for delivery of sterols to the TGN in exchange for PI4P (Mesmin *et al.*, 2013; Mesmin *et al.*, 2017). Others propose Osh4p regulates secretory vesicle formation at the *trans*-Golgi (Fang *et al.*, 1996). Osh4p was initially identified as Kes1p, standing for *kre11-1* suppressor (Brown *et al.*, 1993; Jiang *et al.*, 1994). Kre11p is otherwise known as TRS65 and is a component of TRAPPII, which, as previously discussed, localizes to the *trans*-Golgi and is speculated to be the GEF for the Rab GTPase Ypt32p (Figure 1.2; Sacher *et al.*, 2000). Because Ypt32p is required for exit from the TGN, Osh4p may play a non-essential role in regulating vesicle formation. In addition, Osh4p is a negative regulator of Sec14p, an essential PC/PI transfer protein with a role in isotropic secretion (Bankaitis *et al.*, 1989; Fang *et al.*, 1996). Though Osh protein function does not significantly affect invertase-marked isotropic secretion (Smindak *et al.*, 2017), inactivation of Sec14p can be bypassed by Osh4p inactivation (Fairin *et al.*, 2007).

Although the function of Osh4p at the TGN and whether Osh4p plays a role in isotropic growth is unclear, multiple studies demonstrated Osh4p is involved in polarized growth. Kozminski *et al.* (2006) demonstrated that in cells lacking Osh protein function, Cdc42p and Sec4p localization were perturbed and polarized secretory vesicles

accumulated, indicating Osh proteins are required for polarized growth. Furthermore, Osh4p localizes to Sec4p-marked vesicles (Alfaro *et al.*, 2011; Ling *et al.*, 2014; Smindak *et al.*, 2017). As previously discussed, in the current model for polarized secretion, vesicle maturation depends on a loss of vesicle-associated PI4P. Ling *et al.* (2014) proposed Osh4p extracts vesicle-associated PI4P and deposits it at the PM or at the cortical ER through transient unstable interactions with the vesicle. Although there is no direct evidence to support Osh4p-mediated loss of vesicle-associated PI4P, Osh4p must be capable of binding PI4P to localize to Sec4p-marked vesicles to the extent seen in WT cells (Smindak *et al.*, 2017). In addition, in cells where the only Osh protein is lipid-binding deficient Osh4p (i.e., Osh4p cannot bind PI4P and/or sterol), vesicles were less likely to associate with the PM and form *trans*-SNARE complexes (Smindak *et al.*, 2017). From these observations we can conclude Osh4p and potentially other members of the Osh protein family are involved in regulating polarized secretion. Smindak *et al.* (2017) proposed that, in addition to decreasing vesicle-associated PI4P, Osh4p mediates a lipid exchange between the vesicle and PM that is required for *trans*-SNARE formation. It is unknown whether another Osh protein family member, such as Osh6p, which binds PI4P and PS, is also capable of independently supporting polarized secretion in a lipid-dependent manner.

Current Model of Polarized Secretion and Remaining Questions

The current model of polarized secretion in *S. cerevisiae* hinges on the existence of a PI4P gradient in which vesicles are enriched in PI4P at the *trans*-Golgi relative to when they arrive at the PM (Figure 1.6). In this model, Osh4p-mediated loss of vesicle-associated

PI4P is a driving factor of vesicle maturation. However, there is no direct evidence demonstrating Osh4p regulates a loss of vesicle-associated PI4P. This dissertation addresses several major questions regarding the current model of polarized secretion. Can Osh6p substitute for Osh4p to independently support polarized secretion? Do vesicle-associated PI4P levels change as vesicles traffic? Lastly, what is the role of Osh4p in polarized secretion?

Data presented in this dissertation provide novel insights into the role of the Osh protein family during polarized secretion and support a new model for vesicle maturation in *S. cerevisiae*. These are important steps towards deciphering the mechanisms of polarized secretion and the function of ORPs in mammalian cells.

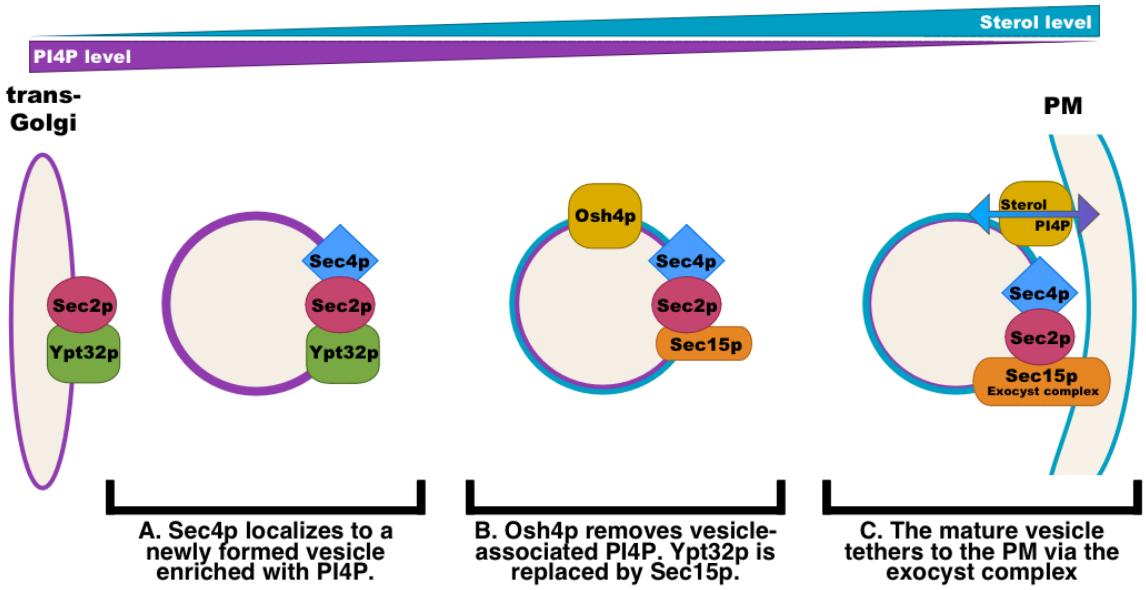


Figure 1.6: Existing model of polarized secretion in *S. cerevisiae*. (A) The Rab GTPase Ypt32p is bound to the Rab GEF Sec2p on newly formed vesicles enriched in PI4P. The Rab GTPase Sec4p is recruited and binds Sec2p at a distinct site. (B) As the vesicle traffic to the PM, Osh4p extracts vesicle-associated PI4P. Loss of PI4P results in the dissociation of Ypt32p and the recruitment and activation of exocyst complex subunit Sec15p, an effector of Sec4p which binds Sec2p at the same site as Ypt32p. (C) The exocyst complex is fully formed. For the mature vesicle to dock at the PM (i.e., for *trans*-SNARE complexes to form), Osh4p mediates a lipid exchange where vesicle-PI4P is deposited on the PM and PM-sterol is deposited on the vesicle. Model adapted from Ling *et al.* (2014) and Smindak *et al*. (2017).

CHAPTER 2

Osh6p Can Independently Support Polarized Secretion In a Lipid-dependent Manner

Data presented in this chapter were part of the following publication:

> Smindak RJ, Heckle LA, Chittari SS, Hand MA, Hyatt DM, Mantus GE, Sanfelippo WA, Kozminski KG (2017). Lipid-dependent regulation of exocytosis in *S. cerevisiae* by OSBP homolog (Osh) 4. *Journal of Cell Science* 130, 3891-3906.

Figures and tables were re-numbered in accordance with the dissertation formation. All sections except for the 2.1 figure description were written by L. Heckle for this dissertation.

Author Contributions

Lindsay A. Heckle: Data collection for and figure construction of Figure 8, which is re-numbered and presented below as Figure 2.1 with a new figure title.

Abstract

Polarized secretion is an essential process whose proteins and key steps are conserved across eukaryotes. In the budding yeast *S. cerevisiae*, polarized secretion is required for cell growth and proliferation. Previous studies in *S. cerevisiae* demonstrated that at least one member of the oxysterol binding protein homologue (Osh) family of lipid transfer proteins is required for survival. This indicates the Osh protein family shares a yet unknown essential function despite members displaying different lipid binding capabilities. For example, Osh4p can exchange PI4P and sterol, while Osh6p can bind PI4P and PS. Prior data from our lab demonstrated that Osh4p alone can support polarized secretion in a lipid-dependent manner (Smindak *et al.*, 2017). Here, I demonstrate that in the absence of all other Osh proteins, Osh6p can also independently support polarized secretion in a lipid-dependent manner. This implies that the role of Osh proteins in polarized secretion is not directly dependent on sterol or PS, though sterol and PS, respectively, are required for Osh4p and Osh6p to independently support polarized secretion.

Introduction

Lipids are essential components that not only confer structure and identity to cellular membranes but are also directly involved in processes such as intracellular signaling and protein recruitment. The synthesis of lipids and the establishment and maintenance of asymmetric lipid distribution are highly regulated processes (reviewed by Chauhan *et al.*, 2016; Balla *et al.*, 2020). Many lipids are synthesized in one compartment then transferred to their final destination through vesicle-dependent and -independent mechanisms (Baumann *et al.*, 2005). Lipid transfer proteins mediate lipid exchange between membranes independent of vesicles (reviewed by Wong *et al.*, 2018).

Oxysterol binding protein (OSBP)-related proteins (ORPs) comprise a large, conserved family of lipid transfer proteins. In the budding yeast *Saccharomyces cerevisiae*, at least one member of the seven-protein oxysterol binding protein homologue (Osh) protein family is required for survival indicating Osh proteins share a yet unknown essential function (Beh *et al.,* 2001). This is particularly interesting because members of the Osh protein family localize to different cellular locations, possess different lipid- and protein-binding domains, and do not necessarily bind the same ligands (reviewed by Tong *et al.*, 2016; Delfosse *et al.*, 2020). For example, Osh1p is the only Osh protein known to localize to nuclear-vacuole junctions and Osh4p is known to localize to the Golgi and secretory vesicles, though both Osh1p and Osh4p bind PI4P and sterol in a mutually exclusive manner (Levine and Munro, 2001; Li *et al.*, 2002; Im *et al.*, 2005; Alfaro *et al.*, 2011; de Saint-Jean *et al.*, 2011).

Previous studies in *S. cerevisiae* demonstrated that Osh proteins are required for polarized secretion, a process that involves the release of exocytic cargo at the PM from

trans-Golgi-derived vesicles (Kozminski *et al.*, 2006; Alfaro *et al.*, 2011). Prior data collected by our lab and presented in Smindak *et al.* (2017) showed that in the absence of all other Osh proteins, Osh4p can independently support polarized secretion in a lipid-dependent manner (i.e., Osh4p must be capable of binding both PI4P and sterol). If Osh4p cannot bind PI4P and/or sterol, *trans*-SNARE complexes fail to assemble to the extent seen in WT cells. Here, we demonstrate that Osh6p, which binds PI4P and PS, is also able to independently support polarized secretion in a lipid-dependent manner in the absence of all other Osh protein function (de Saint-Jean *et al.*, 2011; Maeda *et al.*, 2013). This result implies that despite possessing different structural domains and lipid binding capabilities and, together with prior data, led to a new model for polarized secretion in which Osh4p mediates a non-specific lipid exchange between the vesicle and PM.

Materials and Methods

Yeast Strains and Growth Conditions

S. cerevisiae strains used in this chapter are listed in Table 2.1. Strains were grown at 25˚C in synthetic complete medium (SC), a minimal medium, or in SC lacking one or more amino acids (e.g., SC-TRP) or uracil (Sherman *et al.*, 1986) with 2% glucose.

Bgl2p Accumulation Assay

Quantification of intracellular Bgl2p accumulation was performed as described in Harsay and Schekman (2007). To detect Bgl2p, we probed Bgl2p with a polyclonal rabbit antibody (1:5,000; Alfaro *et al.*, 2011) and a 680LT goat anti-rabbit secondary antibody (1:10,000; catalogue no. 926-68021, Li-Cor, Lincoln, NE). Tubulin served as a loading control and was detected with AA2, a mouse monoclonal antibody against amino acids 412-430 of bovine brain β-tubulin (1:10,000; kind gift of A. Frankfurter, University of Virginia) and IRDye 800CW goat anti-mouse secondary antibody (1:10,000; catalogue no. 926-32210, Li-Cor, Lincoln, NE). Immunoblots were imaged on an Odyssey Infrared Imaging System (Licor, Lincoln, NE). Band intensity was quantified with Image Studio (catalog no. 9202-500, Licor, Lincoln, NE).

Table 2.1: *S. cerevisiae* strains used in Chapter 2.

Strain	Relevant genotype	Source
SEY6210	*MATα leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1Δ901 suc2Δ9*	Robinson *et al.*, 1988
JRY6320	SEY6210 *osh1Δ::URA3 osh2Δ::URA3 osh3Δ::LYS2 OSH4 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3*	Beh *et al.*, 2001
JRY6324	SEY6210 *osh1Δ::URA3 osh2Δ::URA3 osh3Δ::LYS2 osh4Δ::HIS3 osh5Δ::LEU2 OSH6 osh7Δ::HIS3*	Beh *et al.*, 2001
CBY926	SEY6210 *osh1Δ::kanMX4 osh2Δ:: kanMX4 osh3Δ::LYS2 osh4Δ::HIS3 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3* [pRS314 (*osh4-1*^{ts})]	Beh and Rine, 2004
KKY1311	CBY926 [pRS316]	This study
KKY1312	CBY926 [pRS316 (*OSH6*)]	This study
KKY1313	CBY926 [pRS316 (*osh6*^{L69D})	This study
KKY1314	CBY926 [pRS316 (*osh6*$^{H157A/H158A}$)]	This study

Strains with the source "this study" were generated by R. Smindak or K. Kozminski as part of the publication Smindak *et al.* (2017).

Results

Osh6p can substitute for Osh4p to independently support polarized secretion

Prior data in our lab demonstrated Osh4p, in the absence of all other Osh protein function, is sufficient for cell growth and polarized secretion in a lipid-dependent manner (i.e., Osh4p must be capable of binding both PI4P and sterol; Smindak *et al.*, 2017). To determine whether Osh6p, which binds PI4P and PS, can also independently support polarized secretion, we quantified the fold change of internal Bgl2p, a cargo protein specific to vesicles involved in polarized secretion, to tubulin, after shifting from 25˚C to 37˚C (Harsay and Schekman, 2007; Dighe and Kozminski, 2008; Johansen *et al.*, 2016; see Materials and Methods). Accumulation of Bgl2p indicates a failure to execute polarized secretion. Consistent with previous results, in cells expressing all members of the Osh protein family (WT) and cells expressing only Osh4p (*osh*Δ *OSH4*), there was no significant accumulation of Bgl2p post-shift to the non-permissive temperature (Figure 2.1A). Similarly, in cells where the only functioning Osh protein is Osh6p (*osh*Δ *OSH6*), there was no significant increase in internal Bgl2p post-shift to the non-permissive temperature. This is consistent with prior results demonstrating any one member of the Osh protein family is sufficient for survival as polarized secretion is an essential process (Beh *et al.*, 2001).

To determine whether Osh6p supports cell growth in a lipid-dependent manner, we expressed mutant *osh6* alleles deficient in PS binding ($osh6^{L69D}$) or PI4P binding ($osh6^{H157A/H158A}$) in *osh*Δ [*osh4-1*] cells (Figure 2.2B; Maeda *et al.*, 2013; von Filseck *et al.*, 2015a). At a permissive temperature, expression of *osh4-1* alone in cells lacking all other Osh proteins (*osh*Δ) was sufficient to support cell growth (Beh and Rine, 2004).

However, at the non-permissive temperature, *osh4-1* is nonfunctional. Consistent with this, at the permissive temperature, in *osh*Δ [*osh4-1*] cells, expression of WT *OSH6*, PS-binding deficient *osh6*^{L69D}, or PI4P-binding deficient *osh6*$^{H157A/H158A}$ permitted cell growth (Figure 2.1B). However, at the non-permissive temperature, only cells expressing WT *OSH6* supported cell growth. This indicates that, in the absence of all other Osh protein function, Osh6p must be capable of binding both PS and PI4P to support cell growth.

Next, to determine whether failure to grow in cells reliant on lipid binding deficient *osh6* alleles resulted from a failure to execute polarized secretion, we quantified Bgl2p accumulation in *osh*Δ [*osh4-1*] cells carrying WT *OSH6* or a lipid binding deficient *osh6* allele (Figure 2.1C). At the permissive temperature, no strains accumulated Bgl2p, indicating the successful completion of polarized secretion. Consistent with prior results, at the non-permissive temperature, in *osh*Δ [*osh4-1*] cells expressing WT *OSH6*, there was no significant increase in internal Bgl2p post-temperature shift, while *osh*Δ [*osh4-1*] cells expressing an empty vector saw a significant increase in internal Bgl2p (Figure 2.1C; compare to Figure 2.1B). In strains where the only functional Osh protein at the non-permissive temperature was deficient in lipid-binding, *osh6*^{L69D} or *osh6*$^{H157A/H158A}$, there was a significant increase in Bgl2p accumulation. Together, these results indicate that, like Osh4p, Osh6p must bind both lipid ligands to independently support polarized secretion in the absence of all other Osh protein function.

