SPATIAL REGULATION OF EXOCYTOSIS BY *RHO* FAMILY SMALL GTPASES in *Saccharomyces cerevisiae*

Hao Wu

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell and Developmental Biology.

Chapel Hill

2009

Approved by

Advisor: Patrick Brennwald

Committee member: Con Beckers

Committee Member: Doug Cyr

Committee Member: Richard Cheney

Committee Member: Channing Der

ABSTRACT

Hao Wu: Spatial Regulation of Exocytosis by Rho Family Small GTPases
in *Saccharomyces cerevisiae*
(Under the direction of Patrick Brennwald)

Polarized exocytosis is a fundamental cellular process which mediates the delivery of intracellular lipids and proteins to the plasma membrane and secreted to the extracellular compartment. In budding yeast *Saccharomyces cerevisiae*, the Rho family small GTPases are important regulators of exocytosis. However, the precise mechanism by which they regulate exocytosis is not well understood. Earlier work from our laboratory suggested that Rho3 and Cdc42 have direct roles in exocytosis during different stages of the cell cycle. Here we present evidence that the functional specificity of Rho3 and Cdc42 is determined by two elements at the N terminus of Rho3.This region contains elements thatare important for the localization of Rho3 and the interaction with the exocyst component Exo70. We show that Exo70 has the biochemical and genetic properties expected of a direct effector for both Rho3 and Cdc42. Surprisingly we find that C-terminal prenylation of these GTPases both promotes the interaction and influences the sites of binding within Exo70. We identified gain-of-function mutants in *EXO70* that potently suppress mutants in *RHO3* and *CDC42* defective for exocytic function. Taken together, these data suggests that Exo70 is the common

downstream effector for both Rho3 and Cdc42. The localization of the Rho3 and Cdc42 and their interaction with the Exo70 component of the exocyst are the key determinants for their functional specificity in regulating exocytosis.

To my parents

ACKNOWLEGEMENTS

My first and most earnest acknowledgment goes to my advisor Dr. Patrick Brennwald. I started graduate school six years ago with very little background in cell biology. Dr. Brennwald taught me from the very beginning and had spent a lot of time and effort to ensure my academic and professional wellbeing. My research had encountered difficulties at various times, and Dr. Brennwald was always made himself readily available for helpful discussion and trouble shooting. I deeply appreciate his encouragement, patience and mentoring throughout my study. None of this work would have been possible without him.

I would like to thank my committee members, Dr. Con Beckers, Dr. Doug Cyr, Dr. Richard Cheney, and Dr. Channing Der for asking me constructive questions, having insightful comments, and encouraging me in various ways during my study.

Special acknowledgement goes to Dr. Brenda Temple for her assistance with the computer modeling of dominant Exo70 mutations on the crystal structure of Exo70. I also like to thank Hal Mekeel for his technical support with the electron microscopy during the characterization of the novel Exo70 mutants.

It has been a great pleasure to work with members in the Brennwald lab, especially Guendalina Rossi and Leah Watson. I enjoy every moment we have worked together to overcome technical difficulties and share research ideas. I appreciate Leah's friendship and

encouragement in finishing this dissertation. I am particularly thankful to Guendalina for critical reading of my manuscript, providing very useful inputs and helping me improving my presentation skills. I would also like to thank Courtney Turner, Jimmy Gardner, Garrett Thompson, Mallory Demonch and Marianne Lennon for their technical support. Courtney Turner and Jimmy Gardner made great contributions in both of my research papers. I am very grateful for their effort.

My graduate studies would not have been the same without my dear friends: Yang Liu, Qi Zhang, Ran Jin, Wenqi Pan, Jianfu Chen and Xinming Cai. They made my life fun and enjoyable outside of Taylor Hall.

My ultimate thank-you goes to my wonderful parents Banghua Wu and Xiuyun Zhao, and my loving husband Liang Cai. My mother loved me and supported me unconditionally with her whole life.She will forever be the angel that stands by me and guides me. I miss her every day. My father has always been there when I needed him most, and never once complained about how infrequently I visited home. I could not find the right words to express how much I appreciate what he had done for me. My husband has been my best friend over the past couple of years. I enjoyed walking home with him and telling him about my day. I know there are happiness and frustrations only he can understand.

