A Role for the Sterol-Binding Protein Osh4/Kes1 in Polarized Growth

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ABSTRACT

In the budding yeast *Saccharomyces cerevisiae*, seven OSBP-homologues (Osh) proteins are implicated in the transfer of sterol lipids between membranes within cells. To test whether Osh4p acts as a sterol transfer protein, a sterol-binding deficient mutant was analyzed. The mutant Osh4 protein was hyperactivated despite its inability to bind sterols, contrary to what is predicted for a sterol transporter. *OSH* genes also have an established role in vesicle docking during polarized exocytosis. To determine how Osh4p might affect vesicle docking, genetic interactions between *OSH4*, actomyosin-related genes, and regulatory genes involved in vesicle docking were analyzed. Genome-wide screens were performed to identify novel *OSH* genetic interactions and to better understand the functional interrelationship between the seven *OSH* genes. These results suggested that a primary role of the *OSH* genes is to regulate vesicle docking during polarized exocytosis, and not for the direct transfer of sterols between membranes.

Keywords: ORPs, polarity, sterol-binding, Smy1p
DEDICATION

For Ryan, who always believed in me.
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LIST OF ABBREVIATIONS:

Bgl2 - β-1,3 glucanase
ECM – extracellular matrix
ER – Endoplasmic reticulum
FunSpec - Functional specification
GARP - Golgi associated retrograde protein
GOLD – Golgi dynamics
GEF - Guanine nucleotide exchange factor
LE – Late endocytic compartment
MCS – Membrane contact sites
MM – Minimal media
OSBP – Oxysterol binding protein
ORP- OSBP-related protein
OSH – OSBP homologue
ORD – OSBP related domain
PAK – p21-activated kinase
PC – Phosphatidylcholine
PH- pleckstrin homology
PI – Phosphatidylinositide
PIP2 - phosphatidylinositol 4,5-biphosphate
PM- Plasma membrane
RILP – Rab6-interacting lysosomal protein
SGA – Synthetic genetic arrays
SMY1 – Suppressor of Myosin
SNARE - soluble N-ethylmaleimide-sensitive fusion attachment protein receptor
TAP – Tandem affinity purification
VAMP – vesicle associated membrane protein
VAP – VAMP associated protein
1: INTRODUCTION

1.1 Cell Polarization Requires the Asymmetric Trafficking of Proteins and Lipids

The establishment of cell polarity involves both the sorting of lipids and proteins to specific regions within the cell, and their retention once delivered to these regions (reviewed in Schuck and Simons, 2004; Vicente-Manzanares, 2000). The mechanisms that establish and maintain cell polarity generate functionally distinct and separate domains within membranes where specific functions and reactions, such as cytoskeletal nucleation or membrane growth, lead to cell specialization (reviewed in Vicente-Manzanares, 2000; Schuck and Simons, 2004). Key cell processes such as cell migration, division, and differentiation are dependent on the asymmetric growth of polarized cells (Fig.1-1) (Schuck and Simons, 2004). As an example, polarized epithelial cells can divide into two functionally distinct domains, the apical domain, which faces an open lumen and is the site of absorption and secretion, and the basal domain, which interacts with the extracellular matrix in order to anchor cells (Tsukita et al., 2001). Tight junctions physically separate the apical and basal domains in order to prevent mixing between membrane components (Tsukita et al., 2001). In addition to the restricted localization of some proteins to the basolateral or apical membranes, specific lipids also exhibit membrane specificity. The asymmetric distribution of cholesterol in plasma membrane (PM) provides a mechanism for
Figure 1-1  Cell Polarity Plays an Essential Role in Cell Function

The mechanisms that establish and maintain cell polarity generate functionally distinct and separate domains within membranes where specific functions and reactions, such as cytoskeletal nucleation or membrane growth, lead to cell specialization. Key cellular processes are dependent on correct polarization of cells. For example, (A) during development, cell fate determinants localize asymmetrically in order to create two daughter cells with different cell fates; (B) during cell migration, the actin cytoskeleton polarizes towards the leading edge, thereby allowing for movement of the cell; (C) neurons are highly polarized cells with specialized regions with regards to the dendrite and axon; (D) epithelial cells establish and maintain apical-basal polarity in order to function.
Figure 1-1

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http://www.eb.tuebingen.mpg.de/research-groups/silke-wiesner
the polarized sorting of biosynthetic cargo and is therefore important for the
development of the apical membrane (Schuck and Simons, 2004). Lipids such as
cholesterol, together with membrane signalling proteins and the cytoskeleton, are
therefore also integral to cell polarization.

Knowledge of how cells establish and maintain cell polarity is key to the
understanding of many different cellular functions such as cell growth and
development as well as the diseases that occur when these processes are
interrupted. Failure to maintain proper cell polarity is a common characteristic of
several types of cancers making the study of proteins that establish and regulate
cell polarity essential to understanding the pathology of cancers and tumour
progression (reviewed in Tanos et al., 2008, Etienne-Manneville, 2008). For
example, Scribble (Scrib), which forms a complex with the tumour suppressor,
lethal giant Larva (Lgl) and discs large (Dlg) and plays an important role in
maintaining baso-lateral polarity in epithelial cells through a number of signalling
pathways, by restricting the apical polarity complex, the Par complex, to the
apical cell surface (reviewed Humbert et al., 2006). Although the mechanism is
not clear, decreased expression or loss of Scribble has been found in colorectal
cancers and malignant melanomas (reviewed in Mellman and Nelson, 2008;
Gardiol et al., 2006), suggesting that the regulation of the expression of proteins
that maintain cell polarity affects carcinogenesis. In retinitis pigmentosa, a defect
in protein sorting results in the improper localization of Rhodopsin, a key protein
in photoreceptor cell function, to the apical membrane of the photoreceptor
(reviewed in Nelson et al., 2008; Sung et al., 2000) supporting the idea of that
polarized localization is necessary for specific function. The establishment and regulation of cell polarity is a complex and dynamic process involved in many different aspects of cell functions, the understanding of which could lead to better comprehension of a multitude of diseases.

1.1.1 Yeast Cell Polarization is Mediated by a Cascade of Small Rho GTPases

Basic mechanisms of cell polarization are conserved in all eukaryotes, including the budding yeast, *Saccharomyces cerevisiae*. During the process that leads to cell division, budding yeast promotes polarized exocytosis to a specific site on the PM from which a daughter cell will grow (reviewed in Nelson 2003). Yeast cell polarization is regulated by a cascade of small GTPases, which is homologous to mammalian cell polarization (Fig.1-2) (reviewed in Brennwald and Rossi, 2007). Localized at the actomyosin ring during cytokinesis, septins disappear after cytokinesis is complete and are thought to act as landmark proteins for the previous bud site. Mutations in septins as well as the associated proteins, Bud3p, Bud4p and Axl2p results in improper bud site selection suggesting that these proteins act as spatial landmarks for the incipient bud site (Nelson et al., 2003; Casamayor and Synder, 2002). Early in the G1 stage of the cell cycle, the Ras-like GTPase Rsr1p/Bud1p recognizes these landmark proteins and establishes a new bud site adjacent to the bud scar (Adams et al., 1990; Kozminski et al., 2003). Rsr1p then helps to recruit Cdc24p, the Cdc42p guanine nucleotide exchange factor (GEF), which in turn recruits and activates the Rho family GTPase, Cdc42p (Zheng et al., 1994). Cdc42p is the so-called
Yeast cell polarization is regulated by a cascade of small GTPases homologous to mammalian cell polarization (reviewed in Brennwald and Rossi, 2007). A group of proteins including septins, Axl2p, Bud3p and Bud4p associated with the previous bud site, a region referred to as the bud scar act as a landmark for novel bud site selection (Nelson et al., 2003). Early in the G1 stage of the cell cycle, the Ras-like GTPase Rsr1p/Bud1p recognizes these landmark proteins and establishes a new bud site adjacent to the bud scar (Adams et al., 1990; Kozminski et al., 2003). Rsr1p then recruits a Cdc42p guanine nucleotide exchange factor (GEF), Cdc24p, which activates the small Rho family GTPase, Cdc42p (Zheng et al., 1994). Cdc42p concentrates to sites of polarization and mediates the assembly and orientation of the actin cytoskeleton towards the incipient bud site. During late G1/S phase, Cdc42p along with Rho1p promotes polarized secretion to the bud site resulting in vesicular fusion and membrane growth at the site of polarization (Kozminski et al., 2003; Guo et al., 2001) thereby regulating the formation of the daughter bud.
master regulator of cell polarity because it establishes an axis of polarity that is directed towards the new bud site (Adams et al., 1990). Cdc42p concentrates to sites of polarization and mediates the assembly and orientation of the actin cytoskeleton towards the incipient bud site. During late G1/S phase, Cdc42p along with Rho1p promotes polarized secretion to the bud site resulting in vesicular fusion and membrane growth at the site of polarization (Kozminski et al., 2003; Guo et al., 2001). In another Rsr1p-independent mechanism, Cdc42p can establish and maintain cell polarization at a random ectopic site, which involves an actomyosin-dependent Cdc42p “positive-feedback loop” whereby Cdc42p promotes its own polarization (Adamo et al., 2001). Secretory vesicles containing Cdc42p are able to dock and fuse at the PM, independent of Rsr1p, thereby promoting their own polarized localization and cell growth to that site (Adamo et al., 2001). The process of establishing cell polarity in yeast is analogous to that in mammalian cells with many of the proteins sharing mammalian homologues, such as Rho1p (homologous to mammalian RhoA), Cdc42p (Cdc42), and Sec4p (homologous to mammalian Rab8). This makes the study of cell polarity in yeast pertinent to understanding cell polarization in more complex systems.

1.1.2 The Docking of Polarized Secretory Vesicles to the PM is Mediated by the Exocyst Complex

At sites of polarized cell growth, the octomeric exocyst complex mediates the docking of secretory vesicles to the PM (Fig. 1-3) (reviewed in Hsu et al., 2004;
Figure 1-3  Secretory Vesicle Docking in the Bud

An octomeric protein complex known as the exocyst mediates the docking of secretory vesicles to the plasma membrane (PM) at sites of polarization. The vesicular component of the exocyst (Sec5p, Sec6p, Sec8p, Exo84p and Sec15p) are attached to vesicles through the Rab GTPase, Sec4p. The membrane component of the exocyst (Sec3p and Exo70p) interacts with Rho GTPases on the PM. Secretory vesicles move along actin filaments into the daughter bud with the aid of the myosin motor, Myo2p. A kinesin-related protein, Smy1p binds to Myo2p and may play a yet unknown role in polarized secretion. As the two components of the exocyst come into the same vicinity, they bind together docking the vesicle to the PM allowing for membrane fusion and growth mediated by SNARE proteins.
Adapted by C.T. Beh with permission from authors. Munson and Novick, Nature Structural and Molecular Biology, 2006©.
The exocyst complex is divided into two components based on membrane associations. The vesicular component of the exocyst (Sec5p, Sec6p, Sec8p, Sec10p, Exo84p and Sec15p) is attached to vesicles through interactions between Sec15p and the Rab GTPase, Sec4p, found on vesicles (Boyd et al., 2004). Sec4p is a known regulator of post-Golgivesicular transport and plays a role in regulating the exocyst along with Cdc42p, Rho1p, Rho3p, and Rho4p (Lipschutz and Mostov, 2002; Hsu et al., 2004). The membrane component of the exocyst complex (Sec3p, and Exo70p) is tethered to sites of polarization through interactions with Rho GTPases (Zhang et al., 2008). Sec3p directly interacts with the GTP-bound Cdc42p and Rho1p, while Exo70p interacts with GTP-bound Rho3p (reviewed in Hsu et al., 2004). It has been proposed that Sec3p and Exo70p act as spatial landmarks for the vesicular components of the exocyst complex (reviewed in Hsu et al., 2004; Finger et al., 1998). Both Sec3p and Exo70p interact with phosphoinositides (He et al., 2007; Zhang et al., 2008) and Rho GTPases (Guo et al., 2001, Robinson et al., 1999) found at sites of cell polarization and localize to sites of cell growth independent of vesicular transport and actin (He et al., 2007, Guo et al., 2008). It is still unclear how Sec3p and Exo70p are targeted to sites of polarization or the relationship between the two proteins.

The process of polarized exocytosis involves three distinct events, including actomyosin transport of vesicles to sites of polarized growth, exocyst complex-dependent vesicle docking, and membrane fusion between vesicles and the PM (reviewed in Mellman and Nelson, 2008). First, post-Golgi vesicles move
along polarized actin cables into the daughter bud with the aid of the type V myosin motor, Myo2p (Johnston et al., 1991; Schott et al., 1999). As the vesicle-bound exocyst subunits come to the same vicinity as their PM-bound counterparts, they bind together docking the vesicle to the PM. Finally membrane fusion is mediated by SNARE (soluble N-ethylmaleimide-sensitive fusion attachment protein receptor) proteins found on the vesicle and target membranes, which bind together bridging the two membranes allowing for lipid bilayer fusion (Pruyne et al., 2000; Guo et al., 2001; Sutton et al., 1998). The fusion of vesicles at sites of cell growth results in polarized membrane growth and bud formation.

1.1.3 The Actomyosin Cytoskeleton Targets Vesicular Transport

In yeast, before the exocyst complex initiates vesicle docking, post-Golgi vesicles must be delivered from the mother cell into the budding daughter cell. This polarized movement of vesicles is dependent on filamentous actin cables that are oriented towards the bud tip. The actin cytoskeleton is a highly dynamic system that plays a role in many essential cellular functions such as endocytosis, polarized exocytosis, cytokinesis, cell polarization, and the maintenance of cell morphology (Mosely and Goode, 2006). There are three different functional actin structures in yeast, actin patches, actin filaments, and cytokinetic actin rings that contribute to different cellular functions (reviewed in Mosely and Goode, 2006). The transport of post-Golgi vesicles requires myosins, which are molecular motor proteins that facilitate transport along actin filaments (reviewed Mosely and Goode, 2006). In yeast, there are three different classes of myosins; type I, II,
and V, which mediate plus-end, actin-dependent transport of organelles, vesicles, and mRNAs (reviewed Brown, 1999; Moseley and Goode, 2006). The homologous type I myosins, Myo3p and Myo5p are associated with cortical actin patches and play a role in endocytosis (Geli and Riezman, 1996). The type II myosin, Myo1p, functions during cytokinesis and localizes to the actomyosin ring (Tolliday et al., 2003). Specifically, the type V myosin, Myo2p has been implicated in the transport of polarized secretory vesicles along actin filaments to the bud tip during cell growth/division (Johnston et al., 1991; Schott et al., 1999).

A number of auxiliary proteins contribute to Myo2p function during polarized exocytosis, including Smy1p (suppressor of myosin). Smy1p is a kinesin-related protein implicated in actin-dependent polarized transport through its interactions with Myo2p, however its exact role has yet to be determined (reviewed in Brown, 1999; Beningo et al., 2000). Smy1p was initially discovered in a screen for genes that rescued defects in myo2-66 mutations by restoring its localization to sites of cell polarization, suggesting it may play a role in polarized exocytosis (Lillie and Brown, 1992). Smy1p physiologically binds to Myo2p (Beningo et al., 2000) and they both co-localize to sites of polarized cell growth (Lillie and Brown, 1998). Smy1p shares homology in its N-terminal with kinesins (Lillie and Brown, 1992), however it lacks microtubule-based motor activity (Lillie and Brown, 1998), suggesting it may play a role as an adaptor protein for Myo2p by regulating its interactions with actin (Lillie and Brown, 1998; Beningo et al., 2000).
In a structural analysis looking at the conservation of function among related kinesin families, Smy1p was identified as a divergent member of the kinesin-I subfamily, along with the mouse kinesin, KHCX (Lawrence et al., 2002), which has also interacts with type V myosin (Huang et al., 1999). KIF5, a closely related human kinesin to Smy1p, plays an essential role in axonal transport and also belongs to the kinesin-I subfamily (reviewed in Hirokawa and Noda, 2008). Mutations in KIF5A result in the motor neuronal disease, hereditary spastic paraplegia (Reid et al., 2002), suggesting it disrupts neurofilament transport in vivo (reviewed in Hirokawa and Noda, 2008). In yet another paper, Smy1p was classified based on the structure of its catalytic core (Miki et al., 2005). In this case, Smy1p was grouped with a subfamily of unique kinesins, referred to as the kinesin-11 subfamily, which contains a divergent catalytic core and are thought to play a role in signal transduction (Miki et al., 2005). Both structural analyses of Smy1p suggest a role in regulating cell signalling and vesicular transport, potentially through interactions with type V myosins, though their exact mechanism has yet to be defined (Miki et al., 2005).

1.1.4 The Role of Lipids in Regulating Cellular Function

In addition to the cytoskeleton, the lipid composition of membranes plays an important role in polarized vesicular transport. The role of phospholipids has already been established as important for regulating cell signalling and transport (Reviewed in Guo et al., 2008; Bagnat and Simons, 2002). Recently it was shown that the function of the PM component of the exocyst, Sec3p is dependent on its interactions with phosphatidylinositol 4,5-biphosphate (PIP$_2$) in the PM
(Zhang et al., 2008). Disruption of this interaction resulted in an accumulation of secretory vesicles representing defects in polarized exocytosis, specifically vesicle docking (Zhang et al., 2008). Lipids also play a role in regulating vesicular transport in mammalian cells. In neuronal cells, phosphoinositides play a key role in mediating membrane trafficking through interactions with different complexes and regulators (reviewed in Cremona and De Camilli, 2001). Both the clathrin adaptor complex, AP-2, which is involved in clathrin coat recruitment (Beck and Keen, 1991) and synaptojanin 1, a polyphosphoinositide phosphatase thought to regulate clathrin uncoating (McPherson et al., 1996; Woscholski et al., 1997) interact with phosphoinositides. Historically, phosphoinositides have been thought to be the most important lipid involved in regulating cell polarity and exocytosis. However, there is increasing evidence for a role for sterol lipids in regulating polarized cell growth both as a critical structural component of membranes, but also in the capacity of being a signalling molecule (reviewed in Bagnat and Simons, 2002; Rajemdran and Simons, 2005).