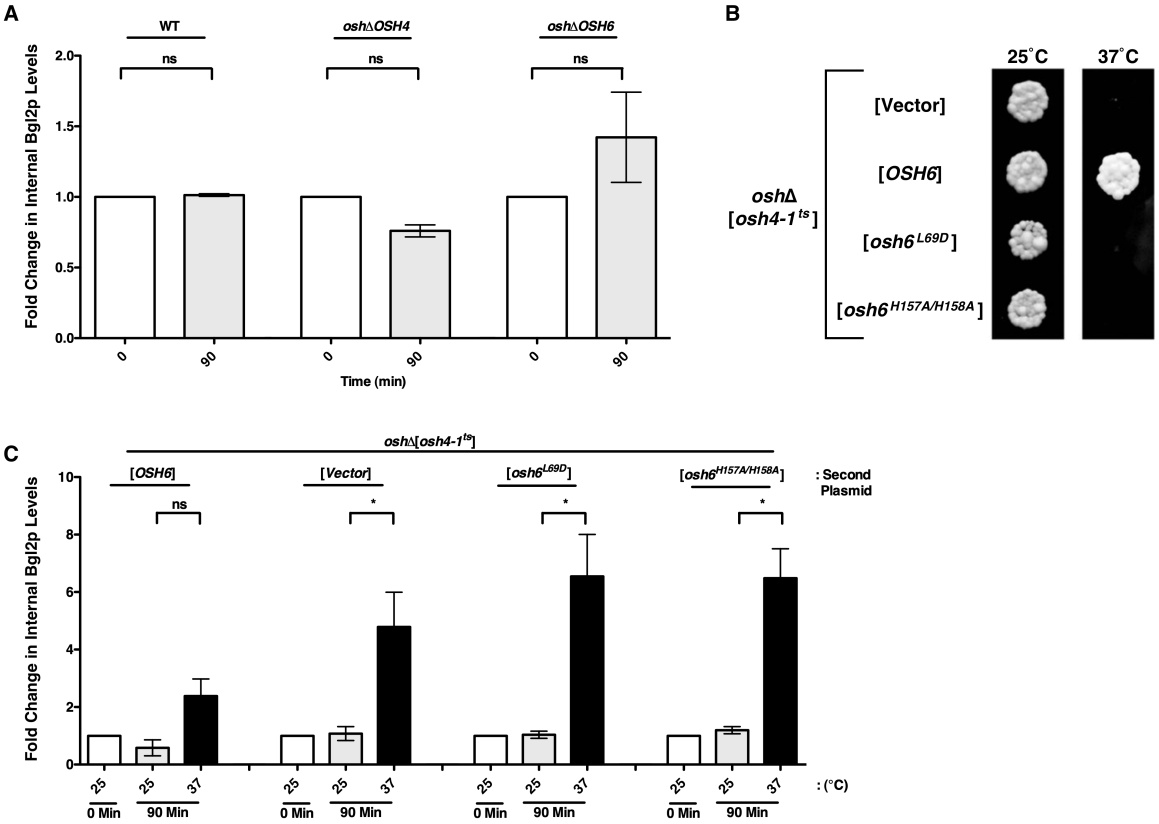


Figure 2.1: Osh6p promotes polarized secretion in a lipid-dependent manner. (A) Fold change in the amount of internal Bgl2p in log-phase WT (SEY6210), *oshΔ OSH4* (JRY3620), and *oshΔ OSH6* (JRY3624) strains, relative to time 0, after 90 min growth at 25°C, as measured by immunoblotting (*n*=2). Tubulin served as a loading control. Total Bgl2p levels were approximately equal among the strains. (B) Equivalent dilutions of an *oshΔ* [*osh4-1*] strain containing a second *CEN* plasmid with either an empty vector (KKY1311), WT *OSH6* (KKY1312), PS binding deficient $osh6^{L69D}$ (KKY1313), or PI4P binding deficient $osh6^{H157A/H158A}$ (KKY1314) grown on minimal medium for 5 days at 25°C or 37°C. (C) Fold change in the amount of internal Bgl2p in log-phase *oshΔ* [*osh4-1*] cells containing a second plasmid with or without an *OSH6* allele, as shown in B, at 25°C or after shift to 37°C for 90 min, relative to time 0 at 25°C, as measured by immunoblotting (*n*=3). Tubulin served as a loading control. Total Bgl2p levels were

approximately equal among the strains. Data were analyzed using one-tailed Student's *t*-test. Figure adapted from Smindak *et al.* (2017; Figure 8). For this dissertation, the figure title was changed, KKY numbers were added to the figure description, and the panels were rearranged without changing the order (i.e., A, B, C).

Discussion

In this study of polarized secretion, we demonstrated that in the absence of all other Osh protein function, Osh6p, like Osh4p, can independently support polarized secretion in a lipid-dependent manner. This is the first study to demonstrate the necessity of either Osh4p lipid ligand binding or Osh6p lipid ligand binding in polarized secretion. These results are consistent with the model of polarized secretion proposed in Ling *et al.* (2014) wherein vesicle maturation is dependent on Osh4p-mediated loss of vesicle-associated PI4P and suggests that Osh6p may serve a similar role in the absence of other members of the Osh protein family. This study also brings insight into the currently unknown essential function shared by the Osh protein family, as Osh4p and Osh6p bind different ligands. Osh4p binds PI4P and sterol, whereas Osh6p binds PI4P and PS, yet neither protein can independently support cell growth without binding both ligands.

As any one member of the Osh protein family is sufficient for survival, we can infer that all seven Osh proteins are capable of supporting polarized secretion despite displaying differences in protein structure and lipid ligands (see Figure 1.5A). It is likely that the shared essential function of the Osh protein family is not directly related to Osh-mediated PS distribution at a cellular level, as Osh4p alone would not support polarized cell growth because it does not bind PS. Similarly, the shared function cannot be related to sterol distribution because Osh6p does not bind sterol. Rather, Smindak *et al.* (2017) proposed a model in which Osh4p mediates a lipid exchange between the vesicle and PM that facilitates the formation of *trans*-SNARE complexes. This was based, in part, on data that demonstrated, in cells reliant on Osh4p, Osh4p must bind both PI4P and sterol or *trans*-SNARE complexes do not form as efficiently compared to WT cells. Because Osh6p can

also independently support polarized secretion in a lipid-dependent manner, we conclude that Osh4p mediates a non-specific lipid exchange in which PI4P and sterol or PS are transferred.

Osh4p may be responsible for altering the composition and/or charge of either the vesicle or PM to recruit proteins that trigger *trans*-SNARE complex formation. This hypothesis is not unreasonable because prior studies demonstrated lipid composition directly affects protein localization at sites of polarized secretion (i.e., the bud tip and bud neck). For example, PI(4,5)P_2 and PS are enriched at sites of polarized secretion (He *et al.*, 2007; Fairn *et al.*, 2011). However, the lipid ligands of Osh6p, PI4P and PS, are both negatively charged. It is possible the lipid exchange exists to transfer Osh4p from the vesicle surface to the PM, though why this would be a lipid-dependent process is unclear.

Alternatively, Osh proteins may interact with SNAREs directly. Weber-Boyat *et al.* (2021) demonstrated three Osh proteins, Osh1-3p, interact with the t-SNARE Sec9p, which is involved in but is not specific to polarized secretion. This interaction was conserved in mammals between ORP2 and SNAP-25. Weber-Boyat *et al.* (2021) determined this interaction in yeast requires SNARE motifs on Sec9p, but they did not identify which region of Osh1-3p is required. This is unfortunate because Osh1-3p are long Osh proteins with N-terminal domains that both Osh4p and Osh6p lack. Additional studies are required to elucidate the relationship between the Osh protein family and SNAREs and the significance of an Osh-mediated vesicle-PM lipid exchange.

CHAPTER 3

Osh-dependent and -independent Regulation of PI4P Levels During Polarized Cell Growth in *Saccharomyces cerevisiae*

Chapter 3 was submitted for publication and is in revision.

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Figures and tables were re-numbered in accordance with the dissertation formation and supplemental videos were converted to time lapse images (see Supplemental Figures 3.3-4). Supplemental Video 3 is excluded from this dissertation because the time lapse is shown in Figure 3.3A. Supplemental Video 4 is excluded because a comparable time lapse is shown in 3.3B.

Author Contributions

Lindsay A. Heckle: Experimental design, data collection, data analysis, data interpretation, figure construction, and drafting of manuscript.

Keith G. Kozminski: Experimental design, data interpretation, drafting of manuscript.

Abstract

Polarized secretion facilitates polarized cell growth. For a secretory vesicle to dock at the plasma membrane, it must mature with a progressive association or dissociation of molecules that are, respectively, necessary or inhibitory to vesicle docking, including an exchange of Rab GTPases. In current models, oxysterol binding protein homologue 4 (Osh4p) establishes a PI4P gradient along the secretory trafficking pathway such that vesicles have higher PI4P levels after budding from the *trans*-Golgi relative to when vesicles arrive at the plasma membrane. In this study, using the lipid binding domain P4M and live cell imaging, we show that secretory vesicle-associated PI4P levels remain constant when vesicles traffic from the *trans*-Golgi to the plasma membrane. We also show that deletion of *OSH4* does not alter vesicle-associated PI4P levels, though loss of any individual member of the *OSH* family or complete loss of *OSH* family function alters the intracellular distribution of PI4P. We propose a model in which the Rab GTPases Ypt32p and Sec4p, remain associated with a secretory vesicle during trafficking, independent of PI4P levels and Osh4p. Together these data indicate the necessity of experiments revealing the location and timing of events required for vesicle maturation.

Introduction

Polarized cell growth depends in part on polarized secretion, a process that involves the exocytic release of cargo from vesicles at one pole of the cell after they traffic from the *trans*-Golgi network (TGN) to the plasma membrane (PM). Notable examples include the release of neurotransmitters from axon terminals and the release of zymogen from the apical surface of pancreatic acinar cells (reviewed by Morris, 2020; Low *et al.*, 2010). In the budding yeast *Saccharomyces cerevisiae*, polarized secretion is essential for bud and eventual daughter cell formation. Among eukaryotes there is commonality among many of the key events and molecules that support this process (reviewed by Zeng *et al.*, 2017).

A key event within the process of polarized secretion is vesicle maturation, whereby different types of molecules associate or dissociate with a vesicle, allowing vesicle trafficking and conversion of the vesicle from a PM docking incompetent to a docking competent state. Examples of vesicle maturation include the Rab27-dependent maturation of insulin granules in pancreatic islet cells (reviewed by Izumi, 2021), the Rab26-dependent regulation of secretory granule maturation in *Drosophila* salivary gland cells (Boda *et al.*, 2023), and the Rab2-dependent maturation of presynaptic precursor vesicles (Götz *et al*., 2021). Most of this research has focused on proteins, for which molecular probes exist in abundance. Much less is known about changes in the lipid composition of vesicles as they traffic from the TGN to PM due to the compositional heterogeneity of isolated vesicles (Harsay and Bretscher, 1995), the promiscuity of early generation lipid probes (reviewed in Wills *et al.*, 2018 and Hammond *et al.*, 2022), and the challenges of high spatial and temporal resolution imaging.

The aforementioned lack of knowledge is unfortunate because there exist models in which vesicle lipid composition is critical for vesicle maturation (Sturek *et al.*, 2010; Ling *et al.*, 2014). In *S. cerevisiae*, for example, a PI4P gradient along the secretory trafficking pathway, where vesicles have higher PI4P levels immediately after budding from the *trans*-Golgi relative to when vesicles arrive at the PM, has been posited to further vesicle maturation, specifically the replacement of a Rab GTPase, Ypt32p, with another Rab GTPase, Sec4p (Mizuno-Yamasaki *et al*., 2010). In addition, the vesicle acquires Sec15p, an exocyst complex subunit, in a Sec4p-dependent manner (TerBush *et al.*, 1996; Guo *et al.*, 1999). Without these maturation events, the vesicle is unable to dock at the PM at sites of polarized growth, the bud tip and bud neck. In this model, vesicle maturation is dependent upon vesicle-associated PI4P levels (Ling *et al.*, 2014).

Additional research proposed that a member of the oxysterol binding protein homologue (Osh) family, Osh4p (Kes1p), establishes the vesicular PI4P gradient by extracting PI4P from the vesicle as the vesicle traffics (Ling *et al.*, 2014). Osh proteins are lipid binding proteins that are the homologues of the mammalian oxysterol binding protein (OSBP) and oxysterol binding-related proteins (ORPs; reviewed by Tong *et al.*, 2016; Delfosse *et al.*, 2020; Arora *et al.*, 2022). Osh4p binds both PI4P and sterol and can extract and exchange these lipids between membranes (Im *et al.*, 2005; de Saint-Jean *et al.*, 2011), and is linked to cell polarity (Kozminski *et al.*, 2006). Although there is *in vivo* evidence that strongly supports the association of Osh4p with secretory vesicles (Alfaro *et al.*, 2011; Smindak *et al.*, 2017) and *in vitro* evidence that *trans*-SNARE complex formation at the PM depends upon Osh4p binding PI4P (Smindak *et al.*, 2017), it is unclear whether these vesicles exhibit a change in PI4P level that is Osh4p-dependent as they traffic.

Recent advancements in lipid biosensors (reviewed in Wills *et al.*, 2018 and Hammond *et al.*, 2022) allowed us to directly determine whether secretory vesicles in *S. cerevisiae* display a changing PI4P level while trafficking from the *trans*-Golgi to the PM and to determine the role of the Osh protein family in this process. We show, contrary to a current model, that vesicular PI4P levels do not change significantly during vesicle trafficking to sites of polarized cell growth. Furthermore, we show that despite sharing a yet undefined common function, the seven members of the Osh protein family serve unique roles in maintaining the distribution of PI4P within cells. We propose a model in which vesicle-associated PI4P levels do not change during trafficking and Osh4p is only functionally relevant during vesicle formation and/or just prior to vesicle docking.

Materials and Methods

Yeast Strains and Growth Conditions

S. cerevisiae strains used in this study are listed in Table 3.1. Strains were grown at 25˚C in synthetic complete medium (SC), a minimal medium, or in SC lacking one or more amino acids (e.g., SC-LEU) or uracil (Sherman *et al.*, 1986) with 2% glucose. Transformed strains were generated by the method of Schiestl and Gietz (1989). For all experiments, unless otherwise noted, three independent transformants of each strain were analyzed.

Plasmids used in this study are listed in Table 3.2. All oligonucleotides used are listed in Supplemental Table 3.1. To construct integrants, the following were performed. YIplac211 (*SEC7-mCherry2bx6*), also known as Addgene plasmid #105267, was digested with SpeI and transformed into yeast at the *SEC7* locus. Integration was validated by PCR amplification of genomic DNA using primers oKK431 and oKK432. YIplac211-mCherry-PUS1 was digested with SacII and integrated at the *PUS1* locus in yeast. pRS306 (*pADH1-GFP-YPT32-CYC1term*), also known as NRB1325, was digested with StuI and transformed into yeast at the *ura3-52* locus. Integration of *pADH1-GFP-YPT32-CYC1term* was validated by PCR amplification of genomic DNA using primers oKK437 and oKK438. For construction of strains expressing a lipid probe, see below.

Table 3.1: *S. cerevisiae* strains used in Chapter 3.

Strain	Relevant genotype	Source
SEY6210	*MATα leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1Δ901 suc2Δ9*	Robinson *et al.*, 1988
AAY104	SEY6210 *pik1::HIS3* [pRS314 (*pik1-83*^{ts})]	Audhya *et al.*, 2000[1]
JRY6320	SEY6210 *osh1Δ::URA3 osh2Δ::URA3 osh3Δ::LYS2 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3*	Beh *et al.*, 2001
CBY924	SEY6210 *osh1Δ::kanMX4 osh2Δ:: kanMX4 osh3Δ::LYS2 osh4Δ::HIS3 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3* [pRS314 (*OSH4*)]	Beh and Rine, 2004
CBY926	SEY6210 *osh1Δ::kanMX4 osh2Δ:: kanMX4 osh3Δ::LYS2 osh4Δ::HIS3 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3* [pRS314 (*osh4-1*^{ts})]	Beh and Rine, 2004
NY17	*MATa ura3-52 sec6-4*^{ts}	Novick *et al.*, 1980
KKY1389	SEY6210 *leu2::pTEF1-Clover-P4M-SynTerm21:LEU2 sec7::SEC7-mCherry2bx6:URA3* [pRS314]	This study
KKY1391	AAY104 [YCplac111 (*pTEF1-Clover-P4M-SynTerm21*)] *sec7::SEC7-mCherry2bx6:URA3*	This study
KKY1394	SEY6210 *leu2-3,112::pTEF1-Clover-D4H-SynTerm21:LEU2 sec7::SEC7-mCherry2bx6:URA3* [pRS314]	This study
KKY1396	AAY104 [YCplac111 (*pTEF1-Clover-D4H-SynTerm21)*] *sec7::SEC7-mCherry2bx6:URA3*	This study
KKY1398	SEY6210 *leu2-3,112::pTEF1-Clover-D4H-SynTerm21:LEU2 pus1::PUS1-Cherry:URA3* [pRS314]	This study
KKY1400	SEY6210 *leu2-3,112::pTEF1-mCherry2b-P4M-SynTerm21:LEU2 ura3-52::pADH1-GFP-YPT32-CYC1term:URA3*	This study
KKY1401	SEY6210 *leu2-3,112::pTEF1-mCherry2b-P4M-SynTerm21:LEU2 ura3-52::URA3*	This study
KKY1403	SEY6210 *leu2-3,112::LEU2 ura3-52:: pADH1-GFP-YPT32-CYC1term:URA3*	This study
KKY1404	SEY6210 *leu2-3,112::LEU2 ura3-52::URA3*	This study
KKY1405	SEY6210 [pRS316 (*GFP-SEC4*)] *leu2-3,112::pTEF1-mCherry2b-P4M-SynTerm21:LEU2*	This study
KKY1406	SEY6210 [pRS316] *leu2-3,112::pTEF1-mCherry2b-P4M-SynTerm21:LEU2*	This study
KKY1407	SEY6210 *leu2-3,112::LEU2* [pRS316]	This study
KKY1408	SEY6210 *leu2-3,112::LEU2* [pRS316 (*GFP-SEC4*)]	This study