TABLE OF CONTENTS

ABSTRACT iii

ACKNOWLEGEMENTS vi

LIST OF FIGURES xii

LIST OF TABLES xiv

CHAPTER ONE 1

The Function of the Exocyst during Polarized Exocytosis 1

Discovery and characterization of the Late Secretory Mutants and the Exocyst complex 2

Localization of the exocyst complex 4

Exocyst components talk to each other 7

The exocyst talks to the SNARE complex 10

The structure of the Exocyst complex 11

REFERENCE 13

CHAPTER TWO 17

The Ghost in the Machine: Small GTPases as Spatial Regulators of Exocytosis 17

ABSTRACT 18

INTRODUCTION 19

RabGTPases: Conserved Regulators of Vesicle Tethering to Target Membranes 20

Ral GTPases: Metazoan Regulators of Exocyst Assembly 23

Rho GTPases: Yeast as a Model for Polarity 26

Rho1: Important for the Localization of the Sec3 Component of the Exocyst Complex 27

Rho3: A Direct Regulator of Exocytosis 28

Cdc42: A Cell Cycle-Specific Regulator of Exocytosis 29

TC10: A Cdc42 Family GTPase Involved in Glucose Transporter Trafficking 30

Rho/Cdc42 Regulation of the Exocyst is Distinct from Rab Regulation. 31

The Exocyst as a Landmark or an Activated Machine? Local Activation vs. Local Recruitment Models 33

Concluding Remarks 38

ACKNOWLEDGEMENT 39

REFERENCE 40

CHAPTER THREE 46

The Exo70 Subunit of the Exocyst is an Effector for Both Cdc42 and Rho3 Function in Polarized Exocytosis 46

ABSTRACT 47

INTRODUCTION 48

MATERIALS AND METHODS 51

Yeast Strains, Reagents, and Genetic Techniques 51

Dominant suppressor screen for Exo70 dominant mutants: 52

Isolation of novel Exo70 mutants by random mutagenesis 52

Genetic analysis of mutants 53

Electronic Microscopy 54

Invertase assay and Bgl2 assay 54

Construction of pGEX-6His vector and protein expression, purification, and quantification 55

In vitro binding assays 56

Generation of yeast cell lysate 57

GST pull-down from yeast cell lysate 57

In vitro prenylation assay 58

Immunoprecipitation of the exocyst complex 58

RESULTS 60

Isolation of Dominant Gain-of-Function Alleles of *EXO70* 60

The GTP-Bound Forms of Rho3 and Cdc42, Produced in Yeast, Interact with Exo70 .. 69

Prenylation of Rho3 and Cdc42 is an Important Determinant for Binding to Exo70 70

Binding of Prenylated Rho3 and Cdc42 to Exo70 is Not Dependent on the C-Domain. 76

Isolation of Novel Conditional-Lethal Alleles of *EXO70* 79

DISCUSSION 85

ACKNOWLEDGEMENT 89

REFERENCE 92

CHAPTER FOUR 103

The N-terminus of Rho3 Determines its Function and Localization during Exocytosis 103

ABSTRACT 104

INTRODUCTION 105

MATERIAL AND METHODS 108

Yeast Strains, Reagents, and Genetic Techniques 108

Construction of chimeras 109

Protein purification and quantification 109

Generation of yeast cell lysate 110

GST pull-down from yeast cell lysate 110

Immunofluorescence 111

Subcellular fractionation 111

RESULT 112

Rho3 and Cdc42 have Distinct Localization Patterns on the Plasma Membrane 112

The Rho3 N-terminus Encodes Multiple Specificity Determinants 117

The N-terminal region of Rho3 and Cdc42 determines the Strength of Binding to Exo70 121

DISCUSSION 125

REFERENCE 132

LIST OF FIGURES

Figure 1: A Model for Interactions between Exocyst Subunits 9

Figure 2. Alignment of Mammalian and Yeast Exocyst Subunits which Interact with Rho, Ral, and Rab small GTPases 23

Figure 3:A three step model for vesicle docking, exocyst activation and vesicle fusion regulated by small GTPases. 33

Figure 4:A model for activation of the exocyst complex by Rho family GTPases. 35

Figure 5:Cross suppression of *EXO70* dominant suppressors isolated from *cdc42-6* or *rho3Δ* screens. 62

Figure 6:Dominant mutations of *EXO70* are able to suppress *rho3Δ* and *cdc42-6* mutant 64

Figure 7: Conservation of Dominant gain-of Function Mutations in Exo70 66

Figure 8: Dominant Mutations in Exo70 do not Affect the Assembly of the Exocyst Complex 68

Figure 9:Exo70 binding to Rho GTPases in E.coli or Yeast 72

Figure 10:Prenylation of Rho3 and Cdc42 Promotes the GTP-dependent interaction with Exo70. 75