1.1.5 Sterols Play a Dynamic Role in Cellular Membranes

Within the membrane, sterols concentrate with sphingolipids to form highly ordered membrane regions known as membrane rafts (reviewed in Koradeet al., 2008). Important proteins involved in cytoskeleton dynamics and cell signalling are known to localize to these highly ordered domains in the membrane such as Rho GTPases (Bagnat et al., 2000). In addition to their structural role, there is increasing evidence that sterol lipids may play a role in cell signalling, particularly in regards to yeast polarization and growth (reviewed in Beh et al., 2009).
metazoan epithelial cells, there is a higher concentration of cholesterol in the apical membranes due to an enrichment of lipid rafts (Simon and van Meer, 1988). Depletion of sterols in these cells disrupts the establishment of the apical domain, suggesting a role for sterols in establishing and maintaining cell polarization (Mays et al., 1995). The exact role of sterols in regulating these processes has yet to be elucidated, but it seems that sterols affect cell and membrane polarization through several mechanisms.

In *S. cerevisiae*, the most abundant sterol, a cholesterol-like molecule called ergosterol, is localized predominantly to the PM, but also found as an ester in lipid storage droplets (reviewed Henneberry et al., 2005). Homeostasis of sterols in the cell is maintained by regulating the synthesis, transport, and storage of these sterols within the cell (reviewed Henneberry et al., 2005; Fei et al., 2008). Disruptions in sterol homeostasis result in defects endocytosis, vesicular transport, mating, and the structural integrity of the vacuole (reviewed Henneberry et al., 2005). In yeast, ergosterol is transported via both vesicular transport to the PM and in an alternative pathway, through a process independent of the secretory pathway termed nonvesicular transport (Kaplan et al., 1985; Henneberry et al., 2005).

Little is known about the nonvesicular trafficking of sterols between cellular membranes, however, two possible mechanisms have been proposed (Fig 1-4): (1) soluble lipid transporters; (2) sterol transfer at membrane contact sites (reviewed in Maxfield and Menon, 2006; Holthuis and Levine, 2005).
Two models of nonvesicular transport of sterols between the ER and PM exist: (1) soluble lipid transporters; (2) sterol transfer at membrane contact sites (MCS). The ratio of sterols to phospholipids is much higher in the PM than in the ER. This is due to associations between sterols and phospholipids that form highly ordered domains (membrane rafts) thereby reducing the chemical potential of the sterol ($\mu$). Chemical equilibrium drives the transfer of sterol from high $\mu$ (unordered) to low $\mu$ (ordered). Soluble lipid transfer proteins may ferry sterols through the cytoplasm as depicted, or facilitate sterol exchange between the PM and ER at a membrane contact site.

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Transport of sterols by soluble lipid transfer proteins is suggested based on in vitro studies (Liscum and Munn, 1999; Raychaudhuri et al., 2006). However, it has yet to be proven whether such a mechanism applies in vivo. Alternatively, transfer might occur directly between the ER membrane and PM. Sterols have a higher affinity for the PM, where the majority of sterols are sequestered into highly ordered membrane rafts, which is not the case in other membranes (Wattenberg and Silbert, 1983). This suggests that nonvesicular transport is promoted by the chemical drive for equilibrium of free sterols to move from the ER membrane to the PM at close membrane contact sites observed in vivo (reviewed in Holthuis and Levine, 2005; Levine, 2004). This does not eliminate the idea that soluble transfer proteins may facilitate sterol transfer at sites of membrane contact, however further studies in vivo are needed.

In budding yeast, there is contradictory evidence that ergosterol maintains a polarized localization within the PM. During mating, sterol-rich membrane rafts have been shown to polarize during Shmoo formation; the mating projection that forms between yeast cells of opposite mating type (Bagnat and Simons, 2002). Staining with filipin, a highly fluorescent polyene that specifically binds to sterols shows a concentration of sterols at the tip of the mating projection (Bagnat and Simons, 2002). In addition, many important proteins involved in mating such as Fus1p, required for cell fusion, associate with membrane rafts (Trueheart et al., 1987; Bagnat and Simons, 2002). However, some debate exists regarding these experiments. Valdez-Taubas and Pelham (2003) propose that localized exocytosis and slow diffusion of proteins combined with endocytosis allows for
the polarization of specific proteins, such as Fus1p (Valdez-Taubas and Pelham, 2003). They also showed that the use of filipin staining in live cells is not reliable (Robinson and Karnovsky, 1980) and in fixed cells, filipin stains evenly around the PM (Valdez-Taubas and Pelham, 2003). Despite these conflicting reports, still other reports seem to connect sterols or sterol homeostasis with cell polarization. Genes involved in sterol synthesis genetically interact with genes associated with cell polarization (Tiedje et al., 2007). The p-21 activated kinase (PAK), Ste20p is a downstream effector of Cdc42p and promotes the polarization of cortical actin through the activation of the MAP kinase pathway (Wu et al., 1995). Three proteins involved in sterol synthesis, Erg4p, Cbr1p, and Ncp1p, were identified as Ste20p-interacting proteins suggesting a possible role for sterols in cell polarization (Tiedje et al., 2007). It is probable that this is an indirect effect because Ste20p localizes to the PM at sites of polarized cell growth (Peter et al., 1996), whereas Erg4p, Cbr1p and Ncp1 localize to the ER (Kreibich et al., 1983, Zweytick et al., 2000). Based on this data, it is unlikely that they form a complex; although cytoplasmic pools of Ste20p do exist that could potential interact with these proteins (Peter et al., 1996). If the enrichment of sterols in membrane rafts plays an important role in cell polarization (Rajendran and Simons, 2005), it might be through membrane lipid composition that Erg4, Cbr1p and Ncp1 affect Ste20p function (Tiedje et al., 2007). Although it clear that sterols play a role in cell polarization in budding yeast, the connection between sterols synthesis and regulation of cell polarity has not been defined.
In contrast to budding yeast, in the fission yeast, *Saccharomyces pombe*, both sterols and membrane rafts have been shown to play a key role in cell division and cytokinesis (Wachler et al., 2003). At sites of polarized cell growth such as cell tips and the medial cell division site, sterol-enriched membrane domains accumulate during interphase and late anaphase (Wachler et al., 2003). The depletion of sterols in cells results in defects in cytokinesis, specifically the formation of the contractile ring. This further supports a role for sterols in cell division (Wachler et al., 2003). In mutations of *CDC15*, which encodes an essential protein required for multiple processes in cytokinesis, irregular filipin staining is observed where sterol domains in the PM form spirals down the length of the cell, suggesting a link between sterols and cell division (Takeda et al., 2004). A similar accumulation of sterols/lipid rafts during cell division in budding yeast has not yet been reported, but it is possible that a similar event is conserved in both species.

1.2 ORPs: Sterol Transfer Proteins or Lipid-Dependent Regulators of Intracellular Signalling?

A family of conserved sterol-binding proteins known as the Oxysterol Binding Protein (OSBP)-related proteins (ORPs) have been implicated in the regulation of sterol lipid homeostasis and cell polarization (reviewed in Beh et al., 2009; Fairn and McMaster, 2008; Raychaudhuri et al., 2006). The canonical OSBP was first isolated from mammalian cells (Levanon et al., 1990) and was discovered based on its high affinity for oxysterols, oxygenated derivatives of
cholesterol formed by oxidative degradation of sterols (Levanon et al., 1990; Kandutsch et al., 1984). Oxysterols act as potent feedback inhibitors of the mevalonate biosynthesis pathway in which cholesterol is synthesized, thereby regulating the synthesis of cholesterol (reviewed in Schroepfer, 2000). The ORPs constitute a highly conserved, multi-gene family that encode proteins that bind or are presumed to bind lipids and are found in almost all eukaryotic organisms (reviewed in Beh et al., 2009). In humans, there are twelve ORPs, which encode nineteen proteins that have distinct tissue-specific expression (Lehto and Olkkonen, 2003). ORPs have been implicated in the transfer of sterol lipids between intracellular membranes and cell signalling pathways (reviewed in Beh et al., 2009; Lehto and Olkkonen, 2003). However, the exact mechanism of their function has yet to be determined.

Studies to define the mechanistic role of ORPs are seemingly contradictory. In both in vivo and in vitro studies, the yeast ORP, Osh4p, has been shown to affect sterol transfer between the ER and PM (Beh and Rine, 2004; Baumann et al., 2005; Raychaudhuri et al., 2006). In Osh protein depleted cells, a decrease in sterols at the PM along with an accumulation of sterols internally is observed (Beh and Rine, 2004). In vivo assays looking at both the de novo (ER->PM) transport and uptake (PM->ER) of sterols, show that cells lacking functional Osh proteins show a significant decrease in the transfer of sterols (Beh et al., 2009; Raychaudhuri et al., 2006). In vitro assays confirm that Osh4p facilitates the transfer of sterols between liposomes (Raychaudhuri et al., 2006). Based on these results, ORPs are suggested to be lipid/sterol transfer proteins.
involved in nonvesicular transport (Raychaudhuri et al., 2006). The conclusion that ORPs are soluble sterol transfer proteins is further supported by the structural analysis of Osh4p. In contrast, the mammalian OSBP functions as a cholesterol-induced regulator of ERK, a kinase involved in the key MAP kinase pathway that controls cell growth (Wang et al., 2005). In this case, cholesterol binding induces OSBP association with two protein phosphatases PP2A and PTPPBS. As a complex, OSBP and the phosphatases dephosphorylate ERK and thereby inactivate it (Wang et al., 2005). Other ORPs have also been implicated in cell signalling (reviewed in Lehto and Olkkonen, 2003). It has yet to be determined, which model of ORP function, or whether aspects of both, are correct.

1.2.1 The Sterol-Bound Crystal Structure of Osh4p Suggests it is a Sterol Transporter

The X-ray crystal structure of the yeast ORP, Osh4p, with and without its bound sterol, has recently been determined (Fig.1-5) (Im et al., 2005). The structure revealed that Osh4p contains a β-barrel-like structure made of 19 anti-parallel β-sheets, with a hydrophobic centre and a flexible N-terminal lid structure consisting of a β sheet and three α-helices (Im et al., 2005). Osh4p binds sterols in the centre of the β-barrel structure through interactions with water molecules that in turn interact with the 3-hydroxyl group of sterols. There are no observed direct interactions between the sterol and any residues of the Osh4p protein (Im et al., 2005). Upon binding by Osh4p, the sterol ligand is inaccessible from the outside suggesting a conformational change in the Osh4 protein during the
The x-ray crystal structure of the yeast ORP, Osh4p is composed of 19 anti-parallel β-sheets in a cup-like structure and N-terminal flexible lid region. The binding of a sterol (red) occurs in the centre of the hydrophobic cup causing that causes a conformational change in the lid region to protect the sterol from the hydrophilic environment. Although the x-ray crystal structure suggests a role for Osh4p as a sterol transfer protein, it does not exclude an alternate role in regulating signalling.

Adapted with permission from authors, Im et al., Nature, 2005©.
binding and release of sterols (Im et al., 2005). The lid region of Osh4p is highly flexible and was unable to be crystallized in the absence of sterol binding suggesting that it is involved in the conformational change (Im et al., 2005). In the unbound form, the lid region remains away from the barrel structure and is thought to interact with membranes, along with the basic rim of the barrel (Im et al., 2005). The conservation of the lid region and the basic rim, but not the cholesterol–binding site suggests that not all ORPs physically bind sterols (Im et al., 2005). The crystal structure of Osh4p closely resembles that of other lipid transfer proteins, including the steroidogenic acute regulatory protein (StAR) transport (START) domain proteins MLN64 and StarD4 (Im et al., 2005). The structural data seems to support the concept of ORPs as lipid transporters in nonvesicular transport. However, it does not eliminate the possibility that they have an additional role in signalling mediated through the binding of lipids, especially because not all ORPs necessarily bind sterols lipids (Im et al., 2005).

1.2.2 ORPs Play an Essential Overlapping Function in Yeast

In S. cerevisiae, the multi-gene family encoding the Oxysterol-Binding Protein homologues (OSHs) appears to integrate sterol regulation with cell polarization (reviewed in Beh et al., 2009). The OSH gene family is comprised of seven genes (OSH1-OSH7) all of which encode proteins that either directly bind, and/or affect sterol lipid homeostasis (Jiang et al., 1994; Beh et al., 2001; Im et al., 2005). All seven Osh proteins contain a high degree of homology in their C-terminal domain referred to as the OSBP-related domain (ORD) (Fig.1-6) (Lehto et al., 2005); this domain can be further characterized functionally into two
The canonical mammalian OSBP and seven OSBP homologue (Osh) protein share homology in their C-terminal OSBP related domain (ORD). The Osh protein family can be subdivided into the large Osh proteins (Osh1p-Osh3p), which contain N-terminal extensions and the small Osh proteins (Osh4p-Osh7p). The large Osh proteins contain N-terminal extensions with specific protein motifs such as GOLD domains, ankyrin repeats and FFAT motifs that could mediate protein binding as well as pleckstrin homology (PH) domains that potentially bind phosphoinositides.
distinct regions, known as the OSBP1 and OSBP2 domains (CT. Beh, unpublished). The Osh protein family can be divided into two groups based on molecular weight; the large Osh proteins, Osh1p-Osh3p, and the small Osh proteins, which is comprised of Osh4p- Osh7p (Beh et al., 2001). The large Osh proteins contain N-terminal extensions that include distinct protein domains such as ankyrin repeats and GOLD (Golgi dynamics) domains, which mediate protein-protein interactions and a pleckstrin homology (PH) domain, which interacts with phosphoinositides (reviewed in Lehto and Olkkonen 2003; Fairn and McMasters 2008). Several Osh proteins also contain an FFAT motif that is thought to interact with vesicles through a vesicle-associated membrane protein, VAP (VAMP-associated protein) (Loewen et al., 2003). VAP recruits regulators of phospholipids to the membrane of the ER further implicating ORPs in lipid regulation (Loewen et al., 2003).

Although the deletion of individual OSH genes has little effect on cell growth, the deletion of the entire OSH gene family is lethal (Beh et al., 2001). The presence of any single functional OSH gene, in the absence of the other six, is sufficient to rescue lethality, suggesting that each individual OSH gene can provide the essential overlapping function of the entire OSH gene family (Beh et al., 2001). The depletion of all Osh proteins in S. cerevisiae yields defects in sterol/lipid regulation resulting in vacuole fragmentation, and an accumulation and enlargement of lipid droplets (Fig 1-7) (Beh and Rine, 2004). Osh protein-depleted cells arrest as abnormally large cells, suggesting defects in cell polarity and vesicular transport, which is supported by the observed accumulation of
Depletion of Osh Protein Results in Defects in Lipid Homeostasis and Cell Polarization

Electron micrographs of Osh protein-depleted cells showing: (A) Wild-type, SEY6210 (B) oshΔ P^MET3-OSH2. Inactivation of the last functional Osh gene by growth on methionine depletes cells of Osh protein. Cells exhibit an accumulation of lipid droplets (LD) and secretory vesicles in the daughter bud, uneven deposits on the cell wall (*), a wide bud-neck and fragmentation of the vacuole (V). Cells lacking Osh protein function are abnormally large. This suggests that the Osh protein family functions in the regulation of sterol homeostasis and cell polarization.
secretory vesicles in the daughter cell, irregular cell wall deposits, and a lack of a
defined mother/daughter cell bud-neck (Beh and Rine., 2004). These results
indicate that OSH genes share overlapping essential function(s), which likely
involves the maintenance of sterol homeostasis including synthesis, transport,
regulation and storage and its wider effect on other important cellular processes
such as cell polarization.

In addition to its sterol binding, Osh4p may regulate vesicular transport
through its ability to regulate phosphoinositides in concert with the essential
phosphatidylcholine/ phosphatidylinositol (PC/PI) transfer protein, SEC14.
Sec14p acts as a lipid sensor to regulate lipid metabolism in the cell (Aitken et
al., 1990). Mutations in SEC14 cause defects in vesicular transport from the
Golgi apparatus suggesting that SEC14 regulates vesicular biogenesis from the
Golgi (Bankaitis et al., 1989). The deletion of OSH4 suppresses mutations that
inactivate SEC14, suggesting OSH4 also plays a role in vesicular biogenesis
(Fang et al., 1996). The ability to rescue sec14<sup>ts</sup> seems to be specific to OSH4,
because the deletion of the other OSH genes does not bypass the requirement
for SEC14 (Fang et al., 1996; Beh et al 2001). This suggests that SEC14-
dependent biogenesis of Golgi vesicles is not an essential overlapping function of
the OSH gene family (Beh et al., 2001).
1.3  *OSH* Genes Affect Yeast Cell Polarization

1.3.1  *OSH* Genes Affect Rho GTPase-Dependent Regulation of Polarized Cell Growth

In wild-type cells, Cdc42p localizes and concentrates to sites of polarized cell growth, in order to promote vesicular trafficking to these sites (Adams et al., 1990). The *OSH* genes have been implicated in helping to maintain the Cdc42p positive feedback loop, and appear to affect polarized cell growth as mediated by Rho1p GTPase (Kozminski *et al.*, 2006). In Osh protein-depleted cells, Cdc42p is able to localize correctly, but cannot maintain its polarization. This defect was reversed by *OSH4* over-expression at non-permissive temperature (Kozminski *et al.*, 2006). The over-expression of other *OSH* genes also rescues specific *CDC42* polarization mutants and exacerbates the growth defects of *RHO1* mutants (Kozminski *et al.*, 2006). Osh proteins physically interact with the Rho GTPases Cdc42p and Rho1p, responsible for regulating cell polarity and polarized exocytosis (G. Alfaro and C.T. Beh, unpublished data). These findings suggest that Osh proteins affect cell polarization by integrating the regulation of sterols with Rho GTPase signalling.