KKY1416	SEY6210 *osh1Δ::URA3* *leu2-3,112::pTEF1-Clover-P4M-SynTerm21:TRP1*	This study
KKY1417	SEY6210 *osh2Δ::URA3* *leu2-3,112::pTEF1-Clover-P4M-SynTerm21:TRP1*	This study
KKY1418	SEY6210 *osh3Δ::LYS2* *leu2-3,112::pTEF1-Clover-P4M-SynTerm21:TRP1*	This study
KKY1419	SEY6210 *osh4Δ::HIS3* *leu2-3,112::pTEF1-Clover-P4M-SynTerm21:TRP1*	This study
KKY1420	SEY62010 *osh5Δ::LEU2* *ura3-52::pTEF1-Clover-P4M-SynTerm21:TRP1*	This study
KKY1421	SEY62010 *osh6Δ::LEU2* *ura3-52::pTEF1-Clover-P4M-SynTerm21:TRP1*	This study
KKY1422	SEY62010 *osh7Δ::HIS3* *ura3-52::pTEF1-Clover-P4M-SynTerm21:TRP1*	This study
KKY1424	SEY6210 *ura3-52::pTEF1-Clover-P4M-SynTerm21:TRP1*	This study
KKY1426	SEY6210 *osh4Δ::HIS3* *leu2-3,112::pTEF1-mCherry2b-P4M-SynTerm21:LEU2* *ura3-52::pADH1-GFP-YPT32-CYC1term:URA3*	This study
KKY1427	SEY6210 [pRS314 (*pTEF1-Clover-P4M-SynTerm21 LEU2*)]	This study
KKY1428	JRY6320 [pRS314 (*pTEF1-Clover-P4M-SynTerm21 LEU2*)]	This study
KKY1429	SEY6210 [pRS314] *ura3-52::pTEF1-Clover-P4M-SynTerm21:URA3*	This study
KKY1430	CBY924 *ura3-52::pTEF1-Clover-P4M-SynTerm21:URA3*	This study
KKY1431	CBY926 *ura3-52::pTEF1-Clover-P4M-SynTerm21:URA3*	This study
KKY1432	SEY6210 [pRS314 (*pTEF1-Clover-D4H-SynTerm21 LEU2*)]	This study
KKY1433	JRY6320 [pRS314 (*pTEF1-Clover-D4H-SynTerm21 LEU2*)]	This study
KKY1435	SEY6210 [pRS314] *ura3-52::pTEF1-Clover-D4H-SynTerm21:URA3*	This study
KKY1436	CBY924 *ura3-52::pTEF1-Clover-D4H-SynTerm21:URA3*	This study
KKY1437	CBY926 *ura3-52::pTEF1-Clover-D4H-SynTerm21:URA3*	This study
KKY1438	NY17 *ura3-52::URA3*	This study
KKY1439	NY17 [pRS316]	This study
KKY1440	SEY6210 *leu2-3,112::LEU2 ura3-52::URA3* [pRS314]	This study
KKY1441	AAY104 *ura3-52::URA3* [YCplac111] [pRS314]	This study

[1] Kind gift of S. Emr (Cornell University)

Table 3.2: Plasmids used in Chapter 3.

Plasmid	Relevant genotype	Source
pLT11	*CEN TRP1 CapR*	Cross, 1997
pUT11	*CEN TRP1 KanR*	Cross, 1997
pRS305	*LEU2*	Sikorski and Hieter, 1989
pRS306	*URA3*	Sikorski and Hieter, 1989
pRS314	*CEN TRP1*	Sikorski and Hieter, 1989
pRS316	*CEN URA3*	Sikorski and Hieter, 1989
YCplac111	*CEN LEU2*	Gietz and Sugino, 1988
YIplac211	*URA3*	Gietz and Sugino, 1988
YIplac211-mCherry-PUS1	YIplac211 (*mCherry-PUS1*)	Han *et al.*, 2007[1]
NRB1325	pRS306 (*pADH1-GFP-YPT32-CYC1term*)	Ling *et al.*, 2014[2]
pRC2098	pRS316 (*GFP-SEC4 URA3)*	Calero *et al.*, 2003
Addgene plasmid #105267[3]	YIplac211 (*SEC7-mCherry2bx6 URA3*)	Day *et al.* 2018[3]
pKK2138	pRS316 (*pMET-Clover-P4M-SynTerm21)*	This study
pKK2151	pRS305 (*pTEF1-Clover-D4H-SynTerm21*)	This study
pKK2153	pRS305 (*pTEF1-Clover-P4M-SynTerm21*)	This study
pKK2155	YCplac111 (*pTEF1-Clover-D4H-SynTerm21)*	This study
pKK2157	YCplac111 (*pTEF1-Clover-P4M-SynTerm21)*	This study
pKK2160	pRSR306 (*pTEF1-Clover-D4H-SynTerm21*)	This study
pKK2161	pRS306 (*pTEF1-Clover-P4M-SynTerm21*)	This study
pKK2167	pRS305 (*pTEF1-mCherry2b-P4M-SynTerm21*)	This study
pKK2176	pRS314 (*pTEF1-Clover-D4H-SynTerm21*)	This study
pKK2177	pRS314 (*pTEF1-Clover-P4M-SynTerm21*)	This study

All plasmids confer ampicillin resistance (*AmpR*). *CapR* is chloramphenicol resistance.

KanR is kanamycin resistance.

[1] Kind gift of M. Kupiec (Tel Aviv University)

[2] Kind gift of P. Novick (University of California San Diego)

[3] Kind gift of B. Glick (University of Chicago)

Design and Construction of Lipid Probes

Genes encoding lipid probes were synthesized as gBlocks (Supplemental Table 3.2; Supplemental Figure 3.2; Integrated DNA Technology, Coralville, IA). For *pTEF1-Clover-P4M-SynTerm21*, *pTEF1* is the 200bp 5' of the *TEF1* start codon in SGD (*Saccharomyces* Genome Database) reference strain S288C (Blazeck *et al.*, 2012; Decoene *et al.*, 2019). SacI and EcoRI sites were introduced to the 5' end of *pTEF1*. The nucleotide sequence of the GFP-variant Clover (Lee *et al.*, 2013), lacking the stop codon, was obtained through reverse translation and codon optimization for expression in *S. cerevisiae,* as was the P4M sequence, which was derived from amino acids 544-647 of SidM from *Legionella pneumophila* (Brombacher *et al.*, 2009; Hammond *et al.*, 2014). A SpeI site was introduced at the junction of the Clover and P4M coding sequences, and a stop codon was introduced at the 3' end of the P4M coding sequence. A synthetic terminator (*SynTerm21*; Curran *et al.*, 2015) sequence was introduced 3' of the stop codon. SmaI and XhoI sites were introduced 3' of the terminator. *pTEF1-mCherry2b-P4M-SynTerm21* was synthesized as described above using *mCherry2b* rather than *Clover*. mCherry2b coding sequence was obtained from YIplac211 *(SEC7-mCherry2bx6),* also known as Addgene plasmid #105267 (Day *et al.*, 2018).

To generate pKK2138 (pRS316 (*pMET-Clover-P4M-SynTerm21*)), gbKK6 cut with SacI and XhoI was ligated into pRS316 cut with SacI and XhoI.

To generate pKK2153 (pRS305 (*pTEF1-Clover-P4M-SynTerm21*)), gbKK8 cut with SpeI and SacI, and pKK2138 cut with SpeI and SmaI were ligated into pRS305 cut with SacI and SmaI. DNA sequence was confirmed by sequencing (Integrated DNA Technology, Coralville, IA) using oKK435 and oKK436. For genomic integration,

pKK2153 was cut with AgeI and transformed into *S. cerevisiae* at the *leu2-3,112* locus. Transformants were identified by growth on SC-LEU and validated by PCR, as described below. To generate *LEU2::TRP1* strains, marker swap plasmid pLT11 cut with XhoI and HpaI was used (Cross, 1997).

To generate pKK2157 (YCplac111 (*pTEF1-Clover-P4M-SynTerm21*)), pKK2153 cut with EcoRI and SmaI was ligated into YCplac111 cut with EcoRI and SmaI. *S. cerevisiae* transformants were selected on SC-LEU.

To generate pKK2161 (pRS306 (*pTEF1-Clover-P4M-SynTerm21*)), pKK2153 was cut with SacI and SmaI, and the resulting fragment was ligated into pRS306 cut with SacI and SmaI. For genomic integration at the *ura3-52* locus, pKK2171 was cut with NcoI and transformed into *S. cerevisiae*. Transformants were selected on SC-URA. To generate *URA3::TRP1* strains, marker swap plasmid pUT11 cut with SmaI was used (Cross, 1997).

To generate pKK2167 (pRS305 (*pTEF1-mCherry2b-P4M-SynTerm21*)), gbKK9 cut with SacI and SpeI was ligated into pKK2153 cut with SacI and SpeI to replace *pTEF1-Clover* with *pTEF1-mCherry2b*. DNA sequence was confirmed by sequencing (Integrated DNA Technology, Coralville, IA) using oKK435 and oKK436. For genomic integration at the *ura3-52* locus, pKK2167 was cut with NcoI and transformed into *S. cerevisiae*. Transformants were selected on SC-LEU.

To generate pKK2177 (pRS314 (*pTEF1-Clover-P4M-SynTerm21*)), pKK2153 cut with SacI and XhoI was ligated into pRS314 cut with SacI and XhoI. *S. cerevisiae* transformants were selected on SC-TRP.

For *pTEF1-Clover-D4H-SynTerm21, pTEF1* and the synthetic terminator (*SynTerm21*) were obtained as described above. SacI and EcoRI sites were introduced 5'

of *pTEF1*. The coding sequence of D4H was obtained by reverse translation and codon optimization of amino acids 391-500 of domain four of perfringolysin O (PFO), with the following mutation, D434S (Shimada *et al.*, 2002; Johnson *et al.*, 2012). XhoI and SmaI sites were introduced 3' of the terminator.

To generate pKK2151 (pRS305 (*pTEF1-Clover-D4H-SynTerm21*)), gbKK8 cut with SacI and SpeI, and gbKK7 cut with SpeI and SmaI were ligated into pRS305 cut with SacI and SmaI. DNA sequence was confirmed by sequencing (Integrated DNA Technology, Coralville, IA) using oKK435 and oKK436. For genomic integration at the *leu2-3,112* locus, pKK2151 was cut with AgeI. Transformants were selected on SC-LEU.

To generate pKK2155 (YCplac111(*pTEF1-Clover-D4H-SynTerm21*)), pKK2151 cut with EcoRI and SmaI was ligated into YCplac111 cut with EcoRI and SmaI. *S. cerevisiae* transformants were selected on SC-LEU.

To generate pKK2160 (pRS306 (pTEF1-Clover-D4H-SynTerm21), pKK2151 cut with SacI and SmaI was ligated into pRS306 cut with SacI and SmaI. For genomic insertion at *ura3-52*, pKK2160 was cut with NcoI. *S. cerevisiae* transformants were selected on SC-URA.

To generate pKK2176 (pRS314 (*pTEF1-Clover-D4H-SynTerm21*)), pKK2151 cut with SacI and XhoI was ligated into pRS314 cut with SacI and XhoI. *S. cerevisiae* transformants were selected on SC-TRP.

All integrations of lipid probes were confirmed by PCR amplification of genomic DNA. For pRS305 vectors, oKK433 and oKK434 were used for amplification. For pRS306 vectors, oKK437 and oKK438 were used.

Bgl2p Accumulation Assay

Quantification of intracellular Bgl2p accumulation was performed as described in Harsay and Schekman (2007). To detect Bgl2p, we probed Bgl2p with a polyclonal rabbit antibody (1:5,000; Alfaro *et al.*, 2011) and a 680LT goat anti-rabbit secondary antibody (1:10,000; catalogue no. 926-68021, Li-Cor, Lincoln, NE). Tubulin served as a loading control and was detected with AA2, a mouse monoclonal antibody against amino acids 412-430 of bovine brain β-tubulin (1:10,000; kind gift of A. Frankfurter, University of Virginia) and IRDye 800CW goat anti-mouse secondary antibody (1:10,000; catalogue no. 926-32210, Li-Cor, Lincoln, NE). Immunoblots were imaged on a Bio-Rad Chemidoc MP Imaging System (catalogue no. 17001402; Hercules, CA). Band intensity was quantified with the standard edition of Bio-Rad Image Lab Software.

Fluorescence Microscopy

Log-phase cells in selective minimal medium were imaged on a Nikon Eclipse Ti Spinning Disc Microscope with a 100x 1.45 NA Plan Apo objective and 488nm and 561nm lasers (Nikon, Melville, NY), a CSU-X1 confocal scanner unit (Yokogawa, Houston, Texas), and a Hamamatsu ORCA-Flash4.0 camera (C11440; Bridgewater, NJ). Imaging parameters were consistent for all strains within a figure panel.

Quantified images of KKY1400 and KKY1426 in Figure 3.5C were captured at the Keck Center for Cellular Imaging at the University of Virginia. Log-phase cells in selective minimal medium were imaged on a Zeiss 980 system consisting of an inverted Axio Observer microscope with laser scanning confocal (Zeiss, Dublin, CA) equipped with 32 channel spectral imaging, one 8Y-airyscan detector and two channels non-descan (NDD)

epi-fluorescence detectors, motorized piezo stage for automated scanning, definite focus for focus compensation in time-lapse imaging, and an IR Insight 3X ultrafast Ti:Sapphire laser for multiphoton excitation (Spectra-Physics, Milpitas, California). Cells were exposed to 488nm at 1% power and 561nm at 0.8% power. Acquisition for each channel took approximately 255ms, with a single time point lasting approximately 770ms. Cells were imaged for a total of 45 cycles at 255ms intervals per channel with a 63x 1.4 NA Plan Apo objective. All images were 317x317 pixels.

Vesicle Trafficking and Quantification

Vesicles were tracked and analyzed with Imaris Software (Oxford Instruments, Concord, MA). Vesicles were manually identified as 200nm regions of interest (ROIs) based on size and speed consistent with prior studies (Schott *et al.*, 2002). ROIs were then assembled into tracks representing the movement of a single vesicle. In Imaris, the speed of the object (µm/s) is the scalar equivalent of object velocity. All vesicles, regardless of speed, were quantified in Figure 3.3C. In Figure 3.4A, vesicles with clear GFP-Ypt32p signal and no discernable mCherry2b-P4M signal, vesicles with an average speed < 1 µm/s, and vesicles with less than 3 time points were excluded from intensity analysis.

Results

Lipid-probe P4M is specific to PI4P in S. cerevisiae

Previous observations indicated PI4P is essential for Osh protein-mediated secretory vesicle trafficking and exocytosis in *S. cerevisiae* and suggested that a supporting PI4P gradient exists along the late secretory pathway (Mizuno-Yamasaki *et al.*, 2010; Beh *et al.*, 2012; Ling *et al.*, 2014; Smindak *et al.,* 2017). To visualize and quantify the intracellular distribution of PI4P in the late secretory pathway of *S. cerevisiae*, we adapted P4M, a genetically-encoded lipid probe used previously to detect PI4P levels in mammalian cells. Prior studies *in vitro* and *in vivo* demonstrated P4M specifically binds PI4P in mammalian and other eukaryotic cells but was not tested in *S. cerevisiae* (Brombacher *et al*., 2009; Schoebel *et al.*, 2010; Hammond *et al.*, 2014). To show in *S. cerevisiae* that P4M localizes exclusively to areas enriched with PI4P, we co-expressed Clover-P4M with a fluorescent protein tagged *trans*-Golgi network (TGN) marker, Sec7p, a guanine nucleotide exchange factor for Arf proteins (Novick *et al.*, 1980; Sata *et al.*, 1998), or with a nuclear marker, Pus1p, a pseudouridine synthase (Simos *et al.*, 1996). It is known that the TGN is enriched with PI4P relative to other membranes such as the endoplasmic reticulum (ER) and nuclear envelope (Zinser *et al.*, 1991; Levine and Munro, 2002). We found P4M co-localizes with Sec7p-mCherry2bx6 but does not localize circumferentially with the nuclear marker mCherryb-Pus1p, indicating P4M localizes to PI4P-enriched membranes in *S. cerevisiae* (Figure 3.1A).

To demonstrate P4M distribution reflects PI4P distribution *in vivo*, we compared P4M localization in wild-type (WT) and temperature-sensitive *pik1*Δ [*pik1-83*^{ts}] cells. Pik1p is an essential kinase that localizes to and generates PI4P at the TGN (Flanagan *et*

al., 1993; Walch-Solimena and Novick, 1999). At the permissive temperature (20˚C), expression of Clover-P4M in WT cells did not affect growth, whereas expression in *pik1*Δ [*pik1-83^{ts}*] cells resulted in a slight growth defect (Supplemental Figure 3.1). In WT cells, after 20 min post shift to 37°C, Clover-P4M localization did not change (Figure 3.1B), localizing to the TGN, marked by Sec7p-mCherry2bx6 (Figure 3.1A and C). In contrast, in *pik1*Δ [*pik1-83^{ts}*] cells, Clover-P4M distribution became noticeably diffuse by 10 min post-shift to 37°C (Figure 3.1B). At 20 min post-shift, Clover-P4M showed significantly reduced co-localization with the TGN-marker Sec7p-mCherry2bx6 (Figure 3.1C). These observations are consistent with Audhya *et al.* (2000), who demonstrated that after 10 min at 37°C, *pik1*Δ [*pik1-83^{ts}*] cells exhibit a significant decrease in both PI4P and PI(4,5)P_2 levels throughout the cell. Therefore, the redistribution of P4M at the restrictive temperature in *pik1*Δ [*pik1-83^{ts}*] is due to a decrease in PI4P at the *trans*-Golgi. P4M localization reflects PI4P distribution *in vivo*.