Figure 11:Binding of Prenylated Rho3 and Cdc42 to Exo70 Does Not Depend on Domain C. 78

Figure 12: Characterization of a novel cold-sensitive mutant *exo70-188*. 81

Figure 13:Characterization of a novel temperature sensitive mutant *exo70-113*. 84

Figure 14: Localization of Rho3 and Cdc42 113

Figure 15:The N terminus of Rho3 is necessary and sufficient for its function and localization. 116

Figure 16:The N terminus of *RHO3* contains two elements that are important for its function and localization. 119

Figure 17: The effect of palmitoytransferase deletion on the ability of Cdc42-NT^{R3} to function as Rho3 121

Figure 18: N terminus of Rho3 determines the affinity of the interaction between Rho3 and Exo70. 124

LIST OF TABLES

Table 1: Yeast Strains used in Chapter Three 90

Table 2: Plasmids used in Chapter Three 91

Table 3: Primers used to Construct Chimeric Proteins 129

Table 4: Yeast Strains used in Chapter Four 130

Table 5: Bacterial Strains used in Chapter Four 131

CHAPTER ONE

The Function of the Exocyst during Polarized Exocytosis

Polarized exocytosis is a fundamental cellular process by which intracellular lipid and protein contents get delivered to target membranes and secreted to the extracellular compartment. In budding yeast *Saccharomyces cerevisiae*, polarized exocytosis involves three distinctive steps: delivery of the post-Golgi vesicles towards the target membrane along actin cables; docking of secretory vesicles at the plasma membrane, and the fusion of vesicles with the plasma membrane.

Many proteins or protein complexes have been reported to regulate exocytosis. These include Rab small GTPases and the type V myosin Myo2 for vesicle delivery, the exocyst complex for vesicle tethering at the plasma membrane; Rho family small GTPases for the local activation of the exocyst complex; and the SNARE complex for membrane fusion. This chapter mainly focuses on the function of the exocyst complex in regulating exocytosis, including the discovery and localization of the exocyst, the structure of the exocyst components, the protein-protein interactions within the exocyst components as well as its communication with the SNARE complex.

Discovery and characterization of the Late Secretory Mutants and the Exocyst complex

The original discovery of the components of the exocyst complex can be traced back to a genetic screen in *Saccharomyces cerevisiae* three decades ago (Novick and Schekman, 1979; Novick *et al.*, 1980). This screen identified 23 complementation groups of temperature sensitive mutants defective in the secretory pathway, ten of which function during Golgi to cell surface transport before membrane fusion (Novick *et al.*, 1980; Novick *et al.*, 1981).

These ten genes were classified as 'late-acting' secretory genes, including *SEC1, SEC2, SEC3, SEC4, SEC5, SEC6, SEC8, SEC9, SEC10* and *SEC15*(Novick *et al.*, 1981). Sec1 was found to regulate the SNARE protein interactions during membrane fusion (Scott *et al.*, 2004; Togneri *et al.*, 2006). Sec4 is a Rab family small GTPase that interacts with the type V myosin Myo2 to mediate vesicle transport (Salminen and Novick, 1987; Govindan *et al.*, 1995; Wagner *et al.*, 2002). Sec2 is the exchange factor for Sec4 and is thought to recruit Sec4 on post-Golgi vesicles (Walch-Solimena *et al.*, 1997). Sec9 is the t-SNARE in yeast and is thought to form a ternary complex with Sec4 and Sro7 to promote vesicle docking and membrane fusion (Brennwald *et al.*, 1994; Grosshans *et al.*, 2006). The remaining six late-acting *SEC* genes were later found to be components of a multisubunit protein complex, the exocyst.

The first indication that a subset of the late *SEC* gene products might belong to a common high molecular mass complex came from the characterization of Sec15. Analysis by differential centrifugation of a wild type strain revealed that approximately 23% of Sec15 was associated with the plasma membrane and the remaining fraction was associated with a 19.5S soluble particle (Bowser and Novick, 1991). Using a c-*myc*-tagged Sec8 strain, the Novick group discovered that Sec6 and Sec8 co-exist with Sec15 in this high molecular mass complex which contains eight polypeptides (Bowser *et al.*, 1992; TerBush and Novick, 1995). This complex was rather stable in wild type cells, but became very unstable when isolated from *sec3-2, sec5-24, sec6-4, sec10-2* and *sec15-1* mutants. This suggested that *SEC3, SEC5*, and *SEC10* could be part of the eight subunit protein complex. Biochemical analysis of the purified complex from a detergent yeast lysate confirmed that *SEC3, SEC5*, and *SEC10* were