1.3.2  Osh Proteins Affect Polarized Exocytosis through Interactions with the Exocyst Complex

In Osh protein-depleted cells, secretory vesicles accumulate in the daughter-cell buds, and cells are generally larger suggesting defects in polarized secretion (Beh *et al.*, 2001; Kozminski *et al.*, 2006). Because an accumulation of vesicles is observed in the daughter bud of these cells, transport into the bud must be functional, suggesting defects in later steps involving vesicle motility at
the target membrane, docking or fusion. Consistent with this model, the localization of the vesicular components of the exocyst complex are mislocalized in Osh protein-depleted cells, while the PM components, Sec3p and Exo70p, remain correctly localized to sites of polarized cell growth (Kozminski et al., 2006). In these mutant cells, the Rab GTPase Sec4p no longer accumulates to sites of polarized cell growth, suggesting a defect in the proper localization of Sec4p polarized vesicles (Kozminski et al., 2006). Secretion assays showed that in cells where OSH gene function has been inactivated, there is an accumulation of internalized β-1,3 glucanase (Bgl2p), but not invertase or other secretory proteins (Kozminski et al., 2006). Bgl2p is secreted outside the cell at sites of polarized cell growth to break down the cell wall to accommodate the growing size of the bud (Harsay and Bretscher, 1995). Defects in polarized exocytosis result in the improper internalization of Bgl2p. Because these defects are specific to the secretion of Bgl2p-containing vesicles it indicates that Osh proteins affect polarized vesicular transport (Kozminski et al., 2006). Osh proteins also physically interact in a complex with exocyst components as well as Sec4p (G. Alfaro and CT. Beh, unpublished data), further supporting a direct role for Osh proteins in the regulation of polarized vesicular transport.

### 1.4 ORPs Mediate Transport through Interactions with the Cytoskeleton

Myo2p mutants are synthetically lethal with most exocyst mutations and result in the mislocalization of the vesicular protein Sec4p (Schott et al., 1999).
Sec4p is not only required for tethering exocyst components to vesicles (Guo et al., 1999; Boyd et al., 2004), but Sec4p also binds Myo2p (Park and Bi, 2007) thereby linking vesicles with the actomyosin cytoskeleton. Based on genetic interactions between OSH genes, SEC4, and MYO2 (G. Alfaro and C.T. Beh, unpublished data), it is possible that the Osh protein family plays a role in the docking of vesicles at the PM in part by regulating vesicle/actomyosin associations. This notion is supported by the fact that several mammalian ORPs play important roles in cytoskeletal dynamics.

Recent studies in mammalian cells have shown that ORPs may be involved in regulating the cytoskeleton and vesicular transport (Lehto et al., 2008; Johansson et al., 2005). The minus-end transport of organelles and vesicles along microtubules is mediated by dynein/dynactin motor protein complex (Fig. 1-8) (Hirokawa, 1998). ORP1L interacts with the mammalian homologue of Sec4p, Rab7 and plays a role in regulating the minus end transport of the late endocytic compartment (LE) by mediating the activation of dyenin-dynactin complex (Johansson et al., 2005; Johansson et al., 2007). Rab7 physically interacts with RILP (Rab7-interacting lysosomal protein) and ORP1L, which then recruits the dynein-dynactin complex through interactions between RILP and the C-terminal arm of the dynein-dynactin complex, p150\textsuperscript{GLUED} (Johansson et al., 2007). ORP1L facilitates the transfer of the Rab7-RILP-p150\textsuperscript{GLUED} complex to βIII-spectrin, which acts a general dynein receptor found on the membrane of LE and other organelles (Muresan et al., 2001). siRNA against ORP1L prevented dynein dependent transport of the LE, similar to the siRNA of βIII-spectrin suggesting
In mammalian cells, a homologue of the Rab GTPase Sec4p, Rab7 localizes to the membranes of the late endocytic compartment (LEs) and recruits the effectors RILP and ORP1L. The N-terminal half of RILP binds to the C-terminal domain of p150Glued, a component of the dynein/dynactin motor complex. ORP1L is required to direct the entire complex to the general dynein/dynactin receptor, βIII spectrin, on the membrane of LEs. Dynein motor–driven minus-end transport of LEs can only occur after the specific late endocytic dynein receptor Rab7–RILP has interacted with the general dynein membrane receptor βIII spectrin, a process that is facilitated by ORP1L.
Figure 1-8

that the translocation of the Rab7-RILP-p150Glued complex to βIII-spectrin by ORP1L is required for activation of dynein-dynactin motor activity (Johansson et al., 2007).

Through interactions with the small GTPase R-Ras, the human ORP3 regulates the actin cytoskeleton and affects cell adhesion and formation of membrane protrusions (Ada-Neguma et al., 2006; Lehto et al., 2008). R-Ras promotes the activation of integrins, which are cell surface receptors that function in cell adhesion, migration and growth by linking the extracellular matrix (EMC) with the actin cytoskeleton (Zhang et al., 1996; Liu et al., 2000). Activated R-Ras results in highly adherent cells (Zhang et al., 1996) and promotes cell migration (Keely et al., 1999) through PI3K activation of Rac1 signalling, which promotes actin polymerization and membrane reshaping (Marte et al., 1996; Holly et al., 2005). Silencing of ORP3 in cell culture resulted in defective filopodial extentsions, abnormal actin organization, and affected cell-cell adhesion while the over-expression of ORP3 caused cell surface protrusions (Lehto et al., 2008). ORP3 co-localizes with R-Ras and physically interacts in a complex with a constitutively active form of R-Ras (Lehto et al., 2008). These data implicate ORP3 in the regulation of the actin cytoskeleton either upstream of R-Ras or in a parallel pathway (Lehto et al., 2008). In yeast, the transport of organelles is mediated by actin-based motor proteins, not microtubules (reviewed in Mosely and Goode, 2006). However, if ORPs in mammalian cells have defined roles in the regulation of components of cytoskeleton and motor proteins through physical interactions
with GTPases, it is possible that Osh proteins may also have a similar role in analogous transport systems in yeast.

In my thesis, I tested the hypothesis that yeast Osh proteins play a role in actomyosin function similar to mammalian ORPs. Using a structure/function approach, I also tested the model that Osh4p is a soluble sterol transfer protein. Lastly, I applied a genomic method to determine genetic interactions between the seven OSH genes that define how they work together, and how they functionally differ.
2: THE ROLE OF STEROL BINDING IN ORP FUNCTION

Note regarding contributions:

The following chapter is a composition of a manuscript in preparation and data collected as part of my thesis. The respective contributions of the authors is listed below.

Manuscript in preparation:

Alfaro, G., Duamel, G., Dighe, SA., Johansen, J., Kozminski, KG., and Beh, CT. The Sterol-Binding Protein Kes1/Osh4p is a Regulator of Polarized Exocytosis.

As co-author of this paper, I generated the data for Figures 2-2, 2-3, 2-4, 2-5 and Table 2-3. Figures 2-1, 2-2, 2-3, 2-6 were created by CT. Beh as part of our manuscript in preparation, while I generated the data for and created Figures 2-4, 2-5, and Table 2-3 as part of my thesis. The manuscript in preparation was written by CT. Beh with contributions from myself, and the other authors.

2.1 Introduction

2.1.1 Are ORPs Lipid Transfer Proteins?

ORPs have been implicated in the trafficking of sterol lipids within cells because some ORPs are sterol-binding proteins (Im et al., 2005; Raychaudhuri et al., 2006). However, recent studies suggest that the ORP family of proteins play a role in cell signalling (reviewed in Beh et al., 2009; Fairn and McMaster, 2008). The exact role of sterol binding in ORP function is quite controversial and has led to two proposed models for the mechanism of ORP activities (reviewed in Maxfield and Menon, 2006; Beh et al., 2009). One model proposes that ORPs
function as lipid transfer proteins during nonvesicular sterol transport. The other model hypothesizes that ORPs regulate intracellular signalling, specifically in relation to cell polarization and exocytosis. Using a structure/function approach, we test these models of ORP function using the yeast ORP, Osh4p.

The yeast OSH gene family was originally shown to be required in vivo for the maintenance of the normal distribution of sterols within the PM, which led to the model that Osh proteins are involved in nonvesicular transport (Beh and Rine, 2004). This finding was supported by further studies in which Osh4p, was shown to facilitate sterol transfer between liposomes in vitro (Raychaudhuri et al., 2006). Osh protein-dependent transfer of sterols was studied in vivo by measuring the esterification by the sterol acyl transferases, Are1p and Are2p of exogenous cholesterol transported from the PM to the ER (reverse to the direction of transfer for de novo synthesized sterols) (Raychaudhuri et al., 2006). In Osh depleted cells, a seven-fold decrease in cholesterol esterification was observed suggesting a decrease in the transport of exogenous cholesterol from the PM to the ER (Raychaudhuri et al., 2006). These data have been called into question due to the nature of the assay (Maxfield and Menon, 2006). Osh-depleted cells show a two-fold decrease in sterol acyl transferase activity, which partially contributes to the decrease in esterification measured. In addition, Raychaudhuri et al., (2006) measured cholesterol transport whereas in yeast Osh proteins would bind ergosterol. In yet another in vivo study, the transport of newly synthesized ergosterol from the ER to the PM revealed a twenty-fold decrease in transport in cells lacking Osh protein function (Beh et al., 2009). Because the
inactivation of Osh proteins did not completely eliminate sterol transport in either study, it has been suggested that alternate transporters or mechanisms must exist. Alternatively, Osh proteins affect the transport of sterols indirectly. So does the binding of sterols necessarily mean ORPs are sterol transporters?

To address the role of sterol binding by Osh proteins, mutations in Osh4p that are unable to bind sterol lipids were obtained. We hypothesized that if the primary function of the Osh4 protein is that of a sterol transfer protein, mutations that block its ability to bind sterols would result in a non-functional protein. Contrary to what is predicted for a sterol transporter, this was not observed as shown in this Chapter.

2.2 Materials and Methods

2.2.1 Strains, Plasmids, Microbial and Genetic Techniques

Yeast strains and plasmids used in this study are listed in Table 2-1 and 2-2. pCB743 was made by sub-cloning the \( P^{MET3} \) _OSH4\(^{Y97F}\) _PvuI_ cut fragment from p426MET-OSH4\(^{Y97F}\) (\( P^{MET3}-OSH4^{Y97F} 2\mu URA3\)) into pRS316. Construct was confirmed by restriction enzyme digest and sequencing. All plasmids were transformed into yeast as described in Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, 5th Edition (Amberg et al., 2005). Growth was evaluated by ten-fold serial dilutions of equivalent cultures spotted onto selective media and grown under different conditions as described. Cells containing an inducible methionine promoter were spotted onto media containing 100 mg/ml,
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<td>Im et al., (2005)</td>
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<td>OSH4 2µ URA3</td>
<td>Kozminski et al., (2006)</td>
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<td>Beh and Rine, (2004)</td>
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<td>pRS416</td>
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<tr>
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20 mg/L or 0 mg/L of methionine in order to induce or repress gene expression.

### 2.2.2 Microscopy

10 ml cultures were grown up to log phase (0.6-1.0 OD 600 nm per ml) in selective media. Cells were collected by spinning down in a clinical centrifuge for 5 minutes at 2000 rpm and fixed in 1 ml of methanol:acetic acid (3:1 v/v). Cells were incubated for 45 minutes at room temperature, spun down in a microfuge and washed in 1 ml minimal media (MM). Cells were re-suspended in 1 ml of MM and 1 ug/ml 4’,6’-diamidino-2-phenylindole (DAPI), then incubated for 30 minutes at room temperature. Cells were washed twice with MM and re-suspended in 1 ml MM. Cells were viewed by fluorescence microscopy and DIC to examine cell morphology and nuclei staining. Cells expressing a vector control or \( P^{MET3} \) \( OSH4^{Y97F} \) were measured in Openlab and the significance of the difference of the two populations was evaluated by a student’s t-test.

### 2.3 Results

#### 2.3.1 The Osh4(Y97F)p Sterol-Binding Mutant is Activated and Dominant Lethal

If sterol binding is necessary for Osh4p function as sterol transporters, mutants that can no longer bind sterols should be recessive/loss of function since they can no longer bind their lipid cargo. To test the role of sterol binding and how it affects Osh4p function, we focused on two Osh4p sterol binding deficient mutations, Osh4(L111D)p and Osh4(Y97F)p, that prevent Osh4p association
with sterols (Fig. 2-1) (Im et al., 2005). The sterol-binding mutants are under the control of a $P^{MET3}$ promoter, which in the presence of methionine is repressed, and induced in the absence of methionine (Cherest et al., 1985). It was previously published that neither of these sterol-binding defective OSH4 mutants were able to rescue the growth defects of a temperature-sensitive $osh\Delta$ osh4-1 strain, which eliminates all OSH function (Im et al., 2005). These results, if correct, would indicate that the OSH4 alleles that interfere with sterol binding are recessive/loss-of-function mutations, as predicted by a sterol transporter model. However, we show that the OSH4$^{Y97F}$ mutation caused dominant lethality when expressed in several wild-type strain backgrounds whereas the $osh4^{L111D}$ mutant caused no growth defects.

The mutation of Osh4(L111D)p prevents the extraction of sterols because it inhibits membrane association by the introduction of a charged residue at the opening to the sterol-binding cavity (Im et al., 2005). Consistent with these findings, we observed that Osh4(L111D)p expression neither rescues the growth defect of $osh\Delta$ osh4-1$^{ts}$ cells nor reverses the $osh4\Delta$ bypass suppression of sec14-3$^{ts}$ as previously shown (Im et al, 2005) (Fig. 2-2). The expression of Osh4(L111D)p had no impact on the viability of wild-type cells, confirming that it is a loss-of-function/recessive mutation as previously shown (Im et al., 2005). The Osh4(Y97F)p mutation alters a critical Tyrosine (Tyr) within the protein cavity that coordinates a key water molecule required for interactions with the sterol hydroxyl group resulting in a complete block in hydrogen bonding with the sequestered sterol and prevents ligand binding (Im et al., 2005). Surprisingly, we
Figure 2-1  Model of Sterol-Binding Deficient Osh4p Mutants

The full-length Osh4p structure (left) and the Osh4p cut-away view (right) with bound sterol in red, hydrogen-bonded water in orange, and space-filling representations of residues corresponding to mutations in blue. The G183D mutation corresponds to the temperature-sensitive osh4-1ts allele (Beh and Rine, 2004), L111D is a missense mutation located at the entrance to the sterol-binding cavity, and the Y97F dominant-lethal mutation disrupts the water-mediated hydrogen bonding to the sterol ligand held within the protein core (Im et al., 2005).

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Figure 2-2  Osh4(Y97F)p Exhibits a Dominant Lethal Phenotype

Equivalent culture dilutions were spotted onto selective media with (+Met) or without the addition of methionine (-Met), the latter of which represses transcription of the MET3 promoter (P\textsuperscript{MET3}). Wild-type (WT; SEY6210), sec14-1\textsuperscript{ts} (CTY1-1A), and osh4Δ sec14-1\textsuperscript{ts} (NDY93) cells were transformed with multicopy plasmids containing OSH4 (pCB241), P\textsuperscript{MET3}-osh4\textsuperscript{L111D} (p426MET-OSH4L111D), P\textsuperscript{MET3}-OSH4\textsuperscript{Y97F} (p426MET-OSH4Y97F), or the vector alone control (YEplac195), and cells were incubated at 25 and 37°C. Regardless of temperature or strain, expression of the P\textsuperscript{MET3}-OSH4\textsuperscript{Y97F} construct caused lethality when cells were cultured on methionine-deficient medium. Growth defects were also apparent in all cells even in the presence of methionine, which reduces P\textsuperscript{MET3}-OSH4\textsuperscript{Y97F} expression. P\textsuperscript{MET3}-OSH4\textsuperscript{Y97F} exhibits lethality in wild-type cells with all seven functional OSH genes suggesting that it is a dominant lethal mutation. The multicopy OSH4 control did not affect the sec14-1\textsuperscript{ts} growth. However, multicopy OSH4 reversed the bypass suppression of sec14-1\textsuperscript{ts} growth defects by osh4Δ, thereby reinstating the temperature-sensitive growth defect. Without added methionine in the medium, the P\textsuperscript{MET3}-osh4\textsuperscript{L111D} expression was induced but failed to reverse osh4Δ bypass suppression of sec14-1\textsuperscript{ts}, and cells grew at 37°C. P\textsuperscript{MET3}-osh4\textsuperscript{L111D} expression had no effect when induced in wild-type cells though a modest growth defect was detected at 37°C.
Figure 2-2

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found that the induction of Osh4(Y97F)p expression caused lethality in all wild-type strains tested, regardless of genetic background or temperature tested (Fig.2-2). Growth defects were also observed in the presence of methionine, which reduces $P^{\text{MET3}}$-$OSH4^{Y97F}$ expression. The fact that the expression of Osh4(Y97F)p is lethal in the presence of all seven functional Osh proteins suggests that it is a dominant lethal mutation. This is contrary to what was previously published (Im et al., 2005) and what is expected if Osh4p is functioning as a sterol transfer protein. Thus, these results suggest that Osh4p must have another function other than sterol transport.