In the same experiment, to show P4M redistribution specifically reflects PI4P distribution and not the distribution of another lipid, we compared WT and *pik1*Δ [*pik1-83^{ts}*] cells expressing Clover-P4M to cells expressing Clover-D4H, a genetically encoded sterol probe (Shimada *et al.*, 2002; Johnson *et al.*, 2012). As with expression of Clover-P4M, expression of Clover-D4H did not affect the growth of WT cells but resulted in a slight growth defect in *pik1*Δ [*pik1-83^{ts}*] cells (Supplemental Figure 3.1). At permissive temperature, in both WT and *pik1*Δ [*pik1-83^{ts}*] cells, Clover-D4H localized to the PM, which is sterol-rich relative to intracellular membranes (Maekawa and Fairn, 2015; Solanko *et al.*, 2017). Post-shift to restrictive temperature, Clover-D4H continued to localize primarily to the PM in WT cells. In contrast, in *pik1*Δ [*pik1-83^{ts}*] cells, Clover-

D4H no longer localized with the PM by 20 min post-shift. Thus, Clover-P4M redistribution preceded Clover-D4H redistribution post temperature shift, indicating the redistribution of P4M reflects PI4P distribution and not that of another lipid. Consistent with data demonstrating the P4M probe is specific to PI4P *in vitro* and in other eukaryotic cells *in vivo*, these results demonstrate the specificity of P4M for PI4P in *S. cerevisiae*.

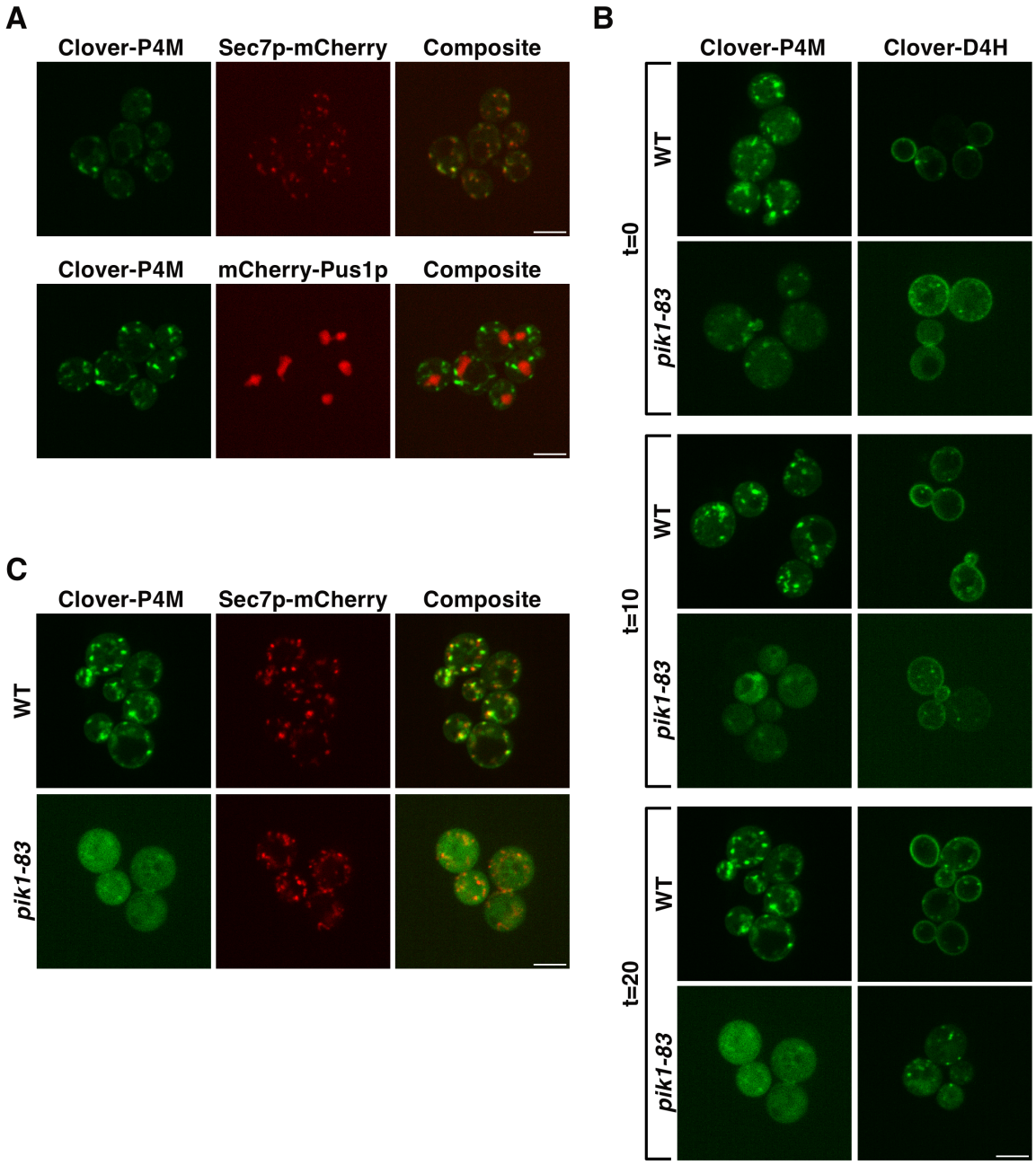


Figure 3.1: P4M localizes to PI4P-enriched membranes in *S. cerevisiae*. (A) Fluorescent micrographs of log-phase WT cells co-expressing Clover-P4M, to detect PI4P, with either the TGN marker Sec7p-mCherry2bx6 (KKY1389), or the nuclear marker mCherry-Pus1p (KKY1398). Scale bar, 4μm. (B) Fluorescent micrographs of WT (KKY1389) and *pik1*Δ [*pik1-83*^{ts}] (KKY1391) cells co-expressing Clover-P4M (shown)

and Sec7p-mCherry2bx6 (not shown), and WT (KKY1394) and *pik1*Δ [*pik1-83*^{ts}] (KKY1396) cells co-expressing Clover-D4H (shown), to detect sterol, and Sec7p-mCherry2bx6 (not shown). Sec7p-mCherry2bx6 fluorescence is shown in 1C. Cells were grown to log-phase in selective minimal medium at 20°C then shifted to 37°C. Images taken at t=0, 10, and 20 min post temperature shift. Scale bar, 4μm. (C) The same cells shown in (B) at 20 min post-shift from 20˚C to 37˚C, but both Clover-P4M and Sec7p-mCherry2bx6 are visualized. Scale bar, 4μm.

P4M expression does not significantly hinder vesicle trafficking or cellular function

Next, we asked whether P4M localizes to secretory vesicles. To our knowledge, no previous study directly demonstrated the association of PI4P with vesicles in *S. cerevisiae* using a genetically-encoded lipid probe specific to PI4P. To assay for the presence of PI4P on secretory vesicles *in vivo*, we co-expressed mCherry2b-P4M with a secretory vesicle marker, either the Rab GTPase Ypt32p or the Rab GTPase Sec4p. In WT cells where P4M was not co-expressed, GFP-Ypt32p primarily localized to distinct cytoplasmic puncta, as well as to known sites of polarized growth, including the bud tip and bud neck (Supplemental Figure 3.3); whereas GFP-Sec4p localized to sites of polarized growth (Supplemental Figure 3.4). In cells co-expressing P4M and one of the vesicle markers, we observed GFP-Ypt32p and mCherry2b-P4M co-localized at distinct cytoplasmic puncta in the mother cell and at sites of polarized growth (i.e., the bud tip and bud neck; Figure 3.2A). In contrast, GFP-Sec4p and mCherry2b-P4M predominantly co-localized at sites of polarized growth (Figure 3.2D). Because P4M expression did not affect localization of GFP-Ypt32p or GFP-Sec4p, we concluded that expression of P4M does not perturb the spatial distribution of secretory vesicles. Consistent with previous studies, these data suggest Ypt32p is present on immature post-Golgi secretory vesicles when vesicular PI4P levels are high; whereas Sec4p is present on late post-Golgi vesicles when vesicular PI4P levels are relatively lower (Mizuno-Yamasaki *et al.*, 2010).

To exclude the possibility that mCherry2b-P4M and GFP-Ypt32p (or GFP-Sec4p) expression significantly affects cellular function, thus potentially altering vesicular PI4P levels from that present in WT cells, we examined the effect of mCherry2b-P4M, GFP-Ypt32p, and GFP-Sec4p expression on i) cell growth; ii) the secretory pathway that

supports polarized cell growth; and iii) vesicle movement. To determine whether expression of these fusion proteins affected cell growth, we serially diluted strains and qualitatively examined growth on solid medium. In comparison to WT cells, co-expression of mCherry2b-P4M and GFP-Ypt32p resulted only in a slight growth defect at 25°C (Figure 3.2B). Co-expression of mCherry2b-P4M and GFP-Sec4p did not noticeably affect cell growth (Figure 3.2E). To determine whether expression disrupted the secretory pathway that supports polarized cell growth, we quantified the fold change in the ratio of internal Bgl2p, a cargo protein specific to vesicles involved in polarized secretion, to tubulin, after shifting from 25˚C to 37˚C for 90 min (Harsay and Schekman, 2007; Dighe and Kozminski, 2008; Johansen *et al.*, 2016; see Materials and Methods). Compared to WT cells, strains co-expressing mCherry2b-P4M and GFP-Ypt32p (Figure 3.2C) or GFP-Sec4p (Figure 3.2F) showed no significant increase in accumulated Bgl2p as determined by a one-tailed Student's *t*-test. In contrast, a strain with a known secretory defect, *sec6-4*^{ts}, exhibited a significant increase in internal Bgl2p accumulation compared to WT cells under the same conditions ($P \leq 0.05$; Novick *et al.*, 1980). Therefore, co-expression of mCherry2b-P4M and vesicle markers GFP-Ypt32p or GFP-Sec4p does not significantly affect polarized secretion or cell growth.

To determine whether fusion protein expression affected vesicle movement in cells co-expressing mCherry2b-P4M with GFP-Ypt32p or GFP-Sec4p, we examined the direction and speed of marked vesicles. Schott *et al.* (2002) tracked GFP-Sec4p vesicles *in vivo* and noted GFP-Sec4p-marked vesicles traveled in linear paths through or along the cortex of the mother cell into the bud. In WT strains expressing mCherry2b-P4M and GFP-Ypt32p or GFP-Sec4p, we observed that vesicles moved linearly through or along the

cortex of the mother cell into the bud (Figure 3.3A and 3.3B). Additionally, dual labeled vesicles moved similarly to vesicles in cells expressing only GFP-Ypt32p or GFP-Sec4p (Supplemental Figures 3.3 and 3.4), further indicating that mCherry2b-P4M expression does not impair the directionality of vesicles trafficking. With respect to velocity, Schott *et al.* (2002) reported Sec4p-marked vesicles traffic with an average velocity of 3 μm/s, with individual vesicles varying from ~ 2 μm/s to 4 μm/s; motion in close proximity to the bud neck, when vesicles slowed, was excluded from measurement. Our analyses included vesicles that were in close proximity to the bud neck, which resulted in a similar though lower rate of vesicle trafficking than previously reported. In cells co-expressing mCherry2b-P4M and GFP-Ypt32p, the average velocity of vesicles was 1.4 μm/s with an average maximum velocity of 2.0 μm/s. In cells expressing GFP-Ypt32p alone, the average velocity of vesicles was 1.2 μm/s with an average maximum velocity of 1.6 μm/s. Taken together, these data indicate that co-expression of mCherry2b-P4M and GFP-Ypt32p or GFP-Sec4p does not significantly affect secretory vesicle trafficking or polarized cell growth.

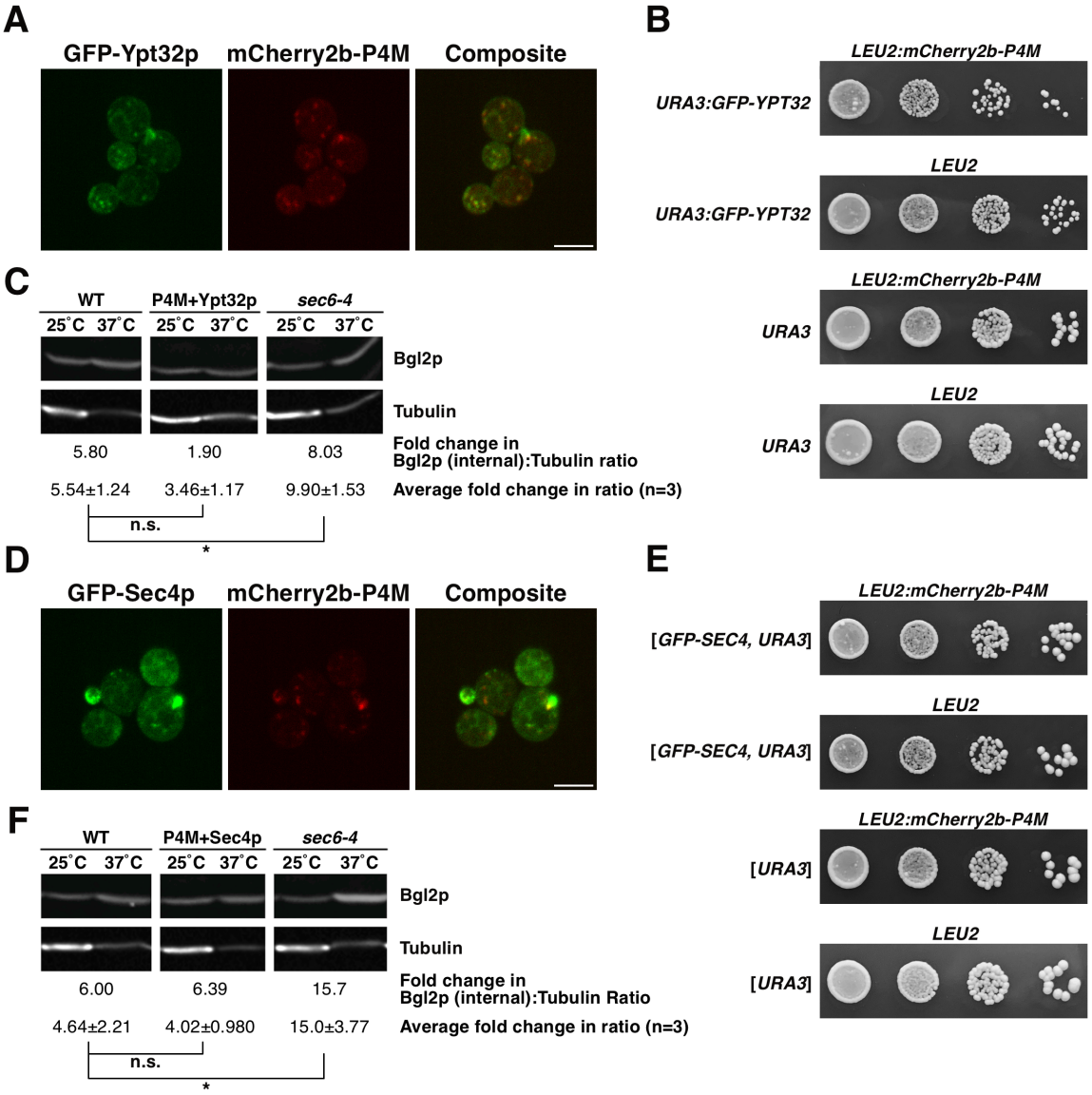


Figure 3.2: mCherry2b-P4M localizes to GFP-Ypt32p-marked regions as well as GFP-Sec4p-marked regions and does not inhibit polarized secretion. (A) Fluorescent micrographs of log-phase WT cells co-expressing mCherry2b-P4M along with vesicle marker GFP-Ypt32p (KKY1400). Scale bar, 4μm. (B) Tenfold serial dilutions, on agar plates prepared with selective minimal medium, of WT cells co-expressing GFP-Ypt32p and mCherry2b-P4M (KKY1400), GFP-Ypt32p (KKY1403), mCherry2b-P4M (KKY1401), or vector controls (KKY1404). Images were taken after a 5-day incubation at

25˚C. (C) Immunoblots of spheroplasted *S. cerevisiae* strains: WT with control vectors (KKY1404), WT with GFP-Ypt32p and mCherry2b-P4M (KKY1400), and *sec6-4*^{ts} (KKY1438). Immunoblots quantified as fold change calculated from the intensity ratio of internal Bgl2p to intensity of tubulin. Average fold change ± SEM, one-tailed Student's *t*-test, $P \leq 0.05$. (D) Same as (A), but with WT cells co-expressing vesicle marker GFP-Sec4p and mCherry2b-P4M (KKY1405). (E) Same as (B), but with WT cells co-expressing GFP-Sec4p and mCherry2b-P4M (KKY1405), GFP-Sec4p (KKY1408), mCherry2b-P4M (KKY1406), or vectors control with selective markers (KKY1407). (F) Same as (C), but with *S. cerevisiae* strains that are WT with control vectors (KKY1407), WT with mCherry2b-P4M and GFP-Sec4p (KKY1405), and *sec6-4*^{ts} (KKY1439).

Post-Golgi Ypt32p-marked vesicles do not lose PI4P while trafficking from the TGN to PM

A prior study in *S. cerevisiae* suggested that vesicular PI4P levels decrease while vesicles traffic from the TGN to the PM, but no direct data support the existence of this gradient (Mizuno-Yamazaki *et al.*, 2010). To directly determine whether secretory vesicles manifest decreasing levels of PI4P as they traffic from the TGN to the PM at sites of polarized growth, we tracked GFP-Ypt32p- and GFP-Sec4p-marked vesicles in cells expressing mCherry2b-P4M and quantified mCherry2b-P4M intensity over time. Firstly, we noted that vesicles marked with GFP-Ypt32p had a mCherry2b-P4M signal at a significantly greater frequency than vesicles marked with GFP-Sec4p (Figure 3.3C). These data are consistent with models in which the PI4P gradient is established before Sec4p is recruited to the vesicle, with the highest levels of PI4P present on vesicles proximal to the TGN.