indeed components of the complex and also revealed another component, *EXO70*. This seven protein complex was named the exocyst since all of the components were required for exocytosis in yeast cells (TerBush *et al.*, 1996). The remaining component, *EXO84*, was initially identified from mammalian cells. Using rat Sec6 and ratSec8 antibody, Hsu *et al* purified the mammalian exocyst complex from rat brain lysates which contain the mammalian homolog of the seven yeast exocyst component, and an additional protein Exo84. The yeast Exo84 was then identified via a database search using the mammalian Exo84 sequence. Temperature sensitive alleles of Exo84 accumulated post-Golgi vesicles and displayed secretory defects, suggesting that Exo84 was also critical for exocytosis (TerBush *et al.*, 1996; Guo *et al.*, 1999a).

Localization of the exocyst complex

In yeast, all components of the exocyst complex are localized at the bud tip where the most active secretion occurs during early bud growth (TerBush and Novick, 1995; Finger and Novick, 1998). The localization of two components of the exocyst complex, Sec15 and Sec3, has been particularly interesting. The first attempt to analyze the localization of Sec15 was problematic due to the low level of Sec15 in the cell. To determine the localization of Sec15, Salminen *et al* overexpressed Sec15 behind a galactose inducible promoter. The result was quite striking in that Sec15 formed concentrated clusters in the bud or adjacent to an emerging bud. Electron microscopy suggested that these bright clusters corresponded to clusters of post-Golgi vesicles. Further analysis of this clustering phenotype indicated that the vesicle clusters would form only when functional Sec2 and Sec4 were present in the cells. This observation positioned Sec15 downstream of the Rab GTPase Sec4, and provided an

important genetic evidence that the exocyst complex function as an effector for Sec4 during the vesicle tethering process (Salminen and Novick, 1989).

The localization of Sec3 was first studied using GFP-Sec3 in cells where the endogenous Sec3 was still present. Fluorescence microscopy suggested that Sec3-GFP protein localized at the bud tip during bud emergence and early bud growth. The Sec3-GFP became less concentrated as the bud enlarged, and was localized at the mother-bud neck region before cytokinesis. While the localization of Sec4 and other components of the exocyst complex required a functional secretory pathway, the localization of Sec3-GFP did not depend on the secretory pathway or a functional actin cytoskeleton. This observation suggested that Sec3 can stably associate with the plasma membrane and help the other components of the exocyst to assemble at sites of vesicle docking and fusion (Finger and Novick, 1998).

Some aspects of these observations were later called into question. Using an affinity purified polyclonal antibody against endogenous Sec3, Roumanie *et al* demonstrated that in mutants defective in actin polarity (*tpm2Δ, tpm1-2*) and in mutants blocked in the secretory pathway, Sec3 was indeed depolarized upon shifting to restrictive conditions (Roumanie *et al.*, 2005). Localization studies using GFP-tagged Sec3 integrated in the chromosome also revealed that the Sec3 localization in the cell required a functional secretory pathway. These data indicated that Sec3 is not the spatial landmark for the exocyst component and there must be another mechanism by which the exocyst assembles and anchors at the plasma membrane (Songer and Munson, 2009).

Recent studies showed that Sec3 and Exo70 directly interact with PI (4, 5) P_2 (phosphotidylinositol 4, 5-biphosphate) at the plasma membrane. Sequence analysis of Exo70 revealed evolutionarily conserved, positively charged residues at the D domain of Exo70 which were clustered as a positively charged surface patch in the C terminus of the Exo70 structure (He *et al.*, 2007b). These charged residues were responsible for interacting with PI(4,5)P_2 at the plasma membrane. Exo70 and other components of the exocyst complex failed to associate with the plasma membrane in mutants where the PI(4,5)P_2 synthesis was reduced at the restrictive temperature, suggesting that the interaction with PI(4,5)P_2 is important in mediating the membrane targeting of the exocyst. Sec3 interacts with PI(4,5)P_2 through a polybasic region at its N terminus (Zhang *et al.*, 2008). Simultaneous disruption of PI (4,5)P_2 interaction with both Exo70 and Sec3 resulted in mislocalization of the exocyst complex, and lead to severe defect in secretion or cell lethality. These observations suggested that Sec3 and Exo70 function together to maintain the exocyst complex at the plasma membrane (He *et al.*, 2007b; Zhang *et al.*, 2008).