There are three types of dominant alleles, each with defining characteristics; a hypermorph, which is a gain-of-function mutation, an antimorph, which works against wild-type protein function and a neomorph, which has a novel function relative to the wild-type protein (Muller, 1932). The addition of wild-type protein in the presence of each of the three types of dominant lethal have different effects on growth. A hypermorph would result in an exacerbation of the dominant lethal phenotype because it would increase the amount of active protein in the cell, while in an antimorph, the addition of wild-type protein would reduce the effects of the dominant lethal phenotype because it would titrate out the function of the mutant protein. There would be no change in growth in a neomorph because the neomorph’s function is independent of wild-type protein function (Muller 1932). To determine how the Osh4(Y97F)p is functioning as a dominant lethal, I have assayed the growth of cells expressing Osh4(Y97F)p in the presence of additional wild-type protein (Fig.2-3). When transcription of the
Figure 2-3  Osh4(Y97F)p is a Hypermorphic Dominant Lethal

Wild-type cells (WT; SEY6210) co-transformed with the P\textsuperscript{MET3}\textsuperscript{-}OSH4\textsuperscript{Y97F} plasmid or its vector control (YEplac195), with multicopy OSH4 (pCB254) or its vector control (YEplac195), were streaked onto synthetic selective media lacking methionine (-Met), or containing methionine concentrations that partially reduced P\textsuperscript{MET3}\textsuperscript{-}OSH4\textsuperscript{Y97F} expression (+Met 20 mg/liter) or conferred a greater repression (+Met 100 mg/liter). The relative positions for each strain are the same on all three plates, as indicated. The growth of two independent P\textsuperscript{MET3}\textsuperscript{-}OSH4\textsuperscript{Y97F}/multicopy OSH4 transformants are shown in comparison with two independent transformants containing the P\textsuperscript{MET3}\textsuperscript{-}OSH4\textsuperscript{Y97F} construct; P\textsuperscript{MET3}\textsuperscript{-}OSH4\textsuperscript{Y97F}/multicopy OSH4 transformants could only be cultured on medium supplemented with higher methionine concentrations suggesting that OSH4\textsuperscript{Y97F} is a gain-of-function mutation.
Figure 2-3

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The **P<sup>MET3</sup>-OSH4<sup>Y97F</sup>** construct was repressed in wild type by addition of methionine (20 µg/ml), a moderate growth defect was still evident indicating that even reduced basal-level expression of Osh4(Y97F)p is detrimental to cells, but not lethal (Fig. 2-3). However, when reduced Osh4(Y97F)p expression was combined with an increased dosage of wild-type **OSH4** on a multicopy plasmid, little or no growth was detected (Fig. 2-3). Even under conditions where **P<sup>MET3</sup>-OSH4<sup>Y97F</sup>** expression was more completely blocked (100 mg/L methionine), the additional expression of **OSH4** on a multicopy plasmid caused a greater growth defect when compared to the expression of Osh4(Y97F)p alone. These findings indicated that the **OSH4<sup>Y97F</sup>** mutation conferred a hypermorphic, gain-of-function mutation as opposed to recessive/loss of function mutation as previously predicted. To determine whether Osh4(Y97F)p requires wild-type protein in order to function as a dominant lethal, Osh4(Y97F)p was expressed in cells lacking wild-type **OSH4**. Induction of Osh4(Y97F)p was still lethal in an osh4∆ strain consistent with the idea that Osh4(Y97F)p is a hypermorphic allele and not does function through wild-type Osh4p (Fig 2-4). These findings established that despite its inability to bind sterols, the Osh4(Y97F)p mutation does not disrupt Osh4p activity but rather increases it suggesting that Osh4p has a sterol-binding independent function, contrary to what is predicted for a sterol-transfer protein.

### 2.3.2 Role of Osh4(Y97F)p in Cell Polarization

To understand how the Osh4(Y97F)p mutation is affecting cell function we examined the morphology of cell expressing the dominant lethal in liquid culture. Although no defects in cell cycle were observed, cells were significantly enlarged
Figure 2-4  *OSH4*<sup>Y97F</sup> Dominant Lethal Phenotype is Not Dependent on Presence of Functional *OSH4*.

Ten-fold serial culture dilutions (left to right) were spotted onto solid medium to compare growth of wild-type (WT; SEY6210) and *osh4Δ* (HAB821) cells transformed with multicopy plasmids containing *OSH4* (pCB241), *P<sup>MET3</sup>osh4<sup>L111D</sup>* (p426MET-OSH4L111D), and *P<sup>MET3</sup>OSH4<sup>Y97F</sup>* (p426MET-OSH4Y97F) or the vector alone control (YEplac195). Transformed strains were incubated at 30°C on selective synthetic medium containing 100 mg/L methionine or no methionine. Deletion of *OSH4* did not have any effect on the *P<sup>MET3</sup>OSH4<sup>Y97F</sup>* dominant lethal phenotype suggesting that *P<sup>MET3</sup>OSH4<sup>Y97F</sup>* does not require functional *OSH4* to exhibit its dominant lethal phenotype further supporting the idea that *P<sup>MET3</sup>osh4<sup>L111D</sup>* *OSH4*<sup>Y97F</sup> is a hypermorphic allele.
similar to Osh depleted cells suggesting possible defects in polarity (Fig.2-5). 
$P^{MET3}$-$OSH4^{Y97F}$ expression caused an accumulation of enlarged cells that were on average 1.6-fold larger than wild type ($p$-value < 0.0001, N=61). This suggests that the gain of function of Osh4(Y97F)p may be related to the regulation of cell polarity. To determine how sterol binding by Osh proteins affects cell polarization, we surveyed specific mutants that affect cell growth to determine if any might rescue or suppress the dominant lethal phenotype.

Investigation by ten-fold serial dilutions of $P^{MET3}$-$OSH4^{Y97F}$ expressed in different mutations known to interact with $OSH$ genes showed that the $P^{MET3}$-$OSH4^{Y97F}$ construct was lethal in $sec14-3^ts$ and $osh4\Delta sec14-3^ts$ cells, $RHO1$ and $CDC42$ temperature-conditional mutants ($rho1l104^ts$ and $cdc42l101^ts$, -118$^ls$, -129$^ls$), and exocyst mutants ($sec3^ls$, $sec4^ls$, $sec5^ls$, $sec6^ls$, $sec8^ls$, and $sec15^ls$). The effects of the dominant lethal Osh4 protein were also tested in ergosterol biosynthetic mutants ($erg3\Delta$ and $erg6\Delta$) and shown to be dominant lethal suggesting the absence of ergosterol does not affect the function of Osh4(Y97F)p.

The expression of $P^{MET3}$-$OSH4^{Y97F}$ construct was originally on a high-copy plasmid ($2\mu$). To determine whether a reduction in gene dosage would affect $P^{MET3}$-$OSH4^{Y97F}$ lethality, the growth of wild-type cells expressing $P^{MET3}$-$OSH4^{Y97F}$ on a low-copy plasmid was investigated. $OSH4^{Y97F}$ was sub-cloned into a low-copy ($CEN$) vector and transformed into wild type. Comparison of growth between $OSH4^{Y97F}$ expressed on high- and low-copy plasmids were assessed by ten-fold serial dilutions of equivalent cell densities on solid medium. This analysis
Figure 2-5  Osh4(Y97F)p Cells Exhibit Enlarged Phenotype Morphology

Cells were visualized using Differential Interference Contrast (DIC) microscopy in wild-type cells expressing (A) a vector alone control (YEplac195) or (B) (C) \( P^{MET3-OSH4^{Y97F}} \) (p426MET-OSH4Y97F). Cell cultures were grown up to log-phase at 30 °C in selective synthetic media with 100 mg/L methionine, spun down and washed twice with synthetic media lacking methionine and resuspended in media lacking methionine. Cells were grown for five hours in the absence of methionine at 30 °C. Cells expressing a vector control or \( P^{MET3-OSH4^{Y97F}} \) were measured in Openlab and the significance of the difference of the two populations was evaluated by a student’s t-test. A population of cells expressing Osh4(Y97F)p were enlarged (32%, N= 237) compared to wild-type suggesting possible defects in polarization (p-value< 0.0001).
Figure 2-5

A

B

C
revealed that induction of $P^{MET3}\cdot OSH4^{Y97F}$ still resulted in lethality regardless of plasmid copy number.

2.4 Discussion

2.4.1 Osh4p Function is Dependent on Membrane Association, Not Sterol Binding

The structure/function analysis of Osh4p indicated that its function was dependent on membrane association, but not sterol binding per se. This conclusion was based on comparing the effects of $osh4^{L111D}$ and $OSH4^{Y97F}$ mutations on cell growth. The Osh4(Y97F) mutant protein is unable to bind sterols due to a specific mutation in a conserved tyrosine that was shown to be hyperactivated, not inactivated as previously shown (Im et al., 2005). This property was unexpected, but such gain-of-function mutations are often associated with mutations in cell signalling genes (Beitel et al., 1990). At this point, it is unclear whether Osh4(Y97F)p is activated because of the lack of sterol binding, or if Osh4(Y97F)p is locked into a conformation that mimics an active sterol-bound state. On the other hand, Osh4(L111D)p cannot make general membrane associations required to extract sterols and it was shown to be a recessive/loss of function mutation (Im et al., 2005). Unlike the wild-type protein, Osh4(L111D)p expression had no effect on $CDC42$ and $SEC14$ mutations, suggesting that Osh4p requires membrane association to function. Consistent with this evidence is the fact that while the lid and rim region of ORPs is highly conserved, the sterol binding region is not (Im et al., 2005), suggesting that it is
the ability to interact with membranes that is conserved and necessary for ORP function.

\[ \text{P}^{\text{MET3-OSH4}^{\text{Y97F}}} \] expressing cells showed morphological defects associated with defects in cell polarity, similar to what is observed in Osh protein depleted cells. Mutations in genes known to genetically interact with \textit{OSH} genes involving polarized exocytosis, were unable to suppress the dominant lethal phenotype suggesting that the function gained in the hypermorphic allele, Osh4(Y97F)p is independent of these functions or functioning upstream.

The tyrosine in Osh4(Y97F)p is highly conserved among all Osh proteins and is located in the ORD domain (Fig 2-6). It is possible that an equivalent mutation in other Osh proteins may result in a similar hyperactivated dominant lethal phenotype suggesting that other Osh proteins have sterol independent functions. This would provide evidence that the Osh protein family and ORPs in general are not solely sterol transfer proteins, but play a role as sterol-dependent regulators of cell signalling. Recent evidence suggesting that Osh proteins physically interact \textit{in vivo} with components of the exocyst complex, Sec4p and the Rho GTPases Cdc42p and Rho1p further support a role for Osh proteins in the regulation of polarized exocytosis (G. Alfaro and CT Beh, unpublished data). The plasma membrane is enriched in membrane rafts and sterols creating a thermodynamic “sink” that effectively traps sterols making the PM the preferred location of sterol release (Baumann et al., 2005). Given that sterols are concentrated in the PM, it is reasonable that Osh4p binds a sterol at an internal membrane (\textit{i.e.} the trans-Golgi network) and then releases it into the PM after
Figure 2-6  Highly Conserved Tyrosine Found in All Seven Osh Proteins

Sequence alignments of the seven Osh proteins (Osh1p-7p) and human OSBP in the region including an adjacent conserved tyrosine (indicated by the asterisk) that corresponds to Osh4p tyrosine (Y) 97, which is mutated to phenylalanine [F] in the dominant-lethal Osh4(Y97F)p. Solid boxes indicate amino acid identity and shaded boxes denote similarity amongst the majority of aligned residues. Note that this alignment differs from Beh et al., (2001) in that with increased gap penalties the tyrosines conserved in all Osh proteins (including Osh6p and 7p) are now aligned.

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vesicular transport into the bud. It is possible that while sterol binding is not required for aspects of Osh4p function, it is necessary for regulation of Osh4 protein function and the release of sterols bound by Osh proteins might potentially act as a trigger for vesicle docking.
3: OSH GENES INTERACT WITH GENES THAT AFFECT THE ACTOMYOSIN CYTOSKELETON

Note regarding contributions:

The following chapter is a composition of a manuscript in preparation and data collected as part of my thesis. The respective contributions of the authors is listed below.

Manuscript in preparation:

Alfaro, G., Duamel, G., Dighe, SA., Johansen, J., Kozminski, KG., and Beh, CT. The Sterol-Binding Protein Kes1/Osh4p is a Regulator of Polarized Exocytosis.

As a co-author of this paper, I generated the data for Figure 3-1, 3-2, 3-3, and 3-4. Figures 3-1, 3-2 and 3-5 were created by CT. Beh as part of our manuscript in preparation. I created Figures 3-3 and 3-4 as part of my thesis. Figure 3-5 is based on data generated by myself, G. Alfaro, KG. Kozminski and CT. Beh. The manuscript in preparation was written by CT. Beh with contributions from myself and the other authors.

3.1 Introduction

3.1.1 The Role of Osh Proteins in Polarized Exocytosis

A role for OSH genes in regulating cell polarization and vesicular transport has been previously established (Kozminski et al., 2006). As an important mechanism that targets polarized exocytosis, we tested whether there is a functional connection between the actomyosin cytoskeleton and the OSH gene family. During the late steps of polarized exocytosis, secretory vesicles
transported along actin cables must be released from the actomyosin cytoskeleton as they dock with the PM at sites of cell polarization (reviewed in Hsu et al., 2004; Mosely and Goode, 2006). Little is known about the transition during which Myo2p-dependent transport leads to vesicle docking with the PM. In neuronal cells, type V myosin motors function with kinesins to facilitate the transition between long-range/short-range transport of vesicles (Langford, 2002). Kinesin motor proteins mediate long-range transport of vesicles along microtubules in axons then, at axon termini, vesicle transport transitions to myosin-based short-range transport along actin filaments (Langford, 2002). In yeast, polarized vesicular transport to the PM is mediated by the type V myosin, Myo2p along actin filaments, instead of along microtubules. None the less, a unique kinesin related protein, Smy1p, binds to Myo2p (Beningo et al., 2000) and has been shown to play a yet unknown role in polarized exocytosis. Although the N-terminus of Smy1p is homologous with other kinesins (Lillie and Brown, 1992), Smy1p does not bind microtubules and its effect on Myo2p does not involve Smy1p kinesin motor activity (Lillie and Brown, 1998). The deletion of SMY1 has no effect on cell growth or polarized exocytosis (Lillie and Brown, 1998). However, the deletion of SMY1 is synthetically lethal with mutations in MYO2, the Rab GTPase, SEC4 and its GEF, SEC2, but not in mutations corresponding to members of the exocyst complex (Lillie and Brown, 1992; Lillie and Brown 1998). These results suggest that SMY1 operates in a parallel pathway that augments MYO2 and SEC4 function. In addition to its role in tethering exocyst subunits to post-Golgi vesicles, Sec4p is also necessary for vesicle attachment to the
actomyosin cytoskeleton via interactions with Myo2p (Park and Bi, 2007). It is possible that Smy1p works to stabilize interactions between type V myosin motors and secretory vesicles through interactions with the Sec4p (Schott et al., 1999). Alternatively, Smy1p may work to regulate the transition between actomyosin-dependent transport and vesicle docking/release.

In cells lacking functional Osh proteins, undocked post-Golgi vesicles accumulate within budding daughter cells resulting in the internal accumulation of protein cargo normally exported via polarized exocytosis (Kozminski et al., 2006). The movement of post-Golgi vesicles into the bud is not dependent on OSH genes suggesting that OSH genes play a role in docking and fusion events (Kozminski et al., 2006). In Osh protein depleted cells, the mobility of Sec4p containing vesicles at the PM in the daughter cell is defective suggesting possible defects in the last actomyosin-dependent events in vesicle transport prior to docking (G. Alfaro and CT. Beh, unpublished). A decrease in vesicle motility at the PM of the daughter cells is also observed (G. Alfaro and CT Beh, unpublished data). To test whether OSH genes play a role in regulating the actomyosin-dependent transport of polarized vesicles, I investigated genetic interactions between OSH4, actomyosin-related genes, and regulatory genes involved in vesicle docking.
3.2 Materials and Methods

3.2.1 Strains, Plasmids, Microbial and Genetic Techniques

Yeast strains and plasmids used in this study are listed in Table 3-1 and 3-2. All plasmids were transformed into yeast as described in Methods in Yeast Genetics: A Cold Spring Harbour Laboratory Course Manual, 5th Edition (Amberg et al., 2005). Growth was evaluated by ten-fold serial dilutions of equivalent cultures spotted onto selective media and grown at different temperatures. Cells containing an inducible galactose promoter were spotted onto media containing 20% glucose (control) or 40% galactose in order to induce expression.

3.2.2 Microscopy

10 ml cultures were grown up to log phase (0.6-1.0 OD 600 nm per ml) in selective media. Cells were collected by centrifugation in a clinical centrifuge for 5 minutes at 2000 rpm and fixed in 1 ml of methanol:acetic acid (3:1 v/v). Cells were incubated for 45 minutes at room temperature, spun down in a microfuge and washed in 1 ml minimal media (MM). Cells were re-suspended in 1 ml of MM and 1 ug/ml 4′,6′-diamidino-2-phenylindole (DAPI), then incubated for 30 minutes at room temperature. Cells were washed twice with MM and re-suspended in 1 ml MM. Cells were viewed by fluorescence microscopy and DIC to examine cell morphology and nuclei staining.
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3.3 Results

3.3.1 Interactions Between Osh4p and the Kinesin-related Protein Smy1p

Over-expression of OSH genes in MYO2 mutations that are specifically defective in polarized exocytosis exacerbates the growth defect, suggesting OSH genes may also play a role in regulating Myo2p-dependent transport (G. Alfaro and CT. Beh, unpublished data). As a proposed regulator of Myo2p-dependent vesicle motility, we tested whether SMY1 genetically interacts with OSH4. When OSH4 was expressed on a multicopy plasmid in smy1Δ cells, a severe growth defect was evident at 37°C (Fig 3-1). In comparison, no defect in growth was observed for smy1Δ cells transformed with multicopy OSH2, OSH5, or the vector control, nor wild-type cells transformed with the same constructs or the multicopy OSH4 plasmid. The reciprocal experiment, over-expressing P$_{GAL}$-SMY1 in a temperature-sensitive oshΔosh4-1ts, did not show any significant genetic interaction at any temperature. This result suggests that SMY1 has an inhibitory effect on OSH4 gene function, where SMY1 functions either upstream or in a parallel pathway. These genetic studies demonstrate a functional connection between the Osh protein family and proteins that mediate actomyosin-dependent vesicular transport.