Loss of vesicle-associated PI4P is hypothesized to drive the functional exchange of Ypt32p for Sec4p, which is required for proper secretory vesicle docking at the PM (Guo *et al.*, 1999; Ling *et al.*, 2014). To directly determine whether secretory vesicles lose Ypt32p or PI4P while trafficking to the bud, we quantified GFP-Ypt32p and mCherry2b-P4M intensity on GFP-Ypt32p-marked vesicles. In WT cells co-expressing GFP-Ypt32p and mCherry2b-P4M, we found vesicles did not exhibit a change in mCherry2b-P4M or GFP-Ypt32p intensity as vesicles traffic from the mother cell to the bud-neck or into the bud (Figure 3.4A).

In the same experiment, to rule out the possibility that vesicles were moving from the plane of movement (X/Y plane) into the Z-axis, thus altering the signal intensity, we

compared the percent change in mCherry2b-P4M and GFP-Ypt32p intensity between time points (Figure 3.4B). The percent change in signal intensities of mCherry2b-P4M and GFP-Ypt32p were not proportional indicating that vesicles remained substantially in the X/Y planes as they trafficked. Movement into the Z-axis would have resulted in equivalent percent changes in mCherry2b-P4M and GFP-Ypt32p signal intensity. Together, these data indicate vesicular Ypt32p levels and PI4P levels remain relatively constant during trafficking from the mother cell to the bud or bud-neck. These data, however, do not exclude the possibility that vesicle-associated PI4P may decrease when the vesicles are formed at the *trans*-Golgi or when vesicles are in close proximity to the PM of the bud.

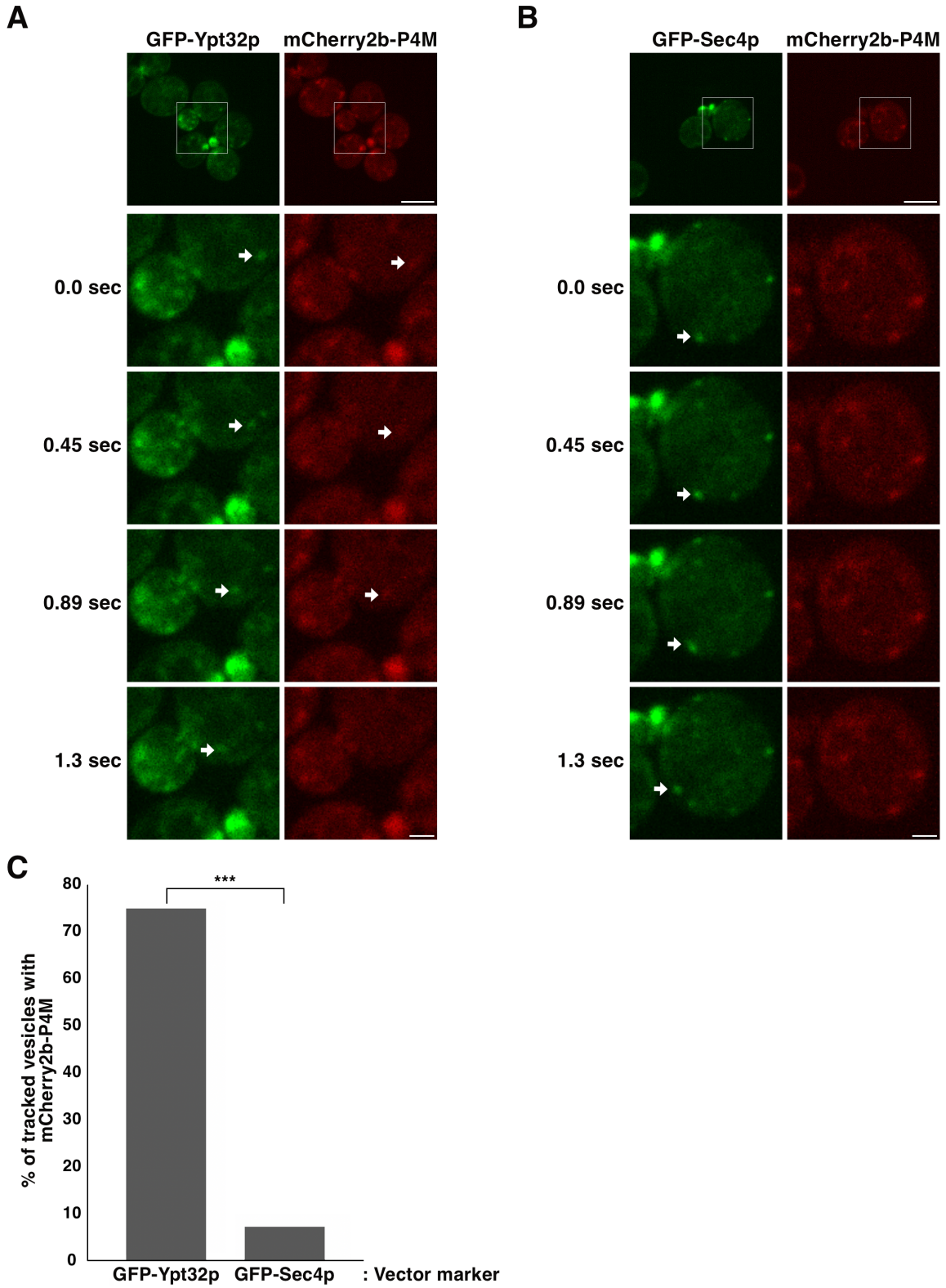


Figure 3.3: GFP-Ypt32p- and GFP-Sec4p-marked vesicles can be tracked *in vivo*. (A) Fluorescent micrographs of log-phase WT cells co-expressing vesicle marker GFP-Ypt32p

and mCherry2b-P4M (KKY1400). Arrows indicate a GFP-Ypt32p-marked vesicle and corresponding mCherry2b-P4M signal. Scale bar: top row 4μm; within time series 1μm. (B) Same as (A), but with WT cells co-expressing vesicle marker GFP-Sec4p and mCherry2b-P4M (KKY1405). Arrows indicate a GFP-Sec4p-marked vesicle with no corresponding mCherry2b-P4M signal. Scale bar: top row 4μm; within time series 1μm. (C) Percent of tracked GFP-Ypt32p-marked (n=32) and GFP-Sec4p-marked vesicles (n=14) with mCherry2b-P4M signal. All GFP-Ypt32p- and GFP-Sec4p-marked vesicles were included in this calculation regardless of speed. Data were analyzed by one-tailed Student's t-test, $P \leq 0.001$.

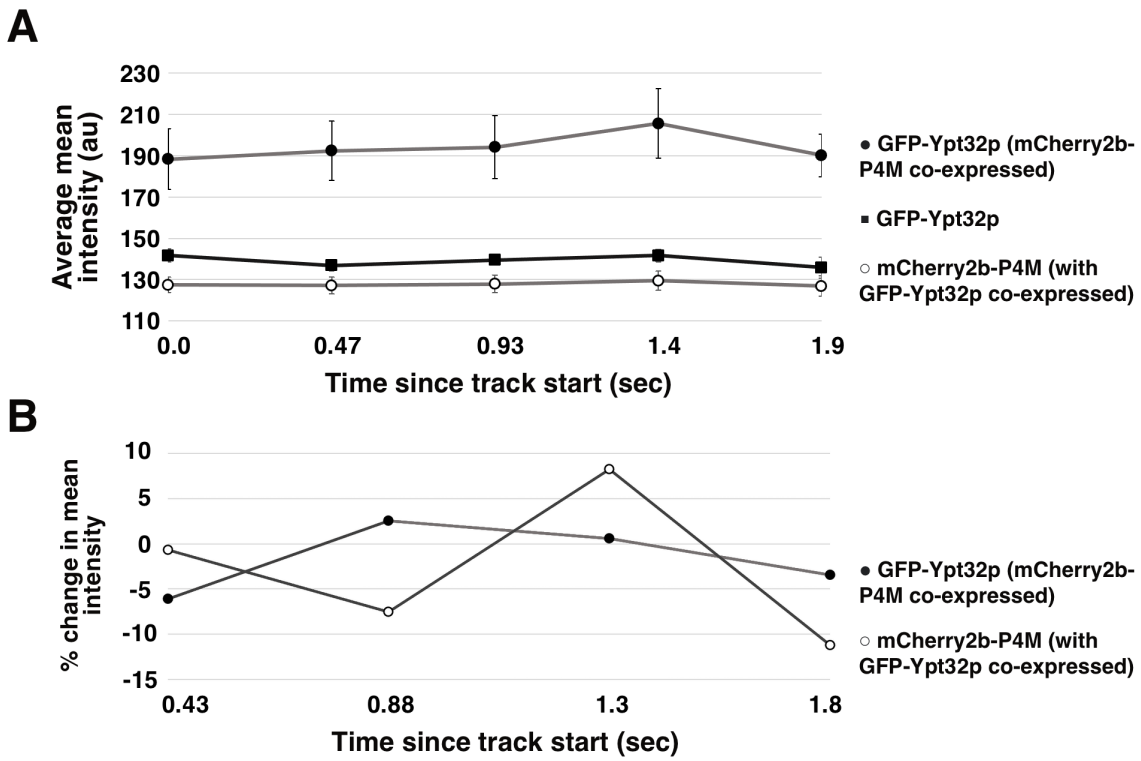


Figure 3.4: mCherry2b-P4M intensity on GFP-Ypt32p-marked vesicles does not change significantly over time. (A) Average mean intensity of fluorescent signal of GFP-Ypt32p-marked vesicles over time in WT cells co-expressing mCherry2b-P4M and GFP-Ypt32p (KKY1400; *n*=11 vesicles at t=0.00, 0.43, 0.88 sec; *n*=8 at t=1.32 sec; *n*=4 at t=1.77 sec across *n*=3 independent transformants), or WT cells expressing GFP-Ypt32p and a selectable marker (KKY1403; *n*=27 vesicles at t=0.00, 0.47, 0.92 sec; *n*=20 at t=1.39 sec; *n*=6 at t=1.85 sec across *n*=3 independent transformants). Only dual-labeled vesicles visible for a minimum of three time points with an average speed ≥ 1 μm/s were quantified. For each time point, mean intensity was quantified as the average signal intensity within a 200 nm-diameter circular region of interest. Data were analyzed by one-tailed Student's *t*-test; no significant changes in mean intensity were detected ($P > 0.05$). (B) Representative percent change in mean intensity of GFP-Ypt32p and mCherry2b-P4M over time

(KKY1400). Representative of *n*=18 quantified vesicles across *n*=3 independent transformants.

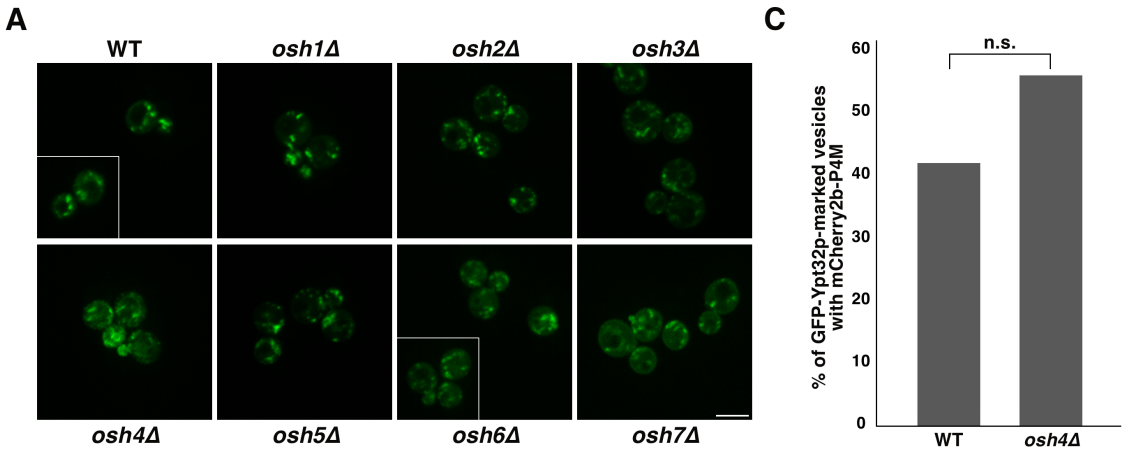
Members of the Osh protein family play unique roles in establishing or maintaining PI4P distribution

Another question we asked concomitantly in our study is what role does the oxysterol binding protein homologue (Osh) family have in PI4P distribution. We asked this question because a prior model of polarized secretion placed Osh4p as the regulator of the PI4P gradient along the trafficking pathway (Ling *et al.*, 2014; Smindak *et al.*, 2017) and any one member of the Osh protein family, all of which are capable of binding PI4P, is sufficient for cell growth (Beh and Rine, 2004; Im *et al.*, 2005; de Saint-Jean *et al.*, 2011; Tong *et al.*, 2013). To this end, we expressed Clover-P4M in WT cells and in a panel of mutant strains, in which each mutant lacked one member of the Osh protein family (Figure 3.5A and B). Consistent with previous results with WT cells (Figure 3.1A), Clover-P4M localized to distinct globular cytoplasmic structures in the mother cell and bud. *osh1*Δ, *osh2*Δ, *osh3*Δ, and *osh7*Δ strains also contained distinct Clover-P4M-marked structures in both the mother cell and bud, though these structures were often more numerous, smaller, or elongated than those in WT cells. In *osh4*Δ, *osh5*Δ, and *osh6*Δ cells, Clover-P4M-marked structures were globular or had a non-distinct morphology. In some but not all *osh3*Δ cells, Clover-P4M localized to the PM of the bud or of both the mother cell and bud, in addition to cytoplasmic localization. In *osh7*Δ cells, Clover-P4M consistently localized to the PM of both the mother cell and bud. To determine if Osh4p affects vesicle-associated PI4P, we expressed GFP-Ypt32p and mCherry2b-P4M in WT and *osh4*Δ cells. mCherry2b-P4M associated with GFP-Ypt32p-marked vesicle with the same relative frequency as in WT cells (Figure 3.5C). Together, these observations indicate that Osh4p does not modulate vesicle-associated PI4P levels and that, despite sharing an essential

function, Osh proteins have unique and necessary roles in maintaining or establishing PI4P distribution *in vivo*.

Osh4p in particular is modeled to mediate an essential PI4P-sterol exchange between secretory vesicles and the PM to allow for docking of secretory vesicles at the PM (Smindak *et al.*, 2017). Thus, our observation that loss of Osh4p produces a change in intracellular PI4P distribution is not entirely surprising. To probe the relationship between Osh4p and PI4P distribution further, we examined PI4P distribution in cells lacking all Osh protein family members (*osh*Δ), except Osh4p. Unlike in WT cells in which Clover-P4M localized to cytoplasmic puncta, we found in *osh*Δ *OSH4* cells that Clover-P4M localized primarily to the PM of the mother cell and bud with fewer cytoplasmic puncta than in WT cells (Figure 3.6A). These results demonstrate although Osh4p alone is sufficient for growth, Osh4p is not sufficient to maintain a WT PI4P distribution, consistent with earlier results (Figure 3.5A). This observation suggests that only a small portion of cellular PI4P needs to be properly localized for growth. To examine whether lethality in the absence of Osh protein family function was a result of further PI4P redistribution, we expressed Clover-P4M in *osh*Δ cells containing plasmid-borne WT *OSH4* or temperature-sensitive *osh4-1* (Figure 3.6B). At 25˚C, a permissive temperature, expression of *OSH4* or *osh4-1* alone in *osh*Δ cells was sufficient to support cell growth (Beh and Rine, 2004). Consistent with this, at permissive temperature, Clover-P4M localized to the PM in both *osh*Δ [*OSH4*] and *osh*Δ [*osh4-1*] cells, mirroring Clover-P4M localization in *osh*Δ *OSH4* cells (compare Figure 3.6A to 3.6B). However, at 90 min post-shift to the restrictive temperature (37˚C), Clover-P4M continued to localize to the PM in both *osh*Δ [*OSH4*] and *osh*Δ [*osh4-1*] cells (Figure 3.6B).

In addition to binding PI4P, Osh4p also binds sterol (Im *et al.*, 2005). To determine whether Osh4p alone is sufficient to maintain sterol distribution in cells, we expressed Clover-D4H in *osh*Δ *OSH4* cells (Figure 3.6C). In WT cells, Clover-D4H localized primarily to the PM, though occasionally Clover-D4H localized to cytoplasmic puncta as well (Figure 3.1B and 3.6C). Similarly, in *osh*Δ *OSH4* cells, Clover-D4H localized to the PM and occasionally to cytoplasmic puncta, indicating that while Osh4p alone is not sufficient to maintain PI4P distribution, Osh4p alone is sufficient to maintain sterol distribution (Figure 3.6C). To determine whether Osh4p, in the absence of other Osh protein family members, is sufficient to maintain sterol distribution, we expressed Clover-D4H in *osh*Δ cells with plasmid-borne WT *OSH4* or temperature-sensitive *osh4-1* (Figure 3.6D). At 90 min post-shift to the restrictive temperature (37˚C), in WT and *osh*Δ [*OSH4*] cells, Clover-D4H localization did not change. However, in *osh*Δ [*osh4-1*] cells, Clover-D4H became noticeably diffuse post temperature shift (Figure 3.6D). Osh4p is therefore sufficient to maintain sterol distribution in the absence of all other Osh proteins. Taken together, these data indicate that multiple Osh family members are needed to maintain the proper cellular distribution of PI4P, but not sterol.