Based on the sequence and structural similarity between yeast and mammalian Exo70, Liu *et al* identified conserved basic residues in mammalian Exo70 at the C terminus. Like yeast Exo70, mutations of these basic residues in mammalian Exo70 also disrupted the interaction with PI(4,5)P_2 at the plasma membrane and blocked exocytosis(Liu *et al.*, 2007). However, the N terminus of Sec3 is not conserved between yeast and mammalian cells. Either there is another mechanism by which the mammalian Sec3 can attach to the plasma membrane, (possibly through interaction with Exo70), or there might be a positively charge patch at other regions of Sec3 that is responsible for interaction with the plasma membrane.

Exocyst components talk to each other

Early research using coimmunoprecipitation experiments have provided links between components of the exocyst complex. For example, in the *sec10-2* mutant, Sec15 was absent when myc tagged Sec8 was used to precipitate the complex, suggesting that Sec15 might associate with the exocyst complex through Sec10. In the *sec5-24* mutant, three components were absent (Sec5, Sec10, and Sec15) and the levels of two components were significantly reduced (Sec3 and Exo70) (TerBush and Novick, 1995), which potentially placed Sec5 at the center of the complex. These observations raised the question of whether there is a core protein in the complex that all the other components are attached to or is it that every component connects with each other to maintain the integrity of the complex? If the exocyst complex has an active and an inactive conformation, is the internal organization of the exocyst different in each state? To better understand these questions, it would be necessary to understand the protein-protein interaction within the exocyst complex.

Using coimmunoprecipitation and yeast two hybrid assays, Guo *et al* identified five pairs of potential interactions between several components of the complex, **Sec6-Sec8, Sec5-Exo70, Sec10-Sec15, Sec5-Sec6,** and **Sec5-Sec3** (Guo *et al.*, 1999b). Dong *et al* performed pull-down experiments both with full length Exo70 and different domains of Exo70 and showed that **Sec6, Sec8, Sec10**, and **Exo84** directly interact with **Exo70**. Interestingly, the interaction between Sec8, Sec10 and Exo84 all require full length Exo70, while Sec6 can interact with Exo70 as long as domain C is present. More recent research using HMM (Hidden Markov Model) modeling identified a domain of Sec10 (residues 145-827) that was soluble, properly folded and functionally important(Croteau *et al.*, 2009). Using this domain

of Sec10, Croteau *et al* performed *in vitro* binding experiments and showed that Sec10 directly interact with **Sec6, Exo70** and the C domain of **Exo84**.

Based on the known interactions within the yeast exocyst complex, shown in Figure 1, there could be an order of function within the exocyst complex. Sec15 interacts with Sec4 on the vesicles and Sec10 in the exocyst, which positions Sec15 as a bridge between the exocyst and the secretory vesicles. Exo70 and Sec3 interact with $PI(4,5)P_2$ at the plasma membrane, which provide a connection between the plasma membrane and the exocyst. Besides the interaction within the exocyst, Sec6 was also found to interact with the t-SNARE Sec9 at the plasma membrane. This observation suggested that Sec6 could potentially function in a similar manner as Sec3 and Exo70 in anchoring the exocyst complex at the plasma membrane. Since Sec8, Sec10 and Exo84 interact with Exo70 only when the full length protein is present, one can imagine these three components pack on the enlongated rod structure of Exo70 and help 'pull' the vesicle close to the plasma membrane during vesicle tethering. The structure analysis of Exo70 and several other components of the exocyst will be discussed in more details later.