3.3.2 OSH4 and SMY1 are Both Dosage Suppressors of CDC42 Mutants

OSH4 was previously identified as a multicopy suppressor of temperature-sensitive CDC42 mutations (Kozminski et al., 2006). Given the genetic
Ten-fold serial culture dilutions were spotted onto solid selective synthetic medium to compare the growth of yeast at 30 and 37°C for \textit{smy1Δ} (CBY4138) and its congenic wild-type strain (BY4741). Strains were transformed with a multicopy plasmid containing \textit{OSH2} (pCB239), \textit{OSH4} (pCB241), \textit{OSH5} (pCB242), or the vector (YEplac195) control and grown at 30 and 37°C. Overexpression of \textit{OSH4}, but not other \textit{OSH} genes tested, resulted in a severe growth defect in \textit{smy1Δ} at 37°C. This suggests that \textit{SMY1} has an inhibitory effect on \textit{OSH4} function.

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interactions between \textit{OSH4} and \textit{SMY1}, we tested whether \textit{SMY1} also affects \textit{CDC42}-dependent polarized growth. Similar to \textit{OSH4}, we found that \( \text{P}^{\text{GAL}}\text{-SMY1} \) was a dosage suppressor of \textit{cdc42-118}^{ts}, a temperature-conditional mutation that is specifically defective in polarity establishment. When induced on galactose-containing medium, \( \text{P}^{\text{GAL}}\text{-SMY1} \) expression rescued the growth defects of \textit{cdc42-118}^{ts} cells incubated at restrictive temperatures (Fig.3-2). Also similar to \textit{OSH4}, \textit{SMY1} did not rescue but actually exacerbated the growth defects of \textit{cdc42-129}^{ts} (Fig. 3-2), an allele that manifests its defect later during the cell cycle after polarity has been established (Kozminski et al., 2000). These results suggested that \textit{OSH4} and \textit{SMY1} play complementary roles during \textit{CDC42}-dependent polarized growth.

Although no significant growth defects were reported in wild-type cells over-expressing \textit{SMY1} (Lillie and Brown, 1992), we found in some genetic backgrounds \( \text{P}^{\text{GAL}}\text{-SMY1} \) expression had a significant negative impact on growth (Fig. 3-3), suggesting an individual role for Smy1p alone. Over-expression by \( \text{P}^{\text{GAL}}\text{-SMY1} \) could not suppress growth defects in \textit{MYO2} mutants, as previously described (Fig. 3-3) (Lillie and Brown, 1992; Schott et al., 1999). Investigations into the morphology of \( \text{P}^{\text{GAL}}\text{-SMY1} \) expressing cells did not reveal any cell cycle defects or morphological abnormalities. Overall, these results suggest that Smy1p plays a role in the essential process of polarized exocytosis, but is not absolutely required.
Figure 3-2  Similar to OSH4, SMY1 is a Dosage Suppressor of CDC42

Ten-fold serial dilutions of wild-type (DDY1300), cdc42-101<sup>ts</sup> (DDY1304), cdc42-118<sup>ts</sup> (DDY1326), and cdc42-129<sup>ts</sup> (DDY1344) cells, transformed with either a vector control (YEplac195) or a P<sup>GAL</sup>-SMY1 plasmid (pCB799), spotted on solid selective media containing either 4% galactose (+Gal) or 2% glucose (+Glc). Cultures were incubated at 25, 34, or 37˚C, corresponding to the permissive, semi-permissive, and restrictive temperatures for the growth of the cdc42<sup>ts</sup> strains. Over-expression of P<sup>GAL</sup>-SMY1 was able to suppress the growth defects of cdc42-101<sup>ts</sup> at all temperatures, which is defective in polarity assembly, while exacerbating the mutant phenotype of cdc42-118<sup>ts</sup>, which is defective in later stages of polarity suggesting a role for SMY1 is promoting polarity establishment.

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Figure 3-3  Over-expression of $P^{GAL}SMY1$ Causes Growth Defects

Ten-fold serial culture dilutions were spotted onto solid medium to compare growth of wild-type (WT; ABY531), myo2-66 (CBY2939), myo2-14 (ABY534) and myo2-16 (ABY536) cells transformed with multi-copy plasmids containing $P^{GAL}SMY1$ (pCB791) or the vector alone control (YEplac195). Transformed strains were incubated at 25°C (permissive temperature) and 37°C (non-permissive temperature) on selective synthetic medium containing 2% glucose or 4% galactose. Although no significant growth defects were reported in wild-type cells over-expressing $SMY1$ (Lillie and Brown, 1992; Lillie and Brown, 1994), we found in some genetic backgrounds $P^{GAL}-SMY1$ expression had an appreciable impact on growth. On galactose medium, expression of $P^{GAL}-SMY1$ resulted in severe growth defects at both 25°C and 37°C in the strains tested, including wild-type.
Figure 3-3

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25°C

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37°C

+ Glc + Gal
3.3.3 **SMY1 Interactions with the Exocyst Complex**

Previous studies showed that deletions in *SMY1* did not have any effect on growth in exocysts mutants other than *SEC4* and *SEC2*, however the over-expression of *SMY1* has not been investigated in these mutants (Lillie and Brown, 1994; Lillie and Brown, 1998). Exocyst mutants have previously been shown to be sensitive to *OSH* gene over-expression (Kozminski et al., 2006; G. Alfaro and CT., unpublished data). To determine whether *SMY1* also interacts with components of the exocyst we tested P\(^{\text{GAL}}\)-*SMY1* expression in exocyst mutants. Ten-fold serial dilutions revealed that P\(^{\text{GAL}}\)-*SMY1* expression in *sec6\(^{ts}\)* mutants exacerbated the growth defect, but not *sec3\(^{ts}\), sec4\(^{ts}\), or sec8\(^{ts}\)* (Fig. 3-4). This suggests that *SMY1* does show genetic interactions with components with the exocyst on vesicles, but not the entire complex. Further investigations into possible *SMY1* genetic interactions with other members of the exocyst complex would also be informative.

3.3.4 **SMY1 Does Not Genetically Interact with a Gene Involved in Vesicle Biogenesis**

*OSH4* represents a unique *OSH* gene in that deletions of *OSH4* bypass mutations in the PI/PC transferase *SEC14* are necessary for vesicle biogenesis from the Golgi, but not other members of the *OSH* gene family (Fairn et al., 2007; Beh et al., 2001). To determine whether *SMY1* plays a role in *OSH4* interactions with *SEC14* dependent vesicle biogenesis or *SEC14* function directly, genetic analysis between *smy1\(\Delta\), osh4\(\Delta\), and sec14-3\(^{ts}\)* was performed. We found that
Figure 3-4  *SMY1* Interacts with the Exocyst Component *SEC6*

Ten-fold serial culture dilutions were spotted onto solid medium to compare growth of wild-type (WT;RSY255), *sec4-2*<sup>ts</sup>(CBY1480), *sec6*<sup>ts</sup>(CBY3093) and *sec8-6*<sup>ts</sup>(CBY3095) cells transformed with multi-copy plasmids containing *p<sup>GAL</sup>SMY1* (pCB791) or the vector alone control (YEplac195). Transformed strains were incubated at 25°C (permissive temperature), and 37°C (non-permissive temperature) on selective synthetic medium containing 2 % glucose or 4% galactose. Over-expression of *p<sup>GAL</sup>SMY1* in *sec6*<sup>ts</sup> resulted in a growth defect suggesting that *SMY1* and *SEC6* genetically interact thereby potentially linking *SMY1* function with the vesicle docking.
Figure 3-4
deletions of SMY1 in sec14-3ts did not bypass the growth defects nor did deletion of SMY1 in a sec14-3 osh4Δ reverse the bypass suppression of osh4Δ. The deletion of SMY1 was not synthetically sick/lethal with a deletion of OSH4 suggesting that they are not redundant. Over-expression of SMY1 in osh4Δ, sec14-3ts or sec14-3 osh4Δ did not have a significant effect on growth, although over-expression of P^GAL-SMY1 resulted in growth defects in the strain background. Altogether these results suggest that SMY1 is not genetically involved in OSH4 function in post-Golgi vesicle biogenesis through SEC14, but rather OSH4 function at the PM.

3.4 Discussion

3.4.1 Osh4p Interacts with Smy1p, a Component of the Actomyosin Cytoskeleton

Here, we provide evidence that Osh4p plays a role in actomyosin-dependent vesicle transport through genetic interactions with SMY1. SMY1 is not an essential gene and deletions in SMY1 do not show any defects in cell polarization or vesicular transport (Lillie and Brown, 1992; Lillie and Brown, 1998). However, we have shown novel genetic interactions suggesting that SMY1 and OSH4 are both cdc42ts dosage suppressors, sharing the same allele specificities for CDC42 polarity establishment mutations. Deletions of SMY1 were only sensitive to increased dosage of OSH4 but not other OSH genes, therefore SMY1 appears to be specific in its inhibitory effect on OSH4. Despite these
genetic interactions, SMY1 and OSH4 had clear differences with respect to their effects on other polarity genes. As described previously, multicopy OSH4 and SMY1 had opposite effects on MYO2 mutants as well (G. Alfaro and CT. Beh, unpublished data). Unlike multicopy OSH4, SMY1 over-expression did not affect the growth of sec14-3ts or sec14-3 osh4Δ cells, nor did deletions in SMY1 bypass sec14-3ts mutations suggesting that SMY1 function does not impact SEC14-dependent vesicle biogenesis. Similar to OSH4, SMY1 also exacerbated growth defects in mutations of SEC6. However, no growth defects were observed in other members of the exocyst suggesting SMY1 genetic interactions with the exocyst are specific to SEC6. It is also possible that SMY1 does interact with other components of the exocyst, however, not all the mutants that were tested showed growth on galactose. Taken together, these results suggest that SMY1 and OSH4 pathways intersect, but do not completely overlap.

Although SMY1 was first identified as a suppressor of MYO2 mutations, we showed that the over-expression of SMY1 by P^{GAL} did not rescue specific mutations in MYO2. (Lillie and Brown, 1994; Schott et al., 1999). The original suppression was observed by multicopy SMY1 expression under its own promoter suggesting that cells are potentially sensitive to the amount of Smy1p (Lillie and Brown, 1992). In fact, the over-expression of P^{GAL}-SMY1, resulted in a growth defect when expressed in the wild-type strain background and certain mutant strains. This represents a novel phenotype for SMY1, previously unobserved, which may lead to a better understanding of SMY1 function in polarized exocytosis.
To determine whether this genetic interaction also represents a physical interaction between Osh4p and Smy1p, Tandem Affinity Purification (TAP) was performed to attempt to purify Osh proteins with TAP-tagged proteins associated with the cytoskeleton. However, the results could not confirm whether or not Osh4p and Smy1p form a complex, \textit{in vivo}. Further studies must be conducted to determine physical interactions between Osh4p, Smy1p and Myo2p.

It is unclear how Smy1p functions to promote \textit{CDC42} function and how it relates to Smy1p function in polarized exocytosis. Other related kinesins, that also bind type V Myosins, are required to facilitate long/short range transport (Huang et al., 1999). However, this is not necessary in small yeast cells, where all vesicular transport is actin based, so Smy1p must have evolved a different function. Perhaps Smy1p functions in cell signalling by regulating polarized vesicular transport by integrating the regulation of Cdc42p, Myo2p and Sec4p through interactions with Osh4p. Alternatively, Smy1p may support the interaction between Sec4p-containing vesicles and Myo2p thereby increasing its processivity during transport and release at the PM.

Combining all the genetic data connecting \textit{OSH4}, \textit{SMY1}, \textit{CDC42}, and \textit{MYO2}, we propose the interaction model summarized in Figure 3-5. Although both \textit{SMY1} and \textit{OSH4} acted as dosage suppressors of \textit{CDC42} mutations, it is likely they act through independent pathways. In this regard, Osh4p and the Osh proteins are upstream regulators of Cdc42p localization (Kozminski et al., 2006), but it is presently unclear how Smy1p affects Cdc42p-dependent polarization. The Osh proteins function to maintain mutant Cdc42p polarization at sites of cell
Both OSH4 and SMY1 are dosage suppressors of cdc42-118ts, suggesting that they are positive-acting regulators of CDC42-dependent cell polarization. OSH4 is an upstream regulator of Cdc42p polarized localization (Kozminski et al., 2006); as indicated by the dashed line, SMY1 might promote Cdc42p polarization either as an upstream regulator or as an effector. SMY1 antagonizes OSH4 function because smy1Δ cells were sensitive to OSH4 over-expression. No reciprocal effect of OSH4 expression on SMY1 function was detected. As indicated, SMY1 was originally identified as a positive-acting multicopy suppressor of MYO2 mutations (Lillie and Brown, 1992; Schott et al., 1999). The same MYO2 mutations were sensitive to increased OSH4 dosage. We infer from the latter result that OSH4 has a negative impact on MYO2 function, as indicated by the dashed line, though this is not the only possibility.

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growth by promoting the Cdc42p positive feedback loop, which is actomyosin
dependent (Adamo et al., 2001, Kozminski et al., 2006). Smy1p may potentially
function in a similar pathway to promote Cdc42p transport and polarization by
supporting actomyosin dependent polarized exocytosis.
4: ANALYSIS OF OSH GENE FAMILY FUNCTIONAL REDUNDANCY

4.1 Introduction

4.1.1 Individual Members of Multi-gene OSH Family Have Unique Functions

The OSH gene family in budding yeast is a highly conserved, multi-gene family comprised of seven homologues, which share one or more essential overlapping function(s) (Beh et al., 2001). Although the deletion of individual OSH genes has little effect on cell growth, the inactivation of all seven OSH genes is lethal (Beh et al., 2001). The identification of overlapping and individual functions in a multi-gene family such as the OSH gene family is extremely difficult due to the redundant function of the seven individual members. To determine if all seven OSH genes have exactly the same function(s), or whether the OSH genes have overlapping and unique functions, multiple gene expression profiles were used to compare the transcriptional consequence of deleting each OSH gene (Beh et al., 2001). This analysis showed that although the seven OSH genes share one or more overlapping essential function(s), they each have a unique expression profile, indicating that each OSH gene has distinct cellular functions (Beh et al., 2001). Although each OSH gene has both unique and overlapping functions, it is still unclear which of the OSH genes act together and what pathways they affect. To address this, a novel genome-wide analysis of
OSH genes was performed to identify novel interactions with genes that define specific functional pathways and then to group the OSH genes into subsets based on shared interactions with the same genes and pathways.

4.1.2 A Novel Approach to the Analysis of a Redundant Gene Family

Synthetic genetic arrays (SGA) analysis allows for high-throughput genetic analysis of a query mutation and a collection of strains representing individual deletions of all non-essential genes in the yeast genome (Tong and Boone, 2006). Identification of such genetic interactions provides valuable information about the functional pathways associated with the query gene. Traditional SGA analysis involves crossing a deletion of a query gene with an array of non-essential gene deletions in order to identify synthetic sick/lethal genetic interactions between the two gene deletions. In order to use SGA to analyze genetic interactions between OSH gene deletions, octuple mutation combinations would need to be created between all the deletions of individual OSH genes, rescued by a conditional OSH gene and the array of non-essential gene deletions known as the nonessential gene deletion collection. However, SGA analysis can also be used to identify genetic interactions using the over-expression phenotype of an individual query gene. In order to identify individual and clusters of overlapping and individual functions of OSH genes, we have developed a novel approach to address the redundant nature of the OSH family. Our approach uses the over-expression phenotype of individual OSH genes in order to identify nonessential gene deletions within the array that are affected by the over-expression of single OSH genes and sub-sets of the OSH gene family.
Many of the known genetic interactions with OSH genes are with essential genes, which are not present in the nonessential gene deletion collection. To identify novel genetic interactions between OSH genes and essential genes in the yeast genome, we will perform SGA analysis on the over-expression of individual OSH genes using a collection of conditional, temperature sensitive mutants of essential genes. Our goal is to characterize genetic interactions of individual OSH genes in order to group the seven OSH genes based on their functionality. This will allow for a better understanding and characterization of the function of individual OSH genes and the entire OSH family. Deletion and conditional mutants affected by all OSH genes, regardless of which one, represent likely candidates for genes involved in the OSH family overlapping functions. If our approach is successful it could potentially provide a novel approach for analyzing the functions of other redundant, multi-gene families.