B

	WT	osh1Δ	osh2Δ	osh3Δ	osh4Δ	osh5Δ	osh6Δ	osh7Δ
Cytoplasmic localization	Globular	Globular Elongated	Globular	Globular Elongated	Non-distinct	Globular Non-distinct	Globular Non-distinct	Globular Elongated
PM localization	No	No	No	Mother and bud (Variable)	No	No	No	Mother and bud

Figure 3.5: Loss of individual Osh family proteins results in altered PI4P distributions. (A) Fluorescent micrographs of log-phase WT (KKY1424), *osh1*Δ (KKY1416), *osh2*Δ (KKY1417), *osh3*Δ (KKY1418), *osh4*Δ (KKY1419), *osh5*Δ (KKY1420), *osh6*Δ (KKY1421), and *osh7*Δ (KKY1422) cells expressing Clover-P4M. Scale bar, 4μm. (B) Location of Clover-P4M signal morphology and location in a WT strain and strains lacking one Osh protein family member each. Globular was defined as nearly spherical puncta marked with Clover-P4M, as seen in WT cells. Elongated was defined as puncta marked with Clover-P4M that were oblong in shape in comparison to the globular morphology seen in WT cells. Non-distinct was defined as Clover-P4M signal that localized with no discernable geometric shape. In *osh3*Δ cells expressing Clover-P4M (KKY1418), Clover-P4M frequently but did not always localize to the PM and, as such, Clover-P4M localization at the PM is described as variable. (C) Percent of tracked GFP-Ypt32p-marked vesicles with visible mCherry2b-P4M signal in WT cells (KKY1400;

n=12 vesicles, *n*=3 independent transformants) and *osh4*Δ cells (KKY1426; *n*=9 vesicles, *n*=2 independent transformants). Data were analyzed by one-tailed Student's *t*-test, $P > 0.05$.

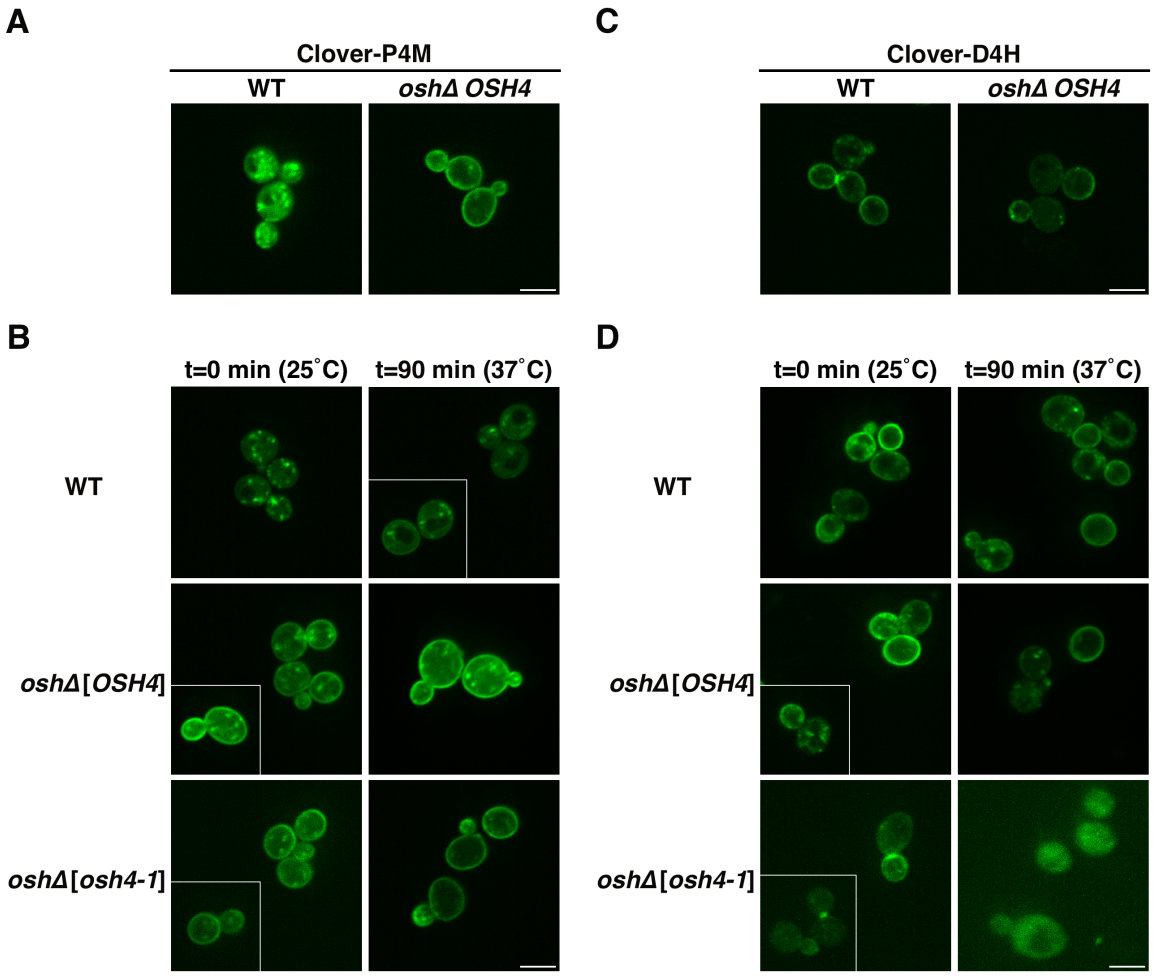


Figure 3.6: Lethality from loss of Osh protein family function in *osh*Δ [*osh4-1^{ts}*] cells is not directly caused by PI4P re-distribution. (A) Fluorescent micrographs of log-phase WT (KKY1427) and *osh*Δ *OSH4* (KKY1428) cells expressing Clover-P4M. Scale bar, 4µm. (B) Fluorescent micrographs of log-phase WT (KKY1429), *osh*Δ [*OSH4*] (KKY1430), and *osh*Δ [*osh4-1^{ts}*] (KKY1431) cells expressing Clover-P4M. Cells were grown in selective minimal medium at 25°C and then shifted to 37˚C at t=0 min. (C) Same as (A), but with WT (KKYKKY1432) and *osh*Δ *OSH4* (KKY1433) cells expressing Clover-D4H. (D) Same as (B), but with WT (KKY1435), *osh*Δ [*OSH4*] (KKY1436), and *osh*Δ [*osh4-1^{ts}*] (KKY1437) cells expressing Clover-D4H.

Discussion

In our study of polarized secretion utilizing the lipid binding domain P4M and live cell imaging we showed that vesicle-associated PI4P levels remain relatively constant as vesicles traffic from the TGN to the PM. Our study is the first to directly quantify vesicle-associated PI4P levels during polarized secretion. We also showed that deletion of *OSH4* does not alter vesicle-associated PI4P levels, although the individual deletion of *OSH4*, as well as the single deletion of other members of the *OSH* gene family, alters the intracellular distribution of PI4P. These results are inconsistent with an existing model (Ling *et al.*, 2014), in which vesicle-associated PI4P decreases, in an Osh4p-dependent manner, as the vesicle traffics from the TGN to the PM. These results are significant because the establishment of a PI4P gradient along the secretory pathway was hypothesized to trigger an exchange of Rab GTPases required for vesicle maturation and docking at the PM. Utilizing the data presented in this study, we propose a model in which vesicle-associated PI4P levels remain constant during trafficking in an Osh4p-independent manner (Figure 3.7).

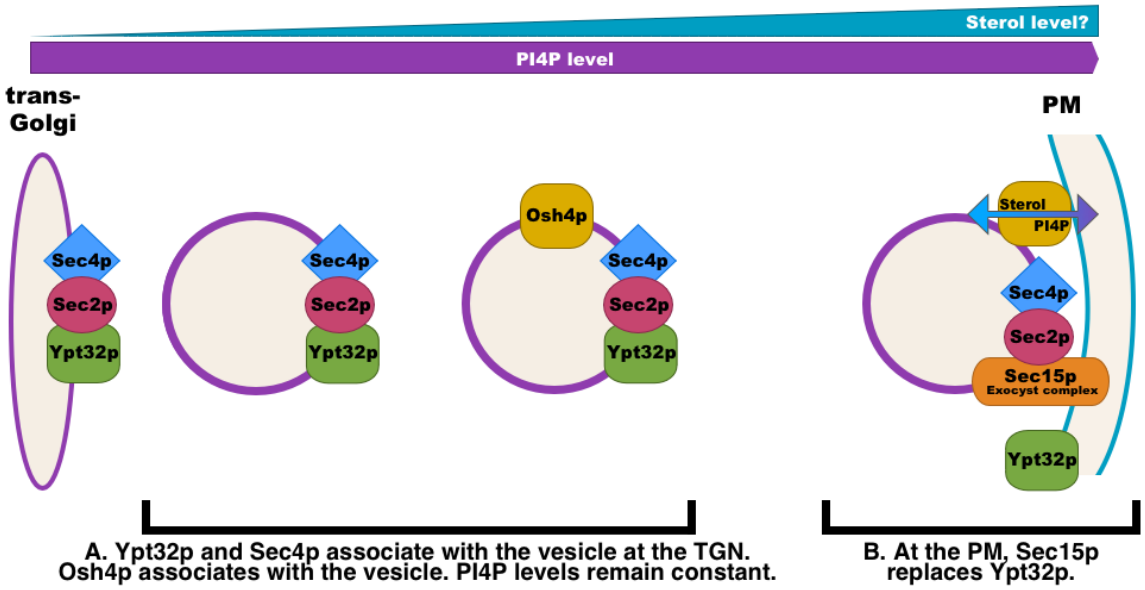


Figure 3.7: Model of secretory vesicle maturation post-Golgi. Ypt32p, Sec2p, and Sec4p are associated with a secretory vesicle when it forms at the *trans*-Golgi. As the vesicle traffics to the PM, Osh4p associates with the vesicle but does not alter vesicle-associated PI4P levels. At the PM, Ypt32p is exchanged for Sec15p, allowing for the formation of the exocyst complex. Osh4p is necessary for vesicle docking through a non-specific lipid exchange between the vesicle surface at the PM. Model adapted from Smindak *et al.*, 2017.

Do vesicle-associated PI4P levels change as vesicles traffic?

For a vesicle to dock at the PM, it must mature by acquiring specific molecules such as the Rab GTPase Sec4p and Sec15p, a subunit of the exocyst complex that is recruited by GTP-bound Sec4p (TerBush *et al.*, 1996; Guo *et al.*, 1999; Medkova *et al.*, 2006). At the beginning the late secretory pathway, vesicle formation at the *trans*-Golgi requires Ypt32p (Benli *et al.*, 1996; Jedd *et al.*, 1997). This Rab GTPases recruits Sec2p, a GEF for Sec4p (Walch-Solimena *et al.*, 1997; Ortiz *et al*., 2002). According to an existing model, as vesicle-associated PI4P levels fall, Sec2p recruits Sec4p to the vesicle, replacing Ypt32p (Mizuno-Yamasaki *et al.*, 2010). In addition to this GTPase exchange, decreasing vesicle-associated PI4P levels preferentially favors the association of Sec2p with Sec15p rather than Ypt32p (Mizuno-Yamasaki *et al.*, 2010). Our data, which show P4M is less likely to associate with Sec4p-marked vesicles than Ypt32p-marked vesicles, is consistent with this model. Yet, we also observed that Ypt32p localized to both vesicles and sites of polarized growth (i.e., bud tip and bud neck), suggesting Ypt32p is not lost from vesicles during trafficking, consistent with other studies (Ling *et al.*, 2014; Gingras *et al.*, 2022). Therefore, Ypt32p may only dissociate from vesicles just prior to vesicle docking at the PM. This idea however is inconsistent with the idea of a GTPase exchange during trafficking, especially in light of evidence for the presence of Sec4p at the Golgi (Gingras *et al.*, 2022). Taken together, our data and that of other studies suggest that both Sec4p and Ypt32p are present on a vesicle as it traffics, and, that for most of the distance a vesicle traffics, vesicle-associated PI4P levels are constant. A loss of vesicle-associated PI4P, when the vesicle is in close proximity to the PM, results in a loss of Ypt32p, freeing Sec2p to allow Sec4p to recruit Sec15p in preparation for vesicle docking at the PM.

Why then does one not observe dual labelled P4M- and Sec4p-marked vesicles frequently? Current models present a single vesicle population, in which vesicles acquire associated proteins at the Golgi and lose these proteins or recruit others when the vesicle is in close proximity to the PM. For example, Sec2p preferentially interacts with Ypt32p when Sec2p is non-phosphorylated and bound to PI4P (Stalder *et al.*, 2013). Loss of PI4P and dephosphorylation of Sec2p causes Sec2p to preferentially interact with Sec15p. This might occur when the vesicle is in close proximity to the PM rather than during trafficking in the mother cell. Our study and that of Gingras *et al.* (2022), in which single vesicles are tracked, support such models. However, this microscopic, single-vesicle approach is not without challenges. As we have found, it is not always possible to generate yeast strains with two or more fluorescently tagged probes of sufficient signal to enable multiplex detection of signal trafficking. Ideally, vesicles would be labeled with both Ypt32p and Sec4p, to determine if and/or when these proteins associate and dissociate relative to one another, as well as P4M, to detect changes in PI4P levels. Our vesicle population data, rather than single vesicle trafficking data, suggests the existence of two independent vesicle populations. One vesicle population has high PI4P and high Ypt32p levels, whereas the other has, relatively speaking, low PI4P and high Sec4p levels. However, such vesicle populations have not yet been recovered by cell fractionation.

What is the role of Osh4p in polarized secretion?

Based on this study, it does not appear that Osh4p, as originally modeled (Ling *et al.*, 2014), has a role in modulating PI4P levels on secretory vesicles as they traffic to the PM. Due to the spatial and temporal resolution of our experiments, we cannot, however,

exclude the possibility that Osh4p modulates PI4P levels at the *trans*-Golgi during vesicle formation or, as hypothesized in Smindak *et al.* (2017), immediately prior to the vesicle docking/tethering at the PM. The former possibility is supported by the observation that Osh4p co-localizes with the TGN marker Kex2p and is demonstrated to counteract the activity of Sec14p, a phosphatidylinositol/phosphatidylcholine transfer protein at the TGN (Fang *et al.*, 1996; Alfaro *et al.*, 2011; Papanikou *et al.*, 2015). With respect to the later possibility, Osh4p functioning in a PI4P-dependent manner at the PM just prior to vesicle docking/tethering is consistent with our previous data which showed that vesicles dependent upon PI4P-binding defective Osh4p had difficulty interacting with the PM, relative to vesicles dependent on wild-type Osh4p (Smindak *et al.*, 2017). These two models of Osh4p function are not mutually exclusive nor necessarily represent two entirely independent events. That is, an Osh4p-dependent vesicle docking defect observable at the PM may have its origin at the Golgi. Previous data suggested this was not the case, though that conclusion depended upon Sec4p associating with vesicles well after they depart the Golgi (Smindak *et al.*, 2017). It now appears that Sec4p is present on vesicles when they are proximal to the Golgi (Gingras *et al.*, 2022). Although Osh4p does not appear to modulate vesicle-associated PI4P levels during vesicle trafficking between the TGN and cell cortex and could well be a passenger on the vesicle, it remains possible that Osh4p has a PI4P-independent function in this context, perhaps interacting with exocyst complex proteins (Alfaro *et al.*, 2011). As P4M localizes to both the TGN and secretory vesicles, additional markers will be necessary to identify secretory vesicles in such conditions with confidence.

Observations in this study indicate that Osh proteins in *S. cerevisiae* have unique roles in maintaining the cellular distribution of PI4P. The loss of individual Osh protein family members generated distinct patterns of PI4P distribution within cells. Subsequent studies will need to determine whether loss of individual Osh protein family members also generates distinct patterns of sterol or phosphatidylserine localization, two lipids that bind certain members of the Osh protein family (Im *et al.*, 2005; de Saint-Jean *et al.*, 2011; Maeda *et al.*, 2013). Together, our observations provide insight into the localization and potential function of specific Osh proteins *in vivo*. For example, we noted an increase in P4M at the PM in *osh7*Δ cells, which is consistent with the hypothesis that Osh7p is involved in the transfer of PS and PI4P between the endoplasmic reticulum and PM (Maeda *et al.*, 2013). Most striking in our *in vivo* analysis of PI4P distribution, however, was the PI4P distribution in cells in which plasmid-borne *OSH4* or *osh4-1*^{ts} is the only functional member of the *OSH* gene family. At permissive temperature, these cells exhibited an intense localization of P4M at the PM, indicating yeast cells can tolerate, with respect to growth, large changes in PI4P distribution. This observation is consistent with findings in which a similar lipid probe, P4C, localized to the PM at non-restrictive temperature in cells in which *osh4-1* is the only functional member of the Osh family (Nishimura *et al.*, 2019). We also demonstrate Osh4p alone is not sufficient to maintain PI4P distribution but is sufficient to maintain sterol distribution. However, we cannot conclude that lethality in the absence of all Osh family members is a result of disrupted sterol distribution, as not all Osh family members bind sterol and any one family member is sufficient for survival. Yet, our observations serve as a caution, considering that both *osh*Δ [*OSH4*] and *osh*Δ [*osh4-1*] strains have been defined, in numerous studies over the last two decades, as wild-type at

permissive temperature. Lastly, we note that ORPs have unique functions in mammalian cells (reviewed by Delfosse *et al.,* 2020), although it is not yet known how these roles relate to PI4P distribution or vice versa. Thus, the study of the Osh protein family is an important step toward deciphering ORP function in mammalian cells.