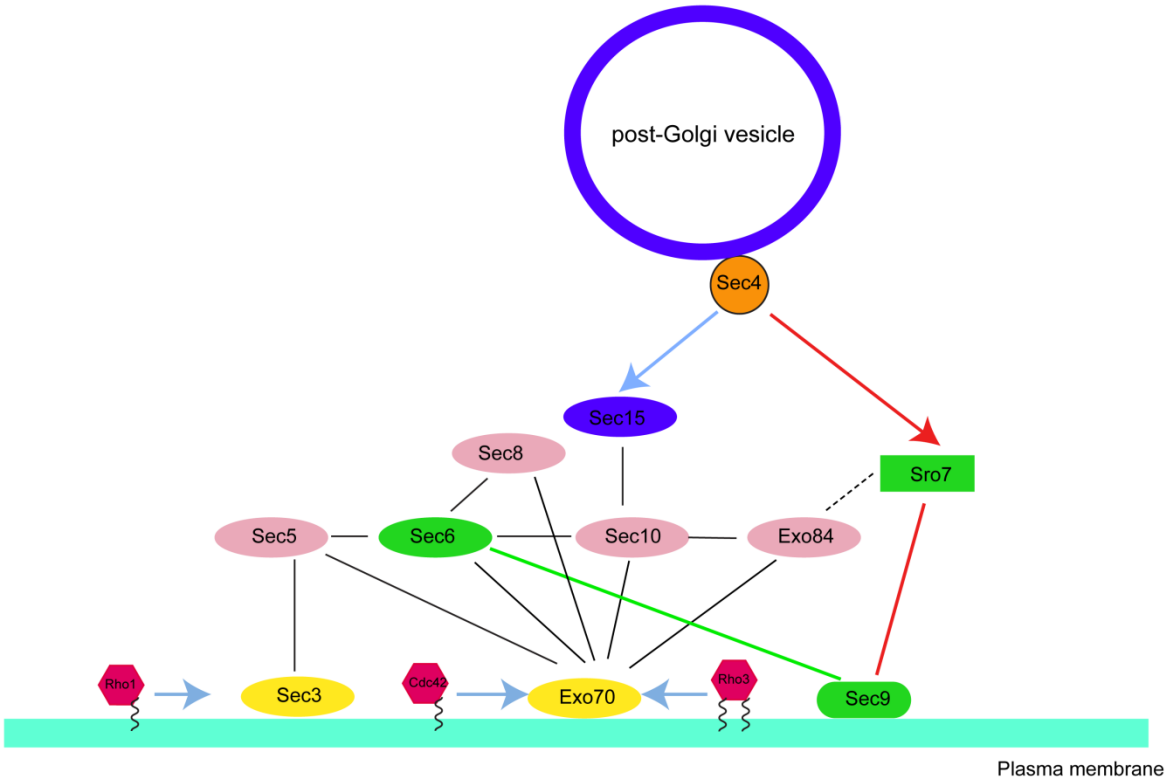


Figure 1: A Model for Interactions between Exocyst Subunits

Proteins labeled with oval shaped pills are components of the exocyst complex. Sec3 and Exo70 (shown in yellow) interact with PIP2 on the plasma membrane and maintain the exocyst at the plasma membrane. Sec6 directly interacts with t-SNARE Sec9, while Exo84 associate with Sec9 through interacting with Sro7. Proteins associates with Sec9 are labeled in green. Sec15 directly interact with Rab GTPase Sec4 (orange) on post-Golgi vesicles (dark blue). Rho family small GTPases (dark pink) regulate the exocyst complex by interacting with Sec3 and Exo70 component of the complex. The interactions within the exocyst complex are marked with solid black lines. The interaction of Sro7 with Sec4 and Sec9 represents a parallel pathway with the exocyst complex during vesicle tethering event. This pathway is marked by solid red line. The regulation of the exocyst complex by Rho or Rab family small GTPases is labeled with blue solid lines.

References: Guo, *et al*, 1999b; Dong, *et al*, 2006; Croteau, *et al*, 2009; Grosshans, *et al*, 2006; Lehman, *et al*, 1999; Sivaram, *et al*, 2005; Zhang *et al*, 2005.

The exocyst talks to the SNARE complex

Assembly of the SNARE complex between the v-SNARE Snc and the t-SNARE Sso and Sec9 is a tightly regulated process. In *sec4-8* and *sec2-41* mutants the SNARE complex failed to assemble due to the defects in vesicle transport (Grote and Novick, 1999). In several mutants in the exocyst components, *sec5-24*, *sec6-4*, *sec8-9* and *sec15-1*, the SNARE complex also failed to assemble, suggesting that the SNARE assembly event functions after exocyst assembly and vesicle tethering (Grote *et al.*, 2000) . Moreover, overexpressing Sec9 can strongly suppress several mutants in the exocyst complex, suggesting that there is a genetic correlation between the exocyst and the SNARE complex (Lehman *et al.*, 1999).

The first connection between the exocyst and the SNARE complex came from the analysis the function of Exo84 in secretion. Although there was no direct interaction between Exo84 and Sec9, Zhang *et al* was able to detect a relatively high affinity interaction between Exo84 and Sro7, which is the yeast homolog of lethal giant larvae protein and was known to interact with the t-SNARE Sec9 (Lehman *et al.*, 1999; Zhang *et al.*, 2005b).

Using GST pull-down and gel filtration chromatography, the Munson group observed that Sec6 specifically bound to plasma membrane t-SNARE Sec9 (Sivaram *et al.*, 2005), which provided a direct interaction between the exocyst complex and the SNARE complex. Songer *et al* later identified novel mutations in the conserved surface patch of Sec6. Unlike conventional sec mutants in which the exocyst complex was disassembled, the novel sec6 mutants contained an intact exocyst complex at the restrictive temperature (Songer and

Munson, 2009), suggesting Sec6 plays an important role in anchoring the exocyst complex at the plasma membrane.