4.2 Materials and Methods

4.2.1 Strains, Plasmids, Microbial and Genetic Techniques

Culture media and genetic techniques were as described (Adams et al., 1997). Yeast strains and plasmids used in this study are listed in Table 4-1 and 4-2. Plasmid pCB741, P^{GAL}-OSH1 URA3 2µ was constructed by PCR amplifying OSH1 from genomic DNA isolated from SEY6210 using forward primer 5’-
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>$MATa\ his3\Delta1\ leu2\Delta0\ met15\Delta0\ ura3\Delta0$</td>
<td>Winzeler et al., (1999)</td>
</tr>
<tr>
<td>BY4741</td>
<td>$MATa\ his3\Delta1\ leu2\Delta0\ met15\Delta0\ ura3\Delta0\ xxx\Delta$</td>
<td>Winzeler et al., (1999)</td>
</tr>
<tr>
<td>CBY 1629</td>
<td>BY4741 $hof1\Delta::KANMX4$</td>
<td>Winzeler et al., (1999)</td>
</tr>
<tr>
<td>DDY0006</td>
<td>$MATa\ his4-619\ ura3-52\ ACT$</td>
<td>Shortle et al., (1994)</td>
</tr>
<tr>
<td>DDY0016</td>
<td>$MATa\ his4-619\ ura3-52\ act1-1$</td>
<td>Shortle et al., (1994)</td>
</tr>
<tr>
<td>Y7092</td>
<td>$MATa\ his3\Delta1\ leu2\Delta0\ met15\Delta0\ ura3\Delta\ can1\Delta::STE2prSp_his5lyp\Delta1$</td>
<td>Tong and Boone, (2006)</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>pBM325</td>
<td><em>HOF1-GFP 2µ URA3</em></td>
<td>Blondel et al., (2005)</td>
</tr>
<tr>
<td>pCB239</td>
<td><em>OSH2 2µ URA3</em></td>
<td>Kozminski et al., (2006)</td>
</tr>
<tr>
<td>pCB241</td>
<td><em>OSH4 2µ URA3</em></td>
<td>Kozminski et al., (2006)</td>
</tr>
<tr>
<td>pCB242</td>
<td><em>OSH5 2µ URA3</em></td>
<td>Kozminski et al., (2006)</td>
</tr>
<tr>
<td>pCB247</td>
<td>$P^{GAL} OSH7 2µ URA3$</td>
<td>Kozminski et al., (2006)</td>
</tr>
<tr>
<td>pCB248</td>
<td>$P^{GAL} OSH6 2µ URA3$</td>
<td>Kozminski et al., (2006)</td>
</tr>
<tr>
<td>pCB249</td>
<td>$P^{GAL} OSH3 2µ URA3$</td>
<td>Kozminski et al., (2006)</td>
</tr>
<tr>
<td>pCB250</td>
<td>$P^{GAL} OSH2 2µ URA3$</td>
<td>Kozminski et al., (2006)</td>
</tr>
<tr>
<td>pCB251</td>
<td>$P^{GAL} OSH4 2µ URA3$</td>
<td>Kozminski et al., (2006)</td>
</tr>
<tr>
<td>pCB691</td>
<td>$P^{GAL} OSH5-HA 2µ URA3$</td>
<td>Gelperin, (2005)</td>
</tr>
<tr>
<td>pCB741</td>
<td>$P^{GAL} OSH1-HA 2µ URA3$</td>
<td></td>
</tr>
<tr>
<td>pKT10-GAL-HA²</td>
<td>$P^{GAL} HA 2µ URA3$</td>
<td>Tanaka et al., (1990)</td>
</tr>
<tr>
<td>YEplac195</td>
<td>2µ URA3</td>
<td>Gietz and Sugino, (1988)</td>
</tr>
</tbody>
</table>
GCGGTACCATGGAACAACCTGATCTATCGTCTGTGGCC-3’ and reverse primer 5’-GCCTCGACTTAGAAAATATCAGCACAATCTTTAAAGTCATG-3’ and cloned into Clonejet (Fermentas) shuttle vector as described by manufacturer. OSH1 fragment was isolated from the clonejet vector using KpnI and SalI, and sub-cloned into pKT10-GAL-HA². Construct was confirmed by restriction enzyme digest and sequencing. All plasmids were transformed into yeast as described in Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, 5th Edition (Amberg et al., 2005). Growth of strains was evaluated by ten-fold serial dilutions of equivalent cultures spotted onto selective media and grown at different temperatures until the first strain had grown out all six dilutions. Cells containing plasmids with galactose-inducible genes were spotted onto media containing either 2% glucose (control) or 4% galactose/2% rafinose in order to induce gene expression.

4.2.2 Synthetic Genomic Array Analysis

Protocol and media based on Tong and Boone (2006) described below.

4.2.3 The Nonessential Gene Deletion Collection and Synthetic Lethal Tester Strain

A total of 4672 different yeast deletion mutants, were generated that represent the total number of viable single mutants from a total of approximately 6200 potential genes in the yeast genome (Winzeler et al., 1999). The genes were disrupted with a Kan-MX4 cassette whose product provides the yeast with resistance to the antibiotic G418 (Invitrogen). The collection of conditional gene
mutations consisted of 752 temperature sensitive alleles marked with a KanMX4 cassette. All strains are derivatives of BY4741 (MAT\textsubscript{a}ura3Δ0 leu2Δ0 his3Δ1 met15Δ0). In Y7092 MAT\textalpha{} his3Δ1 leu2Δ0 met15Δ0 ura3Δ can1Δ::STE2prSp\_his5lypΔ1, the HIS3 open reading frame was integrated at the MFA1 locus such that HIS3 expression is regulated by the mating type specific MFA1 promoter. Thus, the HIS3 gene will be transcribed in haploid yeast strains that are of the ‘a’, but not ‘a’, mating type.

4.2.4 SGA Protocol

Because our query strain was marked with a URA3, the following modifications to the previously described SGA protocol (Tong and Boone, 2006) were made and outlined in Figure 4©1. The query strain was grown on synthetic complete rich media lacking uracil (-URA) and grown 2-3 days at 30°C to create a source plate. The query strain was pinned onto synthetic minimal media (MM) with 2 g/L of histidine, arginine, methionine, leucine, adenine and tyrosine at a density of 1534 using the Singer RoTor (www.singerinstr.co.uk). Selective synthetic media was used to select for the correct diploid selection and grown overnight at 30°C. All media used to select for the haploid was as described except lacking uracil instead of the addition of clonNAT. An additional two steps were included to induce growth on selective synthetic media.
Figure 4-1  Flow chart of Synthetic Genomic Array (SGA) Analysis

A MATα strain carrying a URA3 marked plasmid with a $P^{GAL}$-OSH gene (query gene) and MFA1pr-HIS3, can1Δ and lyp1Δ reporters is crossed to an ordered array of MATa viable yeast deletion mutants, each carrying a gene deletion mutation linked to a kanamycin-resistance marker KanMX that confers resistance to the antibiotic geneticin (G418). Growth of resultant diploids is selected for on medium containing G418 lacking uracil. The diploids are transferred to medium with reduced levels of carbon and nitrogen to induce sporulation. Spores are transferred to selective synthetic medium lacking histidine, which allows for selective germination of MATa haploid because only these cells express the MFA1pr-HIS3 reporter. The MATa haploids are transferred to selective synthetic media containing G418, which selects for the gene deletion mutation (xxxΔ::kanR). Finally, the MATa haploids containing the gene deletion mutation and query gene are grown on selective synthetic media containing 4% galactose/2% rafinose in order to induce expression of the query gene.
Figure 4-1

Adapted from Tong and Boone, (2006)
containing 4% galactose and 2% rafinose. The resulting plates were scanned and saved for analysis.

4.2.5 Bioinformatic and Statistical Analysis

Bioinformatic and statistical analysis was performed in collaboration with Ismael Veraga from the lab of Dr. Jack Chen (SFU). The average colony size was evaluated using the program ColonyScan (University of Toronto), which outputs a raw value based on the number of pixels detected in the image of the colony. The SGA screen for each individual OSH gene was performed in triplicate and the average value of each gene deletion was taken. In order to filter out inconsistent data, we tried using the standard deviation (SD) and 95% confidence intervals; however, the data between the three trials proved to be too variable. Instead, we used a representative value of the population average calculated by taking the value of the average colony size and dividing it by the average SD within a SGA screen to filter out variable data. In the case where one of the three trials was zero due to inconsistency or lack of data, the average of the remaining two values was taken provided they fell into an interval of plus or minus, twice the average colony size divided by the average SD. The average experimental value of each colony was then normalized to the average value of the vector control by taking the ratio of the two. This ratio was used in our clustering analysis that was performed and visualized using TiGRMEV4.0 software (www.tm4.org/mev.html). Relative to the growth of deletion strains transformed with the vector control, the growth of each deletion strain affected by the over-expression of individual OSH genes is represented by colour gradient;
red for increases in growth and green for decreases in growth. The scale was based on the natural distribution of the data from a value of -10 to 5.02. K-means clustering, which groups data points into a pre-determined number of clusters based on the similarities of their means, was used to group the data. The data from all seven individual OSH gene SGA screens was clustered into thirty groups. Functional analysis was completed using a web-based program, FunSpec (an acronym for "Functional Specification") (http://funspec.med.utoronto.ca/), which specializes in functional yeast genetic analysis. Based on a list of input genes, FunSpec creates a summary of functional classes, cellular localizations, protein complexes, etc. by cross-referencing online databases such as MIPS functional classes and GO Biological functions. The output summary from FunSpec groups gene deletions based on general functional similarities within a cluster (referred to as “functional enrichment”). The significance of the enrichment is represented by a statistical p-value, which represents the probability that the enrichment within a given list with any given functional category occurs by chance (Robinson et al., 2002). Enrichment with a p-value of less than 0.01, meaning that there is less than a 1% chance that the enrichment occurred at random, was considered significant.
4.3 Results

4.3.1 Comparison of SGA profiles Reveals Interactions with Genes Involved in the Actin Cytoskeleton and Cell Polarization

To identify novel OSH genetic interactions and to better understand the functional relationship between individual OSH genes, SGA screens were performed wherein plasmids over-expressing each of the seven individual OSH genes were introduced into each deletion strain representing all nonessential genes in the yeast genome. In each plasmid construct, an OSH gene is under the control of a galactose-inducible promoter on a multicopy plasmid. A vector control was also included in a parallel SGA screen as a control for false positives that may arise, such as sterile mutants, which do not mate effectively or mechanical errors in the transfer of colonies. Inconsistent values from the triplicate trials for the SGA screen for each OSH gene were excluded from our final analysis if they did not fall into the range of the population average. This filtering of inconsistent data ensured that our final bioinformatic analysis used only reliable data. The growth for each gene deletion over-expressing each OSH gene was measured relative to the corresponding gene deletion with the vector alone control. It should be noted that the over-expression of individual OSH genes resulted in a negative effect on growth in the majority of the nonessential gene deletions in the array.

To identify groups of gene deletions affected by individual and overlapping OSH genes the results of all seven individual OSH genes SGA screens were
grouped using k-means clustering (TiGERMev). K-means clustering groups similar SGA profiles for gene deletions over-expressing each of the seven OSH genes into a pre-set number of clusters based on the similar effects on growth of each individual OSH gene. After several trials with different pre-set numbers of clusters, an optimal number of thirty groups (designated #1-30) was obtained that optimized clusters of similar genetic effects based on visual inspection. Functional analysis to identify groups of functionally similar genes corresponding to the gene deletions in each cluster (referred to as “functional enrichment”) was performed using FunSpec. The results of the thirty clusters and the functional groups identified in each cluster are summarized in table 4-3. We focused our analysis on three individual clusters where OSH gene over-expression exhibited strong effects on the growth of the gene deletions within the cluster.

Clustering analysis identified a group of sixteen gene deletions located in cluster 3 that grew well when transformed with the vector control, but upon over-expression of any individual OSH gene, these deletion mutants were inviable (Fig. 4-2; Table 4-4). Functional analysis of these sixteen gene deletions using FunSpec software showed that they are enriched in genes involved in the cell cycle and cell polarization (SLA2, CAP2, APM1 and WHI3), specifically the regulation of the cytoskeleton. Given that OSH genes have been shown to be involved in cell polarization (Kozminski et al., 2006) and genetically interact with the actomyosin cytoskeleton (Chapter 3), this seems to be consistent with what is known about the function of the OSH gene family. To determine whether these
Gene clusters of similar functions that interact with individual or distinct subsets of the seven *OSH* genes, as determined by k-means clustering of SGA data. Gene deletion strains affected by the over-expression of each *OSH* genes were grouped, and the groups were then correlated between all *OSH* gene trials. Thirty clusters (designated #1-30) were identified that represent groups of gene deletions affected by the over-expression of one or more *OSH* genes. Each cluster was categorized based on general functional similarities of the genes corresponding to the deletions in each group (defined as “functional enrichment”). In general, *OSH* gene over-expression typically resulted in a negative effect on growth of affected gene deletions. Groups of gene deletion strains whose growth was improved by the over-expression of a specific *OSH* are shown in brackets ( ).
<table>
<thead>
<tr>
<th>Cluster Name</th>
<th>OSH gene overlap</th>
<th>Functional Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1</td>
<td>OSH3</td>
<td>MAPKKK cascade, actin cytoskeleton, ubiquitin-dependent protein catabolic process via the multivesicular body pathway</td>
</tr>
<tr>
<td>Cluster 2</td>
<td>OSH2, OSH4, OSH5</td>
<td>transcriptional control, vacuolar/lysosomal transport</td>
</tr>
<tr>
<td>Cluster 3</td>
<td>OSH1-OSH7</td>
<td>budding, cell polarity and filament formation, cell growth / morphogenesis, protein binding</td>
</tr>
<tr>
<td>Cluster 4</td>
<td>OSH1, OSH2, OSH4, OSH5</td>
<td>retrograde transport, endosome to Golgi, cellular bud site selection</td>
</tr>
<tr>
<td>Cluster 5</td>
<td>OSH1, OSH4, OSH5</td>
<td>ER to Golgi vesicle-mediated transport, establishment of cell polarity, fungal-type cell wall organization and biogenesis</td>
</tr>
<tr>
<td>Cluster 6</td>
<td>(OSH3), OSH4, OSH5</td>
<td>Golgi transport complex, response to unfolded protein</td>
</tr>
<tr>
<td>Cluster 7</td>
<td>OSH5</td>
<td>homeostasis of protons, transport ATPases, vacuolar/lysosomal transport, electron transport</td>
</tr>
<tr>
<td>Cluster 8</td>
<td>(OSH1), (OSH2), (OSH3), (OSH6), (OSH7)</td>
<td>transcriptional control, homeostasis of protons, modification by acetylation, deacetylation, polyphosphoinositol mediated signal transduction, stress response</td>
</tr>
<tr>
<td>Cluster 9</td>
<td>OSH1, OSH4, OSH5</td>
<td>regulation of directional cell growth, modification by phosphorylation, dephosphorylation, protein/peptide degradation, stress response, morphogenesis</td>
</tr>
<tr>
<td>----------</td>
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<td>-----------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Cluster 10</td>
<td>OSH4 OSH5</td>
<td>posttranslational modification of amino acids</td>
</tr>
<tr>
<td>Cluster 11</td>
<td>OSH4 OSH5</td>
<td>regulation of DNA processing, modification by ubiquitination, deubiquitination</td>
</tr>
<tr>
<td>Cluster 12</td>
<td>OSH4</td>
<td>mitochondrion, ribosomal proteins, enzymatic activity regulation, aminoacyl-tRNA-synthetases</td>
</tr>
<tr>
<td>Cluster 13</td>
<td>OSH4 OSH5 OSH6</td>
<td>meiosis</td>
</tr>
<tr>
<td>Cluster 14</td>
<td>OSH1</td>
<td>ABC transporters</td>
</tr>
<tr>
<td>Cluster 15</td>
<td>OSH1, OSH4, OSH5</td>
<td>mitochondrion, ion channels, assembly of protein complexes, budding, cell polarity and filament formation</td>
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<tr>
<td>Cluster 16</td>
<td>OSH1 OSH4</td>
<td>transcription, posttranslational modification of amino acids, pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>Cluster 17</td>
<td>OSH1, OSH5, OSH6</td>
<td>development of asco- basidio- or zygospore, DNA topology</td>
</tr>
<tr>
<td>Cluster 18</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cluster 19</td>
<td>OSH4, OSH5</td>
<td>ribosomal proteins, metabolism of serine, phospholipid catabolism</td>
</tr>
<tr>
<td>Cluster</td>
<td>Genes</td>
<td>Functions</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
<td>-----------</td>
</tr>
<tr>
<td>20</td>
<td><strong>OSH1, OSH4, OSH5</strong></td>
<td>ion channels, meiosis I, chloride transport</td>
</tr>
<tr>
<td>21</td>
<td><strong>OSH1, OSH4, OSH5</strong></td>
<td>induction of apoptosis by intracellular signals, transcription repression</td>
</tr>
<tr>
<td>22</td>
<td><strong>OSH4 OSH5</strong></td>
<td>regulation by modification, modification by phosphorylation, dephosphorylation</td>
</tr>
<tr>
<td>23</td>
<td><strong>OSH4, OSH5</strong></td>
<td>energy conversion and regeneration, polynucleotide degradation, fatty acid transport</td>
</tr>
<tr>
<td>24</td>
<td><strong>OSH1, OSH4, OSH5</strong></td>
<td>biosynthesis of phenylalanine, anaerobic respiration, nucleotide, transport, biosynthesis of arginine</td>
</tr>
<tr>
<td>25</td>
<td><strong>OSH1, OSH4, OSH5</strong></td>
<td>eukaryotic plasma membrane, vesicular transport, pyrimidine nucleotide metabolism, cell cycle and DNA processing</td>
</tr>
<tr>
<td>26</td>
<td><strong>OSH4, OSH5</strong></td>
<td>mitotic cell cycle, biosynthesis of methionine, ribosomal proteins</td>
</tr>
<tr>
<td>27</td>
<td><strong>OSH1, OSH4, OSH5</strong></td>
<td>C-compound and carbohydrate metabolism and transport, cell adhesion</td>
</tr>
<tr>
<td>28</td>
<td><strong>OSH1, OSH4, OSH5</strong></td>
<td>ubiquitin-dependent protein catabolic process via the multivesicular body pathway, intracellular mRNA localization</td>
</tr>
<tr>
<td>29</td>
<td><strong>OSH1, OSH4, OSH5</strong></td>
<td>translational control, amino acid transport, respiration</td>
</tr>
<tr>
<td>Cluster 30</td>
<td>OSH1, OSH4, OSH5</td>
<td>transmembrane receptor protein serine/threonine kinase signalling pathways, sugar, glucoside, polyol and carboxylate catabolism</td>
</tr>
</tbody>
</table>
Figure 4-2  Cluster 3 Involves Cell Polarity and the Cytoskeleton Genes Negatively Affected by OSH Genes