Acknowledgements

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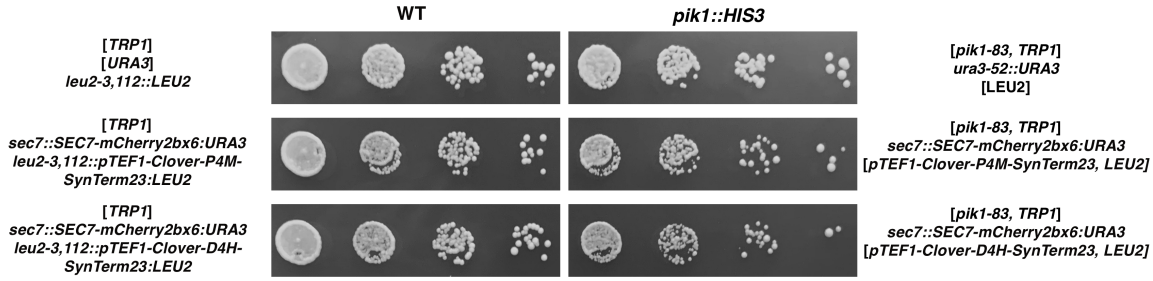
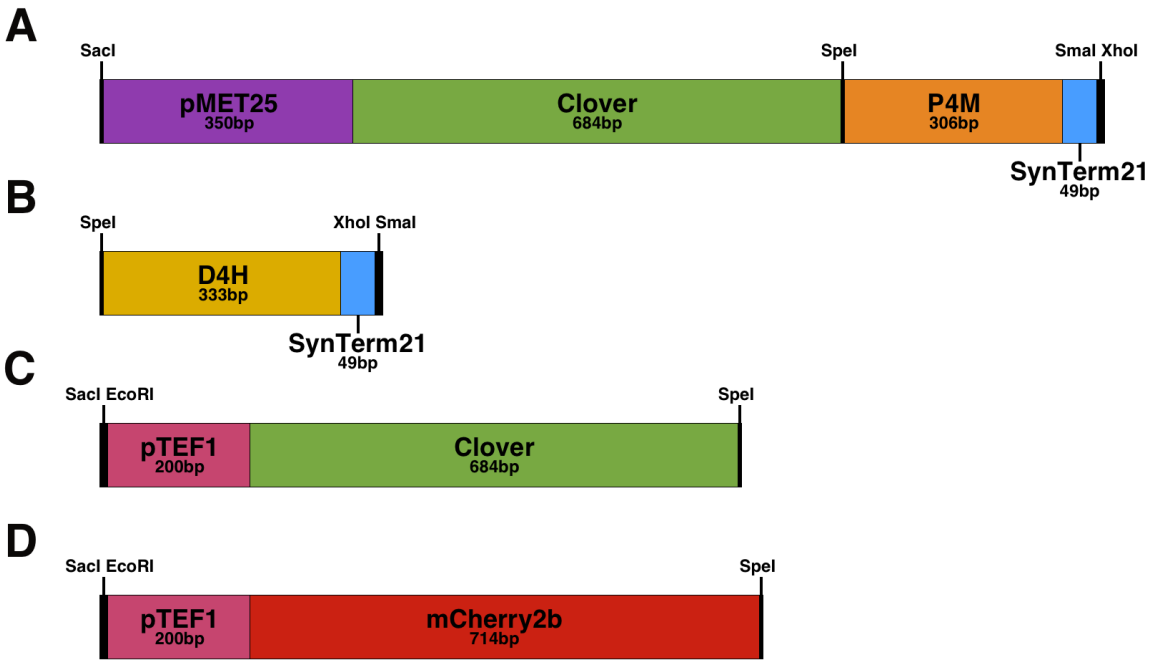
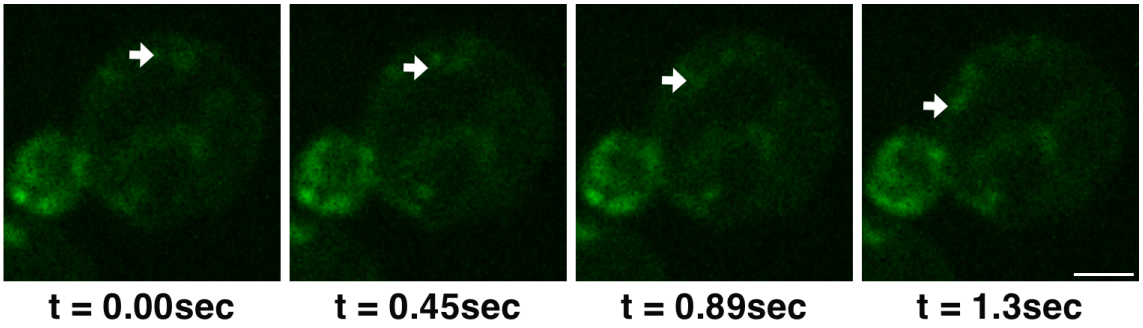


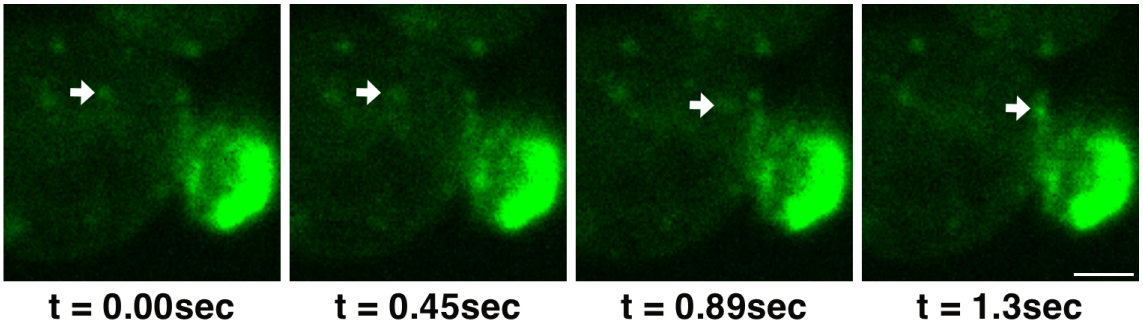
Supplemental Figure 3.1: Expression of Sec7p-mCherry2bx6 with Clover-P4M or Clover-D4H does not significantly affect cell growth. Tenfold serial dilutions, grown on agar plates prepared with selective minimal medium, of WT cells co-expressing vector controls (KK1440), Sec7p-mCherry2bx6 and Clover-P4M (KKY1389), or Sec7p-mCherry2bx6 and Clover-D4H (KKY1394), and *pik1*Δ [*pik1-83*^{ts}] cells co-expressing vector controls (KKY1441), Sec7p-mCherry2bx6 and Clover-P4M (KKY1391), or Sec7p-mCherry2bx6 and Clover-D4H (KKY1396). Image taken after a 5-day incubation at 20˚C.



Supplemental Figure 3.2: Diagram of gBlocks used in this study. (A) gbKK6, *SacI-pMET-Clover-SpeI-P4M-SynTerm21-SmaI-XhoI*. (B) gbKK7, *SpeI-D4H-SynTerm21-XhoI-SmaI*. (C) gbKK8, *SacI-EcoRI-pTEF1-Clover-SpeI*. (D) gbKK9, *SacI-EcoRI-pTEF1-mCherry2b-SpeI*.



Supplemental Figure 3.3: Trajectory of GFP-Ypt32p-marked vesicles. In WT cells, GFP-Ypt32p-marked vesicles localize to sites of polarized growth (i.e., the bud tip; KKY1403). The arrowhead indicates a vesicle trafficking along the cortex of the mother cell towards the bud. Scale bar, 1μm. Adapted from Supplemental Video 1 (Heckle and Kozminski, 2023).



Supplemental Figure 3.4: Trajectory of GFP-Sec4pp-marked vesicles. In WT cells, GFP-Sec4p-marked vesicles localize to sites of polarized growth (i.e., the bud tip; KKY1408). The arrowhead indicates a vesicle trafficking from the mother cell towards the bud. Scale bar, 1µm. Adapted from Supplemental Video 2 (Heckle and Kozminski, 2023).

Supplemental Table 3.1: Oligonucleotides used in Chapter 3.

Name	Sequence
oKK431	CGTATCAAACTAGAATGGACG
oKK432	TCAGTGCTATCCATGCTAGC
oKK433	AGCCTCCAAGAAGGTTGATG
oKK434	CACCTAACTTTTTGTGTGGTGC
oKK435	CAGTCACGACGTTGTAAAAC
oKK436	GAGCGGATAACAATTTCACAC
oKK437	CTCAGTACAATCTGCTCTGATG
oKK438	GCAGAATTGTCATGCAAGGGC

Supplemental Table 3.2: gBlocks used in Chapter 3.

Name	Sequence
gbKK6	GGCTGCGAGCTCTATTTTTTGCTTTTTCTCTTGAGGTCACATGATC GCAAAATGGCAAATGGCACGTGAAGCTGTCGATATTGGGGAACT GTGGTGGTTGGCAAATGACTAATTAAGTTAGTCAAGGCGCCATCC TCATGAAAACTGTGTAACATAATAACCGAAGTGTCGAAAAGGTG GCACCTTGTCCAATTGAACACGCTCGATGAAAAAAATAAGATATA TATAAGGTTAAGTAAAGCGTCTGTTAGAAAGGAAGTTTTTCCTTT TTCTTGCTCTCTTGTCTTTTCATCTACTATTTCCTTCGTGTAATACA GGGTCGTCAGATACATAGATACAATTCTATTACCCCCATCCATAC AATGGTCTCCAAGGGTGAAGAGTTATTCACTGGTGTGGTACCCAT ATTAGTTGAGTTAGATGGCGACGTGAATGGTCATAAATTTAGCGT TAGAGGTGAGGGAGAGGGGGATGCAACCAACGGCAAGCTGACCT TGAAGTTTATATGTACTACAGGAAAGTTGCCGGTCCCCTGGCCCA CTTTGGTAACAACCTTCGGTTATGGAGTGGCCTGCTTCTCTAGGTA CCCAGACCATATGAAACAGCACGACTTCTTCAAGTCTGCCATGCC AGAAGGTTACGTCCAGGAGAGAACAATCAGCTTCAAAGACGACG GTACCTATAAGACACGTGCGGAGGTCAAATTTGAAGGCGATACGT TGGTTAACCGTATCGAACTGAAAGGCATAGACTTCAAAGAAGAT GGCAATATTTTGGGGCATAAGCTAGAATACAATTTCAACTCTCAC AACGTCTACATAACGGCGGACAAACAGAAGAATGGCATAAAAGC AAACTTCAAAATTCGTCACAATGTAGAGGACGGCAGTGTACAATT AGCCGATCACTACCAGCAGAATACCCCTATTGGTGATGGACCTGT ACTACTGCCTGACAACCACTATTTATCCCACCAGAGTGCTCTTTCA AAAGATCCTAACGAAAAGCGTGACCATATGGTGCTGCTGGAGTTC GTGACCGCAGCTACTAGTGCAAGTACAGAAAACTTCAAGAACGT AAAAGAGAAGTACCAACAAATGAGAGGTGACGCATTGAAAACAG AGATACTTGCCGATTTCAAAGATAAATTGGCCGAAGCTACGGACG AGCAAAGTTTAAAGCAAATAGTGGCCGAACTTAAAAGTAAGGAT GAATACCGTATTCTTGCAAAGGGTCAAGGGCTAACTACCCAGTTA CTGGGATTGAAGACATCAAGTGTGAGTTCCTTTGAGAAAATGGTG GAAGAGACTCGTGAGAGTATAAAGTCTCAAGAGAGACAGACAAT TAAGATCAAATAAAATCAAATTCTATATAACTGTCTAGAAATAAA GAGTATCATCTTTCAAACCCGGGCTCGAGATGCCG
gbKK7	GGCTGCGGCTGCACTAGTAAGGGTAAGATTAACCTGGACCACAG CGGCGCATATGTAGCGCAATTTGAGGTAGCCTGGGACGAGGTCTC TTACGATAAAGAAGGAAATGAAGTCTTGACCCACAAGACCTGGG ATGGGAATTACCAGTCCAAAACCGCACACTATTCTACTGTTATAC CACTTGAAGCCAACGCTCGTAACATCAGAATAAAAGCTAGAGAG TGTACCGGGCTGGCATGGGAATGGTGGCGTGACGTTATCTCAGAG TATGATGTTCCGTTGACGAACAACATCAATGTATCAATCTGGGGA ACTACGCTATACCCCGGCTCATCTATCACGTACAACTAAAATCAA ATTCTATATAACTGTCTAGAAATAAAGAGTATCATCTTTCAAACT CGAGCCCGGGATGCCGGGC
gbKK8	GGCGGCTGCCTGCAGGAGCTCGAATTCTTTTATCACGTTTCTTTTT CTTGAAAATTTTTTTTTTGATTTTTTTCTCTTTCGATGACCTCCCA

	TTGATATTTAAGTTAATAAACGGTCTTCAATTTCTCAAGTTTCAGT TTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTA GAAAGAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGG TCTCCAAGGGTGAAGAGTTATTCACTGGTGTGGTACCCATATTAG TTGAGTTAGATGGCGACGTGAATGGTCATAAATTTAGCGTTAGAG GTGAGGGAGAGGGGGATGCAACCAACGGCAAGCTGACCTTGAAG TTTATATGTACTACAGGAAAGTTGCCGGTCCCCTGGCCCACTTTG GTAACAACCTTCGGTTATGGAGTGGCCTGCTTCTCTAGGTACCCA GACCATATGAAACAGCACGACTTCTTCAAGTCTGCCATGCCAGAA GGTTACGTCCAGGAGAGAACAATCAGCTTCAAAGACGACGGTAC CTATAAGACACGTGCGGAGGTCAAATTTGAAGGCGATACGTTGGT TAACCGTATCGAACTGAAAGGCATAGACTTCAAAGAAGATGGCA ATATTTTGGGGCATAAGCTAGAATACAATTTCAACTCTCACAACG TCTACATAACGGCGGACAAACAGAAGAATGGCATAAAAGCAAAC TTCAAAATTCGTCACAATGTAGAGGACGGCAGTGTACAATTAGCC GATCACTACCAGCAGAATACCCCTATTGGTGATGGACCTGTACTA CTGCCTGACAACCACTATTTATCCCACCAGAGTGCTCTTTCAAAA GATCCTAACGAAAAGCGTGACCATATGGTGCTGCTGGAGTTCGTG ACCGCAGCTACTAGTATGCCGGGC
gbKK9	GGCGGCTGCGAGCTCGAATTCTTTTATCACGTTTCTTTTTCTTGAA AATTTTTTTTTTTGATTTTTTTCTCTTTCGATGACCTCCCATTGATA TTTAAGTTAATAAACGGTCTTCAATTTCTCAAGTTTCAGTTTCATT TTTCTTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTAGAAAGA AAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGGATAGCA CTGAGAGCGGCTCCACCGAGTCCGTCATCAAGGAGTTCATGCGCT TCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAG ATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGAC CGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTG GGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGT GAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCC CGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCG GCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGT TCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACG GCCCCGTAATGCAGAAGAAGACTATGGGCTGGGAGGCCTCCTCC GAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAA GCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGG TCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGC GCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAG GACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCA CTCCGGCTCCCAGGGATCTAGCGGCAGCAGTTCTAGCACTAGTAT GCCGGGC

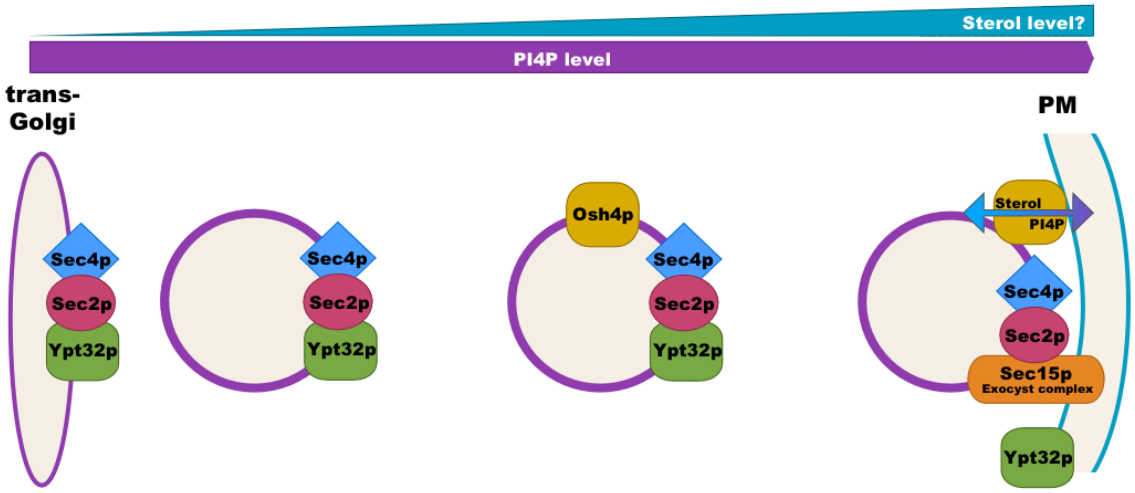
CHAPTER 4

Discussion and Future Directions

Summary

Elucidating the complex relationship between proteins and lipids is crucial to further our understanding of fundamental cellular processes such as polarized secretion. In this dissertation, I demonstrated that Osh6p can independently support polarized secretion in a lipid-dependent manner. I also completed the first study to directly quantify vesicle-associated PI4P levels during polarized secretion using the lipid binding domain P4M. Utilizing the data presented in Chapters 2 and 3, I propose a new model for polarized secretion in which vesicle-associated PI4P levels remain constant during trafficking in an Osh4p-independent manner (Figure 4.1). This is a significant change to the existing model of polarized secretion and highlights the necessity of *in vivo* experiments studying the location and timing of events required for successful polarized secretion.

In this chapter, I will discuss the key findings of my research in the context of the field and propose future studies, in addition to addressing the questions posited in Chapter 1. Can Osh6p substitute for Osh4p to independently support polarized secretion? Do vesicle-associated PI4P levels change as vesicles traffic? Lastly, what is the role of Osh4p in polarized secretion?