The structure of the Exocyst complex

The structures of four exocyst components have been solved: the C terminal domain of yeast Exo84 (residues 523-753)(Dong *et al.*, 2005), the C terminal domain of *Drosophila* Sec15 (residues 382-699)(Wu *et al.*, 2005), and the C terminal domain of yeast Sec6 (residues 411-805)(Sivaram *et al.*, 2006), and nearly full length of both yeast Exo70(residues 67-623)and mouse Exo70 (residues 85-653)(Dong *et al.*, 2005; Hamburger *et al.*, 2006; Moore *et al.*, 2007). Although the subunits of the four exocyst components share very low sequence similarity (<10%), the overall structures of the known components are remarkably similar in their helical bundle structure. The structural similarity between the exocyst components leads to a prediction that the helical bundles pack together to form elongated rod structures (Munson and Novick, 2006). This prediction is consistent with the 'Y' shaped structure observed in electron micrographs of the purified mammalian exocyst complex (Hsu *et al.*, 1998).

Since the structures of both yeast Exo70 and mouse Exo70 are available in nearly full length, the comparison between these two structures and their functional implications deserves further discussion. The overall structure of Exo70 is well conserved despite of the relatively low sequence similarity (12% identity and 35% similarity) between the two species (Moore *et al.*, 2007).Yeast Exo70 and mammalian Exo70 both interact with Rho family small GTPases. Domain truncation studies positioned the interaction site of Rho3 with the C

domain of yeast Exo70 whereas TC10 was reported to interact with the N terminus (domain A) of mammalian Exo70 (Inoue *et al.*, 2003). However, the study identifying the interaction between yeast Exo70 and Rho3 utilized recombinant Rho3 purified from bacterial lysate. Recent studies from our laboratory showed that the interaction between Rho3 and Exo70 requires prenylation of Rho3. Therefore, the mapping result obtained from bacterial lysate could not truly represent the interaction between these two proteins. Using GST pull-down assay from yeast lysate, we found that the domain C could not be the only binding site on Exo70 in that Exo70 deleted for this domain was still able to interaction with Rho3. Further studies using Rho3 from yeast lysate would help identifying the additional binding sites on Exo70.

The structure for the rest of the exocyst components encountered technical difficulties due to the lack of soluble proteins. Croteau *et al* recently used computational HMM (Hidden Markov Model) modeling to predict structural similarities between all of the exocyst components (Croteau *et al.*, 2009). More importantly, this method helped to identify a structural domain of Sec10 that was soluble, properly folded and was predicted to contain helical bundle structures. The HMM computational modeling will be a useful tool to predict structure domains of other exocyst components and help to generate reagents to solve the crystal structure (Croteau *et al.*, 2009). Ultimately, understanding the structure of the individual components, combined with the structural based mutagenesis, will help determining the structure of the exocyst in its active or inactive state, and eventually lead to elucidating the role of the exocyst in polarized exocytosis.

REFERENCE

Bowser, R., Muller, H., Govindan, B., and Novick, P. (1992). Sec8p and Sec15p are components of a plasma membrane-associated 19.5S particle that may function downstream of Sec4p to control exocytosis. J Cell Biol *118*, 1041-1056.

Bowser, R., and Novick, P. (1991). Sec15 protein, an essential component of the exocytotic apparatus, is associated with the plasma membrane and with a soluble 19.5S particle. J Cell Biol *112*, 1117-1131.

Brennwald, P., Kearns, B., Champion, K., Keranen, S., Bankaitis, V., and Novick, P. (1994). Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. Cell *79*, 245-258.

Croteau, N.J., Furgason, M.L., Devos, D., and Munson, M. (2009). Conservation of helical bundle structure between the exocyst subunits. PLoS One *4*, e4443.

Dong, G., Hutagalung, A.H., Fu, C., Novick, P., and Reinisch, K.M. (2005). The structures of exocyst subunit Exo70p and the Exo84p C-terminal domains reveal a common motif. Nat Struct Mol Biol *12*, 1094-1100.

Finger, F.P., and Novick, P. (1998). Spatial regulation of exocytosis: lessons from yeast. J Cell Biol *142*, 609-612.

Govindan, B., Bowser, R., and Novick, P. (1995). The role of Myo2, a yeast class V myosin, in vesicular transport. J Cell Biol *128*, 1055-1068.