An example of a gene deletion cluster as determined by k-means clustering (TiGRMEV) showing the grouping of similar SGA profiles for deletion strains over-expressing each of the seven OSH genes. In cluster 3, sixteen genes affected cell polarity and the actin cytoskeleton show no growth upon over-expression of individual OSH genes. Overall, cluster 3 shows an “enrichment” of genes involved cell polarity, budding and filament formation (SPT3, STE20, CAP2, FAR1, RCY1, VRP1, HOF1, WHI3, DFG16, SUR1, RBD2, CLN2 p-value 8.582e-06) and the cytoskeleton (STE20, FAR1, HOF1, RBD2, CLN2 p-value 0.002561). Relative to growth of deletion strains transformed with the vector control, the growth of each deletion strain affected by the over-expression of individual OSH genes is represented by colour gradient; red for increases in growth and green for decreases in growth. (*) cytoskeleton, (**) cell polarity, budding and filament formation.
Figure 4-2
Table 4-4 Description of Genes Deletions that Show No Growth Upon *OSH* Gene Over-expression

<table>
<thead>
<tr>
<th>ORF</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>YHR051W</td>
<td>COX6</td>
<td>Subunit VI of cytochrome c oxidase, which is the terminal member of the mitochondrial inner membrane electron transport chain;</td>
</tr>
<tr>
<td>YCL008C</td>
<td>STP22</td>
<td>Component of the ESCRT-I complex, involved in protein sorting from the endosome</td>
</tr>
<tr>
<td>YMR002W</td>
<td>MIC17</td>
<td>Mitochondrial intermembrane space cysteine motif protein</td>
</tr>
<tr>
<td>YPL259C</td>
<td>APM1</td>
<td>Subunit of the clathrin-associated protein complex (AP-1); involved in clathrin-dependent Golgi protein sorting</td>
</tr>
<tr>
<td>YHL007C</td>
<td>STE20*</td>
<td>Cdc42p-activated signal transducing kinase of the PAK (p21-activated kinase) family, involved in pheromone response, invasive growth, and vacuole inheritance</td>
</tr>
<tr>
<td>YPL246C</td>
<td>RBD2</td>
<td>Possible rhomboid protease, has similarity to eukaryotic rhomboid proteases including Pcp1p</td>
</tr>
<tr>
<td>YPR189W</td>
<td>SKI3</td>
<td>Ski complex component and TPR protein, mediates 3'-5' RNA degradation by the cytoplasmic exosome</td>
</tr>
<tr>
<td><strong>YIL034C</strong></td>
<td><strong>CAP2</strong></td>
<td>Beta subunit of the capping protein (CP) heterodimer (Cap1p and Cap2p) which binds to the barbed ends of actin filaments preventing further polymerization; localized predominantly to cortical actin patches</td>
</tr>
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<td>---</td>
</tr>
<tr>
<td><strong>YNL197C</strong></td>
<td><strong>WHI3</strong></td>
<td>RNA binding protein that sequesters CLN3 mRNA in cytoplasmic foci; cytoplasmic retention factor for Cdc28p and associated cyclins; regulates cell fate and dose-dependently regulates the critical cell size</td>
</tr>
<tr>
<td><strong>YDL198C</strong></td>
<td><strong>GGC1</strong></td>
<td>Mitochondrial GTP/GDP transporter, essential for mitochondrial genome maintenance; has a role in mitochondrial iron transport;</td>
</tr>
<tr>
<td><strong>YDL073W</strong></td>
<td>Unknown function</td>
<td></td>
</tr>
<tr>
<td><strong>YLR369W</strong></td>
<td><strong>SSQ1</strong></td>
<td>Mitochondrial hsp70-type molecular chaperone, required for assembly of iron/sulfur clusters into proteins at a step after cluster synthesis, and for maturation of Yfh1p</td>
</tr>
<tr>
<td><strong>YEL044W</strong></td>
<td><strong>IES6</strong></td>
<td>Protein that associates with the INO80 chromatin remodeling complex under low-salt conditions; implicated in DNA repair based on genetic interactions with RAD52 epistasis genes</td>
</tr>
<tr>
<td><strong>YLR399C</strong></td>
<td><strong>BDF1</strong></td>
<td>Protein involved in transcription initiation at TATA-containing promoters; associates with the basal transcription factor TFIIID;</td>
</tr>
<tr>
<td>YDR138W</td>
<td><em>HPR1</em></td>
<td>Subunit of THO/TREX complexes that couple transcription elongation with mitotic recombination and with mRNA metabolism and export, subunit of an RNA Pol II complex; regulates lifespan; involved in telomere maintenance</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>YNL243W</td>
<td><em>SLA2</em></td>
<td>Transmembrane actin-binding protein involved in membrane cytoskeleton assembly and cell polarization; adaptor protein that links actin to clathrin and endocytosis; present in the actin cortical patch of the emerging bud tip.</td>
</tr>
</tbody>
</table>

results can be confirmed and are reproducible, a subset of the gene deletions corresponding to cap2Δ, apm1Δ, and whi1Δ were directly transformed with plasmids over-expressing OSH2, OSH4 or OSH5 from the galactose-inducible GAL1 promoter. However, on galactose-containing growth medium, all the deletion strains tested grew as well as the isogenic wild-type control regardless of which OSH gene was over-expressed. If these three deletion strains are any reflection of the other sixteen, then the growth of these deletion strains does not appear to be sensitive to OSH gene dosage.

Cluster 3 contains gene deletions whose growth is significantly negatively affected by over-expression of all individual OSH genes (Fig. 4-2). To determine whether these genes deletions are enriched in a specific biological function or pathway, functional analysis was performed on cluster 3. As a whole, this specific cluster is enriched in genes involved in cell polarity, budding and filament formation (SPT3, STE20, CAP2, FAR1, RCY1, VRP1, HOF1, WHI3, DFG16, SUR1, RBD2, CLN2, p-value 8.582e-06), and the cytoskeleton (STE20, FAR1, HOF1, RBD2, CLN2, p-value 0.002561). A previously established genetic interaction was identified in cluster 3 between HOF1 (homologue of fifteen) and OSH genes. HOF1 encodes a protein involved in regulating actomyosin ring dynamics during cell division (Kamei et al., 1998). Deletions of HOF1 were negatively affected in the SGA profiles of all individual OSH genes, except for OSH3. It has previously been shown that the over-expression of OSH2 and OSH4 in hof1Δ results in a ten-fold decrease in growth at 37°C (Fig 4-3). The growth defect caused by OSH gene over-expression in hof1Δ cells is consistent
Figure 4-3  Over-expression of OSH Genes Negatively Affects Growth in hof1∆

Ten-fold serial culture dilutions (left to right) were spotted onto solid synthetic solid medium to compare growth of wild-type (BY4741) and hof1∆ (CBY1629) cells transformed with multicopy plasmids containing OSH2 (pCB 239), OSH4 (pCB241), GFP-HOF1 (pCB242) or the vector alone control (YEplac195). Transformed strains were incubated at 25°C and 37°C. Over-expression by multicopy OSH2 and OSH4 resulted in a 100-fold growth defect in hof1∆ at 37 °C suggesting that OSH genes play a role in regulating the actomyosin ring during cytokinesis.
with our previous result that OSH genes affect the growth of actomyosin-related mutants (Chapter 3; G. Alfaro and CT. Beh, unpublished data).

4.3.2 Comparison of Individual OSH Gene SGA Profiles

The comparison of individual OSH gene SGA profiles was performed to identify subsets of OSH genes that function in similar pathways. In cluster 4 (Figure 4-4), the over-expression of OSH1, OSH2, OSH4 and OSH5 were shown to have similar effects on a set of gene deletions. Functional analysis revealed that cluster 4 contains an increased number of gene deletions involved in retrograde transport, specifically the GARP (Golgi associated retrograde protein) complex, which regulates retrograde transport from the endosomes to the Golgi and bud site selection. Over-expression of OSH1, OSH2, OSH4, or OSH5 in strains where GARP (VPS52, VPS53, YPT6, p-value 0.001959) or bud-site selection genes (BUD23, BUD31, BUD20, p-value 0.00511) were deleted resulted in significant growth defects. These results suggest that this particular subset of OSH genes might function together in retrograde transport and bud-site selection. However, these genetic interactions must be confirmed by direct transformation of the P^{GAL}-OSH over-expression constructs into each of the identified deletion strain to test the reproducibility of the growth defect.

Although many of the OSH genes shared similar genetic interactions, some OSH genes had unique effects on specific clusters of functionally similar gene deletions. For example, in contrast to the six other OSH genes, the over-expression of OSH3 in cluster 1 significantly negatively affected the growth of gene deletions involved in:(1) actin cytoskeleton regulation
Cluster analysis of a gene deletion cluster as determined by k-means clustering (TiGRMEV) showing the grouping of similar SGA profiles for gene deletions over-expressing each individual OSH genes. In cluster 4, OSH1, OSH2, OSH4 and OSH5 show functional overlap in negatively effecting the growth of a cluster of gene deletions enriched in components of the GARP complex (VPS52, VPS53, YPT6 p-value 0.001959), which is involved in retrograde transport from the Golgi to the endosome and bud site selection (BUD23, BUD31, BUD20, p-value 0.00511). Relative to growth of deletion strains transformed with the vector control, the growth of each deletion strain affected by the over-expression of individual OSH genes is represented by colour gradient; red for increases in growth and green for decreases in growth. (*) GARP complex; (**) Bud site selection.
(SIT4, SPH1, SSK2, p-value 0.00997); (2) the MAPK kinase pathway (SSK2, SMK1, p-value 0.00696) that regulates cell wall biogenesis during cell growth; and (3) ubiquitin-dependent sorting to the endosome (VPS25, SNF7, p-value 0.001611) (Fig 4-5). These results suggest unique roles for OSH3 in these pathways, though all these putative interactions must be confirmed by direct transformation of a P<sup>GAL</sup>-OSH3 construct in each affected deletion mutant.

### 4.3.3 SGA Screen Did Not Identify Suppressors of OSH4<sup>Y97F</sup>

We previously showed that the sterol-binding mutant OSH4<sup>Y97F</sup> is a gain-of-function, dominant lethal, however it is still unclear what pathways are affected by function. To understand how OSH4<sup>Y97F</sup> affects cell function a genomic screen using the nonessential gene deletion collection was performed. By screening for suppressors of the dominant lethal phenotype, we hope to identify specific pathways associated with OSH4<sup>Y97F</sup> function. Deletion mutants that seemed to suppress OSH4<sup>Y97F</sup> lethality appear to slow down metabolism and protein synthesis for example the mitochondrial gene (GGC1) and the vacuolar H+ pump (VMA6). Direct investigation into the growth of these genes by ten-fold serial dilutions of liquid cultures spotted onto solid media did not confirm the results of the SGA screen. It is possible that suppressor mutations arose during the SGA protocol leading to false positives because of
Cluster analysis of a gene deletion cluster as determined by k-means clustering (TiGRMEV) showing the grouping of similar SGA profiles for gene deletions over-expressing each individual OSH genes. Cluster 1 shows a group of gene deletions negatively affected by the over-expression of OSH3, but not the other six OSH genes. Functional analysis shows that gene deletions affected by OSH3 over expression in cluster 1 show functional similarities in genes involved in: (1) the actin cytoskeleton (SIT4, SPH1, SSK2 p-value 0.00997); (2) the MAPK kinase cascade (SSK2, SMK1 p-value 0.00696); which regulates cell wall biogenesis during cell growth; and (3) ubiquitin-dependent sorting to the endosome (VPS25, SNF7 p-value 0.00161). The over-expression of other individual OSH genes in the deletion strains shown in the cluster was minimal, suggesting that OSH3 has a distinct function in these pathways. Effects on growth by over-expression of individual OSH genes relative to a vector control are represented by a colour gradient; red for increase in growth and green for decrease in growth. (*) actin cytoskeleton, (**) MAPK kinase cascade, (^) ubiquitin-dependent sorting to the endosome.
Figure 4-5
the selective pressures against the dominant lethal $OSH4^{Y97F}$ mutation and multiple transferring steps.

### 4.3.4 Over-expression of $OSH5$ Suppresses Mutations in Actin

Of the already known genetic interactions involving the $OSH$ gene family (Kozminski et al., 2006, Li et al., 2002), most involve essential genes, which are not represented in the nonessential gene deletion collection. To identify by SGA novel genetic interactions between individual $OSH$ genes and essential genes in the yeast genome all seven individual $OSH$ genes were over-expressed in novel array of strains containing 752 conditional mutants. In this analysis, we tested whether the growth of any of these conditional mutants was affected (either rescued or exacerbated) by the over-expression of individual $OSH$ genes at permissive (25°C) and non-permissive (37°C) temperature.

A preliminary screen of $OSH5$ over-expression in the conditional mutation array revealed novel interactions with genes associated in cell polarization and the actin cytoskeleton. Several actin alleles were identified as being suppressed by $OSH5$ over-expression. To directly test this result, we measured the growth of the actin allele, $act1-1$ expressing $OSH2$, $OSH4$ or $OSH5$ compared to a vector control. The results confirmed the SGA result that the over-expression of $OSH5$ was able to suppress the growth defect of $act1-1$ at restrictive temperature (Fig.4-6). This suppression was best achieved on glucose using a high-copy vector expressing $OSH5$ under its own promoter.
Figure 4-6  Over-expression of OSH5 Suppresses Growth Defects in Actin Mutants

Ten-fold serial culture dilutions (left to right) were spotted onto selective solid, synthetic medium to compare growth of wild-type (WT; DDY0006) and act1-1<sup>ts</sup> (DDY0016) cells transformed with multicopy plasmids containing OSH2 (pCB239), OSH4 (pCB241), OSH5 (pCB242) or the vector alone control (YEplac195). Transformed strains were incubated at 25°C (permissive growth temperature) and 37°C (restrictive growth temperature). Over-expression by multicopy OSH5, but not OSH2 and OSH4, was able to suppress growth defects in act1-1<sup>ts</sup> at 37°C suggesting OSH5 promotes the function of the actin cytoskeleton.
The fact that increased Osh5p expression suppressed mutations in actin suggests that OSH5 may play a role in the function of the actin cytoskeleton. Interestingly, this phenotype seems to be unique to OSH5 since the other OSH genes directly tested (OSH2 and OSH4) had no effect on the growth of act1-l at any temperature.

4.3.5 Over-expression of OSH Genes Suppresses Defects in Cytoskeleton Associated Genes

In order to identify specific mutations that are rescued or suppressed by the over-expression of any individual OSH gene, all seven P^GAL-OSH genes and the vector control were each introduced by SGA into the array of conditional mutants for analysis. A list of 301 genes that showed growth at non-permissive temperature in the SGA profile of one or more OSH gene was compiled. Gene ontology was performed to identify functional groups of conditional mutant genes affected by over-expression of individual OSH genes. Temperature sensitive mutations suppressed at non-permissive temperature in the SGA profiles of four or more single OSH genes were shown to have significant enrichment in genes involved: (1) in budding and cell polarity (CMD1, CDC28, CDC10, ARP2, CDC1, MSS4, SEC4, MOB2, ACT1, CDC43, CDC24, SEC15, SEC9, YIP1, CDC12, SEC6, PAN1, CDC11, DAD2, COF1, RGR1, SEC10, CDC42, PFY1, LAS17, MYO2, SEC8, p-value 5.424e-08); (2) the cytoskeleton (CDC24, CMD1, CDC10, TCP1, TUB2, ACT1, DUO1, SEC15, DAM1, YIP1, CDC12, CDC11, TOR2, DAD2, CDC42, p-value 3.449e-10); (3) transport of vesicles (CDC48, SEC20, SEC4, SEC15, SEC9, SEC6, SEC10, SEC22, SEC8, p-value 1.572e-06); and (4)
cell division (CDC10, ARP2, SEC4, ACT1, SEC15, CDC12, SEC6, PAN1, CDC11, SEC10, PIK1, LAS17, SEC8, p-value 3.806e-08). Specific alleles of CDC42 have been shown to genetically interact with OSH genes (Kozminski et al., 2006). In our analysis, a novel allele of CDC42, cdc42-1, was suppressed by the over-expression of OSH1, OSH3, OSH4, OSH5, and OSH6. These data suggest that OSH genes play a role in regulating cell polarity and division, consistent with the results of the SGA screens in the nonessential gene deletion collection.