Component	Prior model	New model (above)
Sec4p	Sec4p is recruited to the vesicle.	Sec4p is recruited at the *trans*-Golgi prior to vesicle formation (Gingras *et al.*, 2022).
PI4P	Vesicle-associated PI4P levels decrease as vesicles traffic from the *trans*-Golgi to the PM.	Vesicle-associated PI4P levels do not decrease during trafficking. Levels may change at the TGN or PM.
Osh4p	Osh4p ssociates with the vesicle during trafficking and decreases vesicle-associated PI4P.	Osh4p associates with the vesicle during trafficking, but the function of vesicle-associated Osh4p prior to arrival at the PM is unclear.
Ypt32p	Loss of vesicle-associated PI4P results in the dissociation of Ypt32p from Sec2p to allow Sec15p binding.	Ypt32p remains on the vesicle until the PM, where Ypt32p is exchanged for Sec15p.

Figure 4.1: New model of polarized secretion in *S. cerevisiae*. In the new model of polarized secretion, the Rab GTPase Sec4p arrives at the *trans*-Golgi prior to vesicle formation. During trafficking, Osh4p associates with the vesicle but does not alter vesicle-associated PI4P levels. At the PM, the Rab GTPase Ypt32p is exchanged for Sec15p, a member of the exocyst complex. Osh4p mediates a non-specific lipid exchange between the vesicle and PM that is necessary for docking (i.e., *trans*-SNARE complex formation). This model does not exclude the possibility that vesicle-associated PI4P decreases at the *trans*-Golgi and/or PM. Rather, this model narrows the temporal window during which such a loss would occur, thereby providing a foundation for future studies on the role of PI4P in polarized secretion. The prior model can be seen in its entirety in Figure 1.6.

Can Osh6p Substitute for Osh4p to Independently Support Polarized Secretion?

In Chapter 2, I demonstrated that Osh6p can independently support polarized secretion in a lipid-dependent manner. This is, at a glance, a confounding result because members of the Osh protein family share a yet unknown essential function (Beh *et al.*, 2001). If the shared function of the Osh protein family was related to sterol, then Osh6p, which cannot bind sterol, should not be able to independently support polarized growth. Yet, in the absence of all other Osh proteins, lack of sterol binding for Osh4p is lethal. In the same vein, the opposite holds true for PS.

How do all Osh proteins independently support polarized secretion?

As any member of the Osh protein family is sufficient for survival, we can infer any single Osh protein can support polarized secretion (Beh *et al.*, 2001). However, it is unclear whether all Osh proteins would support polarized secretion in a similar manner. It is possible that an Osh protein may not interact with vesicles but fulfill an essential role elsewhere that indirectly allows for the completion of polarized secretion, such as maintaining a lipid distribution at the TGN to facilitate vesicle formation or protein segregation that affects the recruitment of downstream effectors. Alternatively, PI4P gradients established by the Osh protein family may drive the vesicle-dependent or -independent transport of other lipids. This is not unreasonable as PI4P-enrichment at the TGN is speculated to provide energy to drive sterol transport from the ER to the TGN (de Saint-Jean *et al.*, 2011; Mesmin *et al.*, 2013). Other families of lipid-binding and -transfer proteins may rely on PI4P distribution established by Osh proteins to transfer their respective lipid ligands between membranes (reviewed by Wong *et al.*, 2017; Wong *et al.*,

2018). Examining the distribution of lipids that are not ligands of the Osh protein family would provide insight into vesicle-independent lipid transfer.

If all Osh proteins support polarized secretion through the same function as Osh4p, then all Osh proteins must be recruited to secretory vesicles. While Osh4p is known to associate with Sec4p-marked vesicles in a Sec6p-dependent manner, there are no direct data demonstrating other Osh proteins directly interact with Sec4p-marked vesicles (Alfaro *et al.*, 2011). Are all Osh proteins capable of associating with secretory vesicles and, if so, are they recruited by the same mechanism?

To determine whether all Osh proteins localize to secretory vesicles in WT cells and/or in the absence of all other Osh proteins, all seven members of the Osh protein family need to be labeled. Others have successfully used fluorescently tagged Osh proteins, though many of these constructs are dated and lack the benefits of modern fluorophores, such as increased photostability and brightness (Levine *et al.*, 2001; Schulz *et al.*, 2009; Stefan *et al.*, 2011; Ling *et al.*, 2014). In addition to *in vivo* localization experiments, protein tags will allow us to conduct pull-down assays to identify Osh protein interactors. HaloTag fusions, which can be used for both *in vivo* imaging and protein assays, could prove useful (reviewed by England *et al.*, 2015). Because not all Osh proteins are studied with equal fervor, *in vitro* binding assays between purified recombinant Osh proteins and suspected interactors of Osh4p, such as Sec6p, will provide critical information to those that study the role of the Osh protein family in polarized secretion in both WT and mutant strains.

Much of our current understanding of Osh protein function is derived from studies in which there is only one functional Osh protein. In many cases, this requires us to examine

cells at a non-permissive temperature (i.e., in an *osh*Δ *osh4-1* strain background). This is problematic because *osh*Δ cells are known to have altered lipid composition and distribution relative to WT cells, including 3.5 fold more ergosterol than WT cells (Beh *et al*., 2001) and, as shown in Chapter 3, increased PI4P at the PM (Figure 3.6). Extended exposure to a non-permissive temperature could exacerbate phenotypes that result from altered lipid composition and distribution. In addition, we do not know if a protein function that is essential in a mutant strain is also essential in a WT strain. Therefore, it is crucial that we develop tools to study individual Osh proteins in all strain backgrounds including WT.

Do Vesicle-associated PI4P Levels Change as Vesicles Traffic?

In Chapter 3, I validated the lipid probe P4M in *S. cerevisiae* by demonstrating P4M is specific to PI4P and reflects changes in PI4P distribution *in vivo* (Figure 3.1, 3.2). Using P4M and live cell imaging, I directly quantified vesicle-associated PI4P levels to show vesicular PI4P levels do not change significantly on Ypt32p-marked vesicles during trafficking to sites of polarized cell growth (Figure 3.4). This is contrary to the prior model of polarized secretion that proposed secretory vesicles are enriched in PI4P after budding from the *trans*-Golgi relative to when the vesicles arrive at the PM (Figure 4.1). My data do not rule out the possibility that vesicle-associated PI4P levels change at the *trans*-Golgi or PM, which I will discuss later in this chapter.

Though vesicular PI4P levels do not change during trafficking, I observed P4M is more likely to associate with Ypt32p-marked vesicles than Sec4p-marked vesicles, which

is consistent with the prior model (Figure 3.3; Figure 4.1). This vesicle population data, rather than my single vesicle trafficking data, suggests the existence of two independent vesicle populations. One population has high PI4P and Ypt32p levels, whereas the other has, relatively speaking, low PI4P and high Sec4p levels. Though other vesicle populations have been isolated by cell fractionation (i.e., polarized and non-polarized secretory vesicles), sub-populations of polarized vesicles have yet to be recovered (Harsay and Bretscher, 1995). Ideally, vesicles would be labeled with both Ypt32p and Sec4p, as well as P4M, though it is not always possible to generate yeast strains with two or more fluorescently tagged probes of sufficient signal to enable multiplex detection of signal trafficking.

Is phosphorylation of Sec2p biologically relevant?

In the prior model for polarized secretion, loss of vesicle-associated PI4P was postulated to trigger phosphorylation of Sec2p, which in turn results in the dissociation of the Rab GTPase Ypt32p and the recruitment of the exocyst subunit Sec15p (Elkind *et al.*, 2000; Mizuno-Yamasaki *et al*., 2010; Stalder *et al.*, 2013). While *in vitro* data strongly support the hypothesis that when phosphorylated at serine residues 181, 186, and 188, Sec2p preferentially interacts with Sec15p over Ypt32p, there are no direct in *viv*o data that demonstrate Sec2p phosphorylation affects Sec15p localization. To understand the role of PI4P in polarized secretion, we must identify whether vesicle-associated phosphorylation of Sec2p drives protein association and dissociation during vesicle maturation.

To determine whether the recruitment of Sec15p to polarized secretory vesicles is influenced by Sec2p phosphorylation, what is needed is expression of phosphomimetic and

nonphosphorylatable Sec2p mutants in cells expressing fluorescently tagged Ypt32p and Sec15p. If phosphorylation of Sec2p is not biologically relevant to Ypt32p and Sec15p localization, it would not necessarily exclude the possibility that vesicles undergo a loss of PI4P. However, the results of this experiment will clarify how and when Sec15p is recruited to the vesicle.

What Is the Role of Osh4p in Polarized Secretion?

In the prior model for polarized secretion, Osh4p served two essential functions—decreasing vesicle-associated PI4P during trafficking to drive vesicle maturation and mediating a non-specific lipid exchange between the vesicle and PM to facilitate the formation of *trans*-SNARE complexes (Ling *et al.*, 2014, Smindak *et al.*, 2017). In Chapter 3, I demonstrated vesicle-associated PI4P levels do not change during trafficking (see Figure 3.4). Therefore, if Osh4p mediates an essential loss of vesicle-associated PI4P, then this most likely occurs at the TGN and/or PM, not during trafficking.

Do exocytic vesicles lose vesicle-associated PI4P at the trans-Golgi?

The hypothesis that Osh4p reduces vesicle-associated PI4P at the *trans*-Golgi is supported and contradicted by prior studies. In support of this hypothesis, Osh4p is known to localize to the TGN, though the function of Osh4p at the TGN is debated. Some posit that Osh4p negatively regulates vesicle formation due a genetic interaction with Sec14p, which is involved in non-polarized secretion, while others speculate Osh4p transfers sterol against its concentration gradient to the TGN from the ER like OSBP (Fairin *et al.*, 2007;

de Saint-Jean *et al.*, 2011; Mesmin *et al.*, 2013; Mesmin *et al.*, 2017). Though the function of Osh4p at the TGN is unclear, we know Osh4p is not required for exit from the TGN, as post-Golgi vesicles accumulate in the absence of Osh protein function (Smindak *et al.*, 2017). If Osh4p transfers PI4P from the vesicle to the *trans*-Golgi, then this must occur prior to Sec4p recruitment, as Sec4p interacts with the motor protein Myo2p to facilitate vesicle trafficking (Schott *et al.*, 1999). However, an extensive high-resolution study reported Sec4p is recruited to the *trans*-Golgi prior to vesicle formation, which makes one wonder when Osh4p would be able to mediate a lipid transfer (Gingras *et al.*, 2022). The prior model for polarized secretion hinged on loss of vesicle-associated PI4P triggering the recruitment of Sec15p (Stalder *et al.*, 2013; Ling *et al.*, 2014). If we assume this to be true, then Osh4p mediated lipid exchange at the *trans*-Golgi implies Sec15p is recruited at the TGN, but this is unlikely because data suggest that vesicle-associated components of the exocyst complex are recruited when the vesicle is in close proximity with the PM (Gingras *et al.*, 2022).

To resolve this uncertainty, I reiterate the importance of an experiment proposed earlier in this chapter to determine whether Sec2p phosphorylation is biologically relevant and propose a study to determine whether Osh4p associates with vesicles through direct interaction with Sec6p. The latter is more challenging because we currently do not know any direct interactors of Osh4p. Alfaro *et al.* (2011) isolated polarized secretory vesicles marked with Bgl2p and Sec4p in *sec6-4*^{ts} cells and noted that Osh4p no longer associated with secretory vesicles, but it is unclear whether Osh4p binds Sec6p directly. To determine the specific proteins required to recruit Osh4p to exocytic vesicles, one could examine whether Osh4p localizes to polarized secretory vesicles in cells with temperature-sensitive

alleles of proteins downstream of Sec6p. Identifying how essential proteins are recruited to polarized secretory vesicles is key to understanding the timeline of events and, by extension, protein function.

Does Osh4p mediate a non-specific lipid exchange at the PM?

Smindak *et al.* (2017) demonstrated that cells reliant on lipid binding-defective Osh4p failed to efficiently form *trans*-SNARE complexes when compared to cells reliant on WT Osh4p (*osh*Δ *OSH4*). Together with my data from Chapter 2 that showed Osh6p can support polarized secretion in a lipid-dependent manner, Smindak *et al.* concluded Osh4p mediates a non-specific lipid exchange between the vesicle and PM. This is consistent with the hypothesis that Osh4p mediates loss of vesicle-associated PI4P at the PM. How the proposed lipid exchange relates to *trans*-SNARE complex formation is not readily clear.

One possible explanation is that Osh4p-mediated lipid exchange exists to establish a specific lipid composition on the vesicle or PM. It is well established that PS and PI(4,5)P_2 are enriched at sites of polarized secretion to recruit essential proteins, and it stands to reason that the lipid composition of the vesicle or PM may be altered to allow progression of vesicle tethering, docking, and/or fusion (He *et al.*, 2007; Fairn *et al.*, 2011). However, based on the lipid-binding capabilities of Osh4p and Osh6p, it is unlikely this transfer is related to sterol, PS, or charge. Rather, PI4P is likely the functionally relevant lipid in this exchange.

If PI4P is the essential lipid in the Osh4p-mediated exchange, then one would expect vesicles to tether, dock, and fuse more quickly or more slowly when the PM is

enriched with PI4P. This is because Osh4p will need to transfer less or more PI4P to establish the required lipid composition. In Chapter 3, I demonstrate that the PM is abnormally enriched in PI4P in *osh*Δ *OSH4* cells relative to WT cells (see Figure 3.5 and 3.6). However, Alfaro *et al.* (2011) determined, in *osh*Δ *OSH4*, vesicles spend the same amount of time at the PM relative to vesicles in WT cells, which conflicts with the idea that Osh4p mediates an essential change of PI4P composition on the vesicle or PM. One could conclude there is no Osh4p-mediated lipid exchange between the vesicle and PM. However, we must consider the possibility that the results in Alfaro *et al.* (2011) are inaccurate due to the limitations of technology when the study was conducted. Consistent with this, in a more recent study, vesicles in WT cells were determined to spend approximately 5 sec at the PM, which three times faster than the reported times for WT cells in prior studies (Alfaro *et al.*, 2011; Donovan and Bretscher, 2015; Gingras *et al.*, 2022). It is worth repeating the experiment in Alfaro *et al.* to determine how much time vesicles spend at the PM in WT cells, *osh*Δ *OSH4* cells, and *osh*Δ *osh4-1*^{ts} cells. The result of this experiment will support or contradict a model where Osh4p mediates a non-specific lipid exchange between the vesicle and PM.

If Osh4p does not mediate a vesicle-PM lipid exchange, then how does Osh4p function relate to the formation of *trans*-SNARE complexes? First, we must acknowledge that Smindak *et al.* examined Osh4p-dependent *trans*-SNARE complex formation but not Osh4p-dependent exocyst complex formation, which is a prerequisite for efficient *trans*-SNARE complex formation. This decision was supported in part by a prior study that showed Sec5p, a vesicle-associated member of the exocyst complex, localizes to the PM in the absence of Osh protein function (Alfaro *et al.*, 2011). However, localization of Sec5p

to the PM does not indicate the exocyst complex is fully formed. Therefore, it is possible that Osh4p assists in the assembly or disassembly of the exocyst complex, and/or interacts with SNAREs directly. As discussed previously (see Chapter 1), the exocyst complex is hypothesized to actively regulate the formation of t*rans*-SNARE complexes.

Though it is not entirely clear how lipid binding by Osh4p would be essential for Osh4p-exocyst or Osh4p-SNARE interactions, this hypothesis is supported by existing data demonstrating Osh proteins interact with both exocyst complex subunits and SNAREs. As previously discussed, Osh4p localization to Sec4p-marked vesicles is inferred to be dependent on Sec6p, a component of the exocyst complex that interacts with the t-SNARE Sec9p (Sivaram *et al.*, 2005; Alfaro *et al.*, 2011). Osh4p is also posited to interact with another exocyst complex subunit, Sec8p (Alfaro *et al.*, 2011). Osh4p may stabilize the exocyst complex when in lipid bound state and lipid transfer is required to change the confirmation of Osh4p, which destabilizes the exocyst complex to allow for *trans*-SNARE formation. In regard to direct interaction with SNAREs, there is currently no evidence that Osh4p can directly interact with SNAREs. However, a recent study demonstrated Osh1-3p interact with the t-SNARE Sec9p and suggested the paralog of Osh4p, Osh5p, may also interact with Sec9p (Weber-Boyat *et al.,* 2021). Together, these data suggest Osh4p may regulate the formation of the exocyst complex and/or *trans*-SNARE complexes through direct protein-protein interactions rather than a non-specific lipid exchange. These hypotheses are not mutually exclusive; Osh4p could regulate tethering, docking, and/or fusion through protein-protein interactions and a non-specific lipid exchange. It is also possible that lipid binding by Osh4p is essential for another cellular process that influences polarized secretion. In cells lacking all other Osh proteins,

restricting Osh4p localization to the TGN with an additional domain, such as Fapp1p-PH, could determine whether Osh4p performs an essential function on secretory vesicles.

Concluding Remarks

Together, the data presented in this dissertation present a new model for polarized secretion in *S. cerevisiae*, which challenges the existence of a PI4P gradient in which vesicle-associated PI4P decreases as vesicles traffic and mature. In addition, I validated P4M in *S. cerevisiae*, providing a tool to continue the study of PI4P *in vivo*. Together with probes specific to sterol and PS, we can begin to tackle how intracellular lipid distribution is established and/or maintained by the Osh protein family and how lipid distribution regulates essential cellular processes, such as polarized secretion. As studies in humans tend to focus on the role of ORPs in lipid synthesis, lipid distribution, and the relationship between ORPs and various diseases and viral infections (reviewed by Arora *et al.*, 2022), it is of the utmost importance to continue to study Osh proteins in essential cellular processes to aide ORP-related studies in mammalian cells.

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