In order to identify essential gene mutations that are sensitive to OSH gene dosage, each OSH gene was over-expressed in the strain collection of conditional mutants and inspected for growth defects at 25°C, the temperature that permits growth of all mutants. Gene mutations were grouped using k-means clustering (TiGRMEV) into seven clusters (designated #1-7TS) based on similar SGA profiles of each conditional mutants over-expressing each of the seven OSH genes. Each group was analyzed for functional enrichment of gene mutations affected by OSH gene over-expression summarized in Table 4-5. Several clusters (clusters 1TS, 3TS, 4TS, 5TS, 6TS, and 7TS) showed enrichment in mutants involved in mitotic cell cycle control, spindle formation and cytokinesis. Cluster 4TS, 5TS, and 7TS showed enrichment of genes involved in cell budding and polarity, which parallels what was observed in the SGA screens using the nonessential gene deletion collection. In cluster 2, OSH gene over-expression increased the growth of a cluster of gene mutations at permissive temperature.
Gene clusters of similar functions that interact with individual or distinct subsets of the seven OSH genes, as determined by k-means clustering of SGA data. Gene mutation strains affected by the over-expression of each OSH genes were grouped, and the groups were then correlated between all OSH gene trials. Seven clusters (designated #1-7TS) were identified that represent groups of conditional mutants affected by the over-expression of one or more OSH genes at permissive temperature, 25°C. Each cluster was categorized based on general functional similarities of the genes corresponding to the gene mutations in each group. In general, OSH gene over-expression typically resulted in a negative effect on growth of affected conditional mutants. Groups of mutant strains whose growth was improved by the over-expression of a specific OSH are shown in brackets ( ).
<table>
<thead>
<tr>
<th>Cluster Name</th>
<th>OSH gene Overlap</th>
<th>Functional Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1 TS</td>
<td>OSH1-OSH7</td>
<td>spindle pole body/centrosome and microtubule cycle, mitotic cell cycle and cell cycle control, exocytosis, microtubule cytoskeleton</td>
</tr>
<tr>
<td>Cluster 2 TS</td>
<td>(OSH1-OSH7)</td>
<td>rRNA processing, ATP binding, RNA transport</td>
</tr>
<tr>
<td>Cluster 3 TS</td>
<td>OSH4, OSH5</td>
<td>mitotic cell cycle and cell cycle control, ATP binding, cytoskeleton/structural proteins, pheromone response, ER to Golgi transport, cytokinesis</td>
</tr>
<tr>
<td>Cluster 4 TS</td>
<td>OSH1-OSH7</td>
<td>mitotic cell cycle and cell cycle control, budding, cell polarity and filament formation, actin cytoskeleton, cytokinesis</td>
</tr>
<tr>
<td>Cluster 5 TS</td>
<td>OSH1, (OSH3), (OSH6), OSH7</td>
<td>mitotic cell cycle and cell cycle control, microtubule cytoskeleton, cytokinesis, budding, cell polarity and filament formation, vesicle fusion</td>
</tr>
<tr>
<td>Cluster 6 TS</td>
<td>OSH1, OSH7</td>
<td>mitotic cell cycle and cell cycle control, splicing, transcription initiation, cytoskeleton/structural proteins, transcription initiation, ER to Golgi transport, cytokinesis</td>
</tr>
<tr>
<td>Cluster 7 TS</td>
<td>OSH4</td>
<td>mitotic cell cycle and cell cycle control, ATP binding, proteasomal degradation, exocytosis, microtubule cytoskeleton, vesicle fusion, budding, cell polarity and filament formation</td>
</tr>
</tbody>
</table>
These mutations are enriched in genes involved in RNA transport and processing suggesting a novel role for $OSH$ genes in regulating these processes. These potential genetic interactions still need to be confirmed by direct transformation of $P^{GAL}\text{-}OSH$ gene constructs in each the same temperature sensitive mutants from the conditional array.

### 4.1 Discussion

Our analysis revealed potential interactions between individual and overlapping subsets of $OSH$ genes in regulating cell polarity and cell growth through interactions with the actomyosin cytoskeleton, which supports previous data suggesting such a role for $OSH$ genes (Kozminski et al., 2006). Confirmation of a genetic interaction between $OSH5$ and $ACT1$ suggests a novel role for $OSH5$ in the function of the actin cytoskeleton. Unfortunately, direct analysis into several of the genetic interactions identified in our SGA screens did not reproduce the results of the SGA screen. Further investigation by directly testing for genetic interactions with specific genes of interest should be completed in order to confirm the data from our SGA screens.

#### 4.1.1 A Subset of $OSH$ Genes Function Together to Influence Different Pathways

Genetic analysis of SGA profiles of the over-expression of individual $OSH$ genes has revealed novel pathways and functions of the $OSH$ gene family and provided further evidence to support a role for $OSH$ genes in previously
discussed functions such as transport and cell polarity. Subsets of OSH genes have been shown to have overlapping genetic interactions suggesting that they may function together or in parallel in specific pathways. Here, we have shown in cluster 4, an enrichment of genes involved in retrograde transport and bud site selection suggesting that OSH1, OSH2, OSH4 and OSH5 may work together to regulate these pathways. The genetic interaction between OSH genes and yet another transport complex involved in retrograde transport between the early endosome and the Golgi (reviewed in Oka et al., 2005) is not surprising based on the evidence that Osh proteins function in vesicular transport from the Golgi to the PM (Kozminski et al., 2006; G. Alfaro and CT. Beh, unpublished data). The function of the GARP complex even parallels that of the exocyst in mediating vesicle docking and fusion (Conibear et al., 2003) suggesting Osh proteins may play a role in regulating the docking and fusion of vesicles to the appropriate membranes. Direct investigations into these results have yet to be completed in order to confirm the results of the SGA screen.

Our SGA analysis identified a novel allele of CDC42, cdc42-1, suppressed by the over-expression of OSH1, OSH3, OSH4, OSH5 and OSH6. Direct investigation into these interactions must be completed to verify the results, however OSH genes have been previously shown to be dosage suppressors of specific CDC42 alleles (Kozminski et al., 2006). Inconsistencies in the SGA data were also seen in the over-expression of OSH genes in certain mutant phenotypes. For example, mutations in components of the exocyst complex, sec6-4, sec8-6, exo70-38 and the Rho GTPase, rho3, have both been previously
shown to be sensitive to the over expression of *OSH* genes affected by the over-expression of individual *OSH* genes (G. Alfaro and CT. Beh, unpublished; Kozminski et al., 2006). However, our SGA analysis showed that *OSH3*, *OSH4*, *OSH5* or *OSH6* over-expression actually suppressed the growth defects at the elevated temperature. A similar situation is observed with *myo2-14* and *myo2-16*, where increased *OSH* gene expression exacerbates their growth defects (G. Alfaro and CT. Beh, unpublished data), but these mutations were suppressed in our SGA screen. These inconsistencies are possibly due to technical errors regarding the transfer of yeast or the result of additional unlinked mutations that suppress the conditional alleles and highlight the importance of direct investigation into any potential SGA result.

The conditional mutant array consists of several hundred temperature sensitive mutations, each with its own specific non-permissive temperature. In our SGA screens we analyzed growth at two temperatures, 25°C (permissive) and 37°C (non-permissive). It is possible that potential genetic interactions were missed, if the non-permissive temperature of the mutant was not reached or was passed. In the future it may be beneficial to conduct a large scale SGA screen looking for genetic interactions at a wide range of non-permissive temperatures.

**4.1.2 SGA Screen Identifies Novel Interaction Between *OSH5* and Actin**

SGA profiles of the over-expression of individual *OSH* genes have allowed of the identification of a novel genetic interaction with the actin cytoskeleton. The over-expression of *OSH5* was able to suppress the growth defect in *act1-1*
mutants at non-permissible temperature, a phenotype that seems to be unique to OSH5. This suggests that OSH5 may play a unique role in promoting the function of actin, which is consistent with the data implicating a role for OSH genes in actomyosin-dependent transport discussed in the previous chapter. Other actin alleles were identified in our SGA screen however the ability of OSH5 and other OSH genes to suppress growth defects in other ACT1 alleles still needs to be established. A similar relationship is observed with the mammalian ORP4S and the intermediate filament, vimentin (Wang et al., 2002). Vimentin is thought to influence cell signaling and vesicular transport by creating a scaffolding network for important proteins involved in these processes (Wang et al., 2002). Many of the clusters affected by the over-expression of individual OSH genes were enriched in genes involved in different aspects of regulating the actin cytoskeleton and cell growth. This suggests that the multi-gene OSH family is involved in different pathways working towards establishing cell polarity and vesicular transport through interactions with the actin cytoskeleton. For example, HOF1 is involved in the regulation of the actomyosin ring during cytokinesis (Kamei et al., 1998), deletions of which were affected by the over-expression of all OSH genes except for OSH3 suggesting certain OSH genes play a role in cytokinesis. Many of these results have not be tested directly, however previously studies support the idea of Osh proteins in the regulation of the actin cytoskeleton (reviewed in Beh et al., 2009; Lehto et al., 2008).
4.1.3 Identification of Suppressors of $OSH4^{Y97F}$

Attempts to identify suppressors of the hypermorphic, dominant lethal $OSH4^{Y97F}$ through SGA analysis did not yield any significant results when tested directly. Surprisingly, upon induction of $OSH4^{Y97F}$ a large number of colonies survived suggesting that suppressor mutations arose during the SGA protocol. $OSH4^{Y97F}$ is a robust dominant lethal and strains are prone to suppressor mutations over short periods of time due to selective pressures. It is possible that due to the nature of the SGA protocol, which involved multiple passaging over a long period of time that random suppressor mutations arose biasing our final analysis. Identification of gene deletions that slow down metabolism and protein synthesis in order to survive during times of stress seemed reasonable, however were not confirmed when tested directly. Further investigations into the function of Osh4(Y97F)p needs to be completed.

4.1.4 SGA Screens are Highly Variable and Must be Directly Investigated

There is a large potential of error when working with high-density genetic screens such as technical errors in strain transfer, errors within the array itself, computer errors as well as human errors. For example, deletion strains that have severe mating defects (eg, sterile mutant) or are slow growing mutants would not survive or be adequately transferred during the selection process making a false synthetic lethal/sick hit. Analysis done using SGA technology must be directly investigated in order to confirm potential results and identify false positives. With regards to our direct analysis of the sixteen gene deletions identified to be synthetically lethal with $OSH$ gene over-expression, it was revealed that in this
particular strain background, over-expressing individual *OSH* genes on galactose resulted in growth defects in the wild type strain. This growth defect was comparable to the growth defects observed in the deletion strains tested. *STE20* was excluded from direct analysis because the deletion of *STE20* results in a sterile mutant, and could not have undergone mating during the SGA screen. It is possible that the lack of growth identified upon *OSH* gene over-expression in these sixteen gene deletion is due to an error in colony transfer in the vector screen or computer error failing to detect the spots.

Another issue that arose during our analysis was the concept of high variability between trials. Statistical analysis using the SD or 95% confidence intervals to filter out inconsistent data proved to be too stringent due to the high amount of variability between replicates. Attempts to utilize data across all seven *OSH* gene datasets did not alleviate the problem. Previously published papers have suggested performing three-to-four independent trials of an SGA screen in order to apply statistics with higher confidence intervals (Fairn et al., 2005). Overall, our approach to SGA screens has proven far too variable to reliably identify specific novel genetic interactions.
5: CONCLUSIONS

Note regarding contributions:

The following chapter discusses the results of a manuscript in preparation and data collected as part of my thesis. The respective contributions of the authors is listed below.

Manuscript in preparation:

Alfaro, G., Duamel, G., Dighe, SA., Johansen, J., Kozminski, KG., and Beh, CT. The Sterol-Binding Protein Kes1/Osh4p is a Regulator of Polarized Exocytosis.

The summary Figure 5-1 was created by CT. Beh based on data generated by myself, G. Alfaro, KG. Kozminski and CT. Beh as part of our manuscript in preparation

Here, we have presented evidence for Osh4 protein function in the regulation of the actomyosin dependent transport of vesicles during polarized exocytosis. Novel genetic interactions between the kinesin-related protein, SMY1 and OSH4 support a role for OSH4 in regulating the transition between actomyosin dependent transport and vesicle docking at the PM. Similar to OSH4, SMY1 was found to be a dosage suppressor of specific CDC42 mutations defective in polarization establishment suggesting that OSH4 and SMY1 are both involved in the regulation of cell polarity through CDC42. These data were further supported by genome-wide genetic screens of individual OSH gene function, which identified genetic interactions with components of the actomyosin cytoskeleton and regulators of polarity. Investigations into the role of sterol binding in ORP
function revealed that specific mutants of the yeast ORP, Osh4p that eliminated sterol binding were hyperactivated and dominant lethal, contrary to what is predicted for a sterol transfer protein. Based on our data we propose a model of Osh4p function as a regulator of cell signalling involving vesicular transport and cell polarization triggered by the binding of sterol lipids.

5.1 The Role of Sterol Binding in Osh4p Function

The role of sterol binding in ORP function has long been debated with evidence supporting both the idea of a lipid transfer protein and a lipid-dependent regulator of intracellular signalling (reviewed Beh et al., 2009; Fairn and McMaster 2008). Here we tested the hypothesis that Osh4p functions as a sterol transfer protein by investigating the function of two sterol binding deficient mutations. We have shown that a specific mutant of Osh4p that is unable to bind sterols exhibits a hyperactive, dominant phenotype opposed to what we predicted if it were functioning solely as a sterol transfer protein. Similar dominant lethal phenotypes have been observed in mutations in cell signalling genes, such as ras (Beitel et al. 1990) supporting a role for Osh4p as a regulator of cell signalling versus lipid transport. Mutations in Osh4p that blocked association with cellular membranes resulted in recessive/loss of function phenotype suggesting that the interaction with membranes is essential to Osh4 protein function. Overall, we suggest that the function of Osh4p is dependent on associations with cellular membranes, not sterol binding. This supports the model that the primary function of ORPs is not lipid transfer, but cell signalling involving the regulation of cell
polarity and vesicular transport. Based on the fact that the Osh4(Y97F)p is a gain of function mutant, it is probable that the unbound form of Osh4p is its activated form. This suggests that sterol binding by Osh4p happens early in vesicular transport during vesicle biogenesis at the Golgi and releases its bound sterol when vesicles dock at the PM. We propose that sterol binding acts a trigger for Osh4p dependent regulation of vesicular transport and the release of its bound sterol promotes its function at the PM.

5.2 Interactions Between OSH Genes and the Actin Cytoskeleton

Cells lacking Osh protein function show specific defects in cell polarity and vesicular transport (Beh and Rine 2004; Kozminski et al., 2006), particularly a decrease in vesicle motility at the PM (G. Alfaro and CT. Beh, unpublished data) suggesting defects in the actomyosin dependent transport of vesicles. Here we have identified a novel genetic interactions between OSH4 and the Myo2p-associated protein, SMY1, which has provided a link between Osh4p and the actomyosin-dependent movement of polarized secretory vesicles to the PM. Little is known about the mechanism of Smy1p function, however we have identified two novel genetic interactions involving SMY1, OSH4 and CDC42, supporting a regulatory role for Smy1p in polarized exocytosis. SMY1 also has an inhibitory effect on OSH4 activity suggesting that it represses Osh4p function. While the over-expression of both SMY1 and OSH4 rescue specific CDC42 mutations defective in polarity establishment, SMY1 and OSH4 showed different genetic interactions with regards to SEC14-dependent biogenesis and MYO2 transport.
Based on our genetic analysis, we propose that *OSH4* and *SMY1* function in parallel pathways to promote polarized exocytosis. Because vesicles in yeast do not travel along microtubule filaments, there is no need for Smy1p to act as a bona fide kinesin motor during vesicle transport, other than through its regulatory effects on myosin. It is possible that Smy1p functions with Osh4p to regulate the transition from actomyosin motility to exocyst-dependent vesicle docking through interactions with Sec4p.

Interactions with the actin cytoskeleton have been further supported through genome-wide analysis of *OSH* gene interactions. Gene ontology was performed to identify functional groups of gene deletions affected by the over-expression of individual *OSH* genes. This revealed several clusters enriched in genes involved in regulation of the actin cytoskeleton, cell growth and polarization. Identification of *OSH5* as a dosage suppressor of the actin allele *act1-1* suggests that *OSH5* may promote the function of the actin cytoskeleton. It needs to be established whether or not the over-expression of *OSH5* in *act1-1* restores the formation of the actin cytoskeleton or if it suppresses *act1-1* in another way. Genetic interactions have also been observed in specific mutants of *MYO2* defective in polarized exocytosis suggesting that *OSH* genes may influence actomyosin dynamics. A previously established genetic interaction between *HOF1* and *OSH* genes was identified in our SGA screen, supporting a role of *OSH* genes in the regulation of actomyosin ring during cytokinesis. While many known *OSH* gene interactions were identified in our SGA analysis, other contradictory results were also observed. This suggests that, while there is
potential for reproducible results, there is also a large amount of error in high throughput genetic screens such as SGA and direct confirmation is necessary.

5.3 A Model for Osh4p Activities During Polarized Exocytosis

There is increasing evidence that ORPs play a role in regulating cell signalling by influencing regulatory protein interactions opposed to lipid transport (reviewed in Fairn and McMaster 2008; Beh et al., 2009). It is possible the role of ORPs in nonvesicular transport might involve the ordered assembly of complexes required for lipid transfer at membrane contact sites. Based on the data presented in our thesis, we propose the following model for Osh4p activities during polarized exocytosis summarized in figure 5-1. After vesicle biogenesis from the Golgi and its transfer on vesicles into the bud, Osh4p releases its bound sterol when closely apposed to the PM. We suggest that sterol release spatially restricts Osh4p activity to the PM, ensuring that exocyst docking occurs only when vesicle and PM are juxtaposed, but is not essential for all Osh4p function. In this respect, Osh4p could regulate the transition from actomyosin dependent transport to exocyst-dependent vesicle docking through its interactions with Sec4p and Smy1p.
Figure 5-1  Model for Osh4p Function in Polarized Exocytosis

Osh4p can be viewed as “transport conductor” that regulates the arrival of vesicles at sites of polarized growth by releasing its bound sterol, thereby triggering Osh4p function regulating the transition between exocyst docking and vesicles release from the actomyosin cytoskeleton. Because Osh4p directly interacts with Sec4p (G. Alfaro and CT. Beh, unpublished data), which is central to the attachment of myosin and exocyst components to the vesicle, we propose that Osh4p promotes polarized exocytosis by modulating Sec4p interactions. When vesicles and PM are closely apposed, Osh4p delivers its bound sterol to the PM thereby activating Osh4p function in its role in exocyst complex docking. The actomyosin machinery represses Osh4p activity through the Myo2p-associated protein Smy1p, which also promotes Cdc42p-dependent polarization. As such, Osh4p mediates the mechanistic transfer of vesicles from the actomyosin cytoskeleton to the exocyst docking complex, which promotes targeted membrane fusion between the vesicle and polarized sites on the cell cortex.
G. Alfaro et al., manuscript in preparation
6: REFERENCES


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