Grosshans, B.L., Andreeva, A., Gangar, A., Niessen, S., Yates, J.R., 3rd, Brennwald, P., and Novick, P. (2006). The yeast lgl family member Sro7p is an effector of the secretory Rab GTPase Sec4p. J Cell Biol *172*, 55-66.

Grote, E., Carr, C.M., and Novick, P.J. (2000). Ordering the final events in yeast exocytosis. J Cell Biol *151*, 439-452.

Grote, E., and Novick, P.J. (1999). Promiscuity in Rab-SNARE interactions. Mol Biol Cell *10*, 4149-4161.

Guo, W., Grant, A., and Novick, P. (1999a). Exo84p is an exocyst protein essential for secretion. J Biol Chem *274*, 23558-23564.

Guo, W., Roth, D., Walch-Solimena, C., and Novick, P. (1999b). The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. EMBO J *18*, 1071-1080.

Hamburger, Z.A., Hamburger, A.E., West, A.P., Jr., and Weis, W.I. (2006). Crystal structure of the S.cerevisiae exocyst component Exo70p. J Mol Biol *356*, 9-21.

He, B., Xi, F., Zhang, X., Zhang, J., and Guo, W. (2007). Exo70 interacts with phospholipids and mediates the targeting of the exocyst to the plasma membrane. EMBO J *26*, 4053-4065.

Hsu, S.C., Hazuka, C.D., Roth, R., Foletti, D.L., Heuser, J., and Scheller, R.H. (1998). Subunit composition, protein interactions, and structures of the mammalian brain sec6/8 complex and septin filaments. Neuron *20*, 1111-1122.

Inoue, M., Chang, L., Hwang, J., Chiang, S.H., and Saltiel, A.R. (2003). The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin. Nature *422*, 629-633.

Lehman, K., Rossi, G., Adamo, J.E., and Brennwald, P. (1999). Yeast homologues of tomosyn and lethal giant larvae function in exocytosis and are associated with the plasma membrane SNARE, Sec9. J Cell Biol *146*, 125-140.

Liu, J., Zuo, X., Yue, P., and Guo, W. (2007). Phosphatidylinositol 4,5-bisphosphate mediates the targeting of the exocyst to the plasma membrane for exocytosis in mammalian cells. Mol Biol Cell *18*, 4483-4492.

Moore, B.A., Robinson, H.H., and Xu, Z. (2007). The crystal structure of mouse Exo70 reveals unique features of the mammalian exocyst. J Mol Biol *371*, 410-421.

Munson, M., and Novick, P. (2006). The exocyst defrocked, a framework of rods revealed. Nat Struct Mol Biol *13*, 577-581.

Novick, P., Ferro, S., and Schekman, R. (1981). Order of events in the yeast secretory pathway. Cell *25*, 461-469.

Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell *21*, 205-215.

Novick, P., and Schekman, R. (1979). Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *76*, 1858-1862.

Roumanie, O., Wu, H., Molk, J.N., Rossi, G., Bloom, K., and Brennwald, P. (2005). Rho GTPase regulation of exocytosis in yeast is independent of GTP hydrolysis and polarization of the exocyst complex. J Cell Biol *170*, 583-594.

Salminen, A., and Novick, P.J. (1987). A ras-like protein is required for a post-Golgi event in yeast secretion. Cell *49*, 527-538.

Salminen, A., and Novick, P.J. (1989). The Sec15 protein responds to the function of the GTP binding protein, Sec4, to control vesicular traffic in yeast. J Cell Biol *109*, 1023-1036.

Scott, B.L., Van Komen, J.S., Irshad, H., Liu, S., Wilson, K.A., and McNew, J.A. (2004). Sec1p directly stimulates SNARE-mediated membrane fusion in vitro. J Cell Biol *167*, 75-85.

Sivaram, M.V., Furgason, M.L., Brewer, D.N., and Munson, M. (2006). The structure of the exocyst subunit Sec6p defines a conserved architecture with diverse roles. Nat Struct Mol Biol *13*, 555-556.

Sivaram, M.V., Saporita, J.A., Furgason, M.L., Boettcher, A.J., and Munson, M. (2005). Dimerization of the exocyst protein Sec6p and its interaction with the t-SNARE Sec9p. Biochemistry *44*, 6302-6311.

Songer, J.A., and Munson, M. (2009). Sec6p anchors the assembled exocyst complex at sites of secretion. Mol Biol Cell *20*, 973-982.

TerBush, D.R., Maurice, T., Roth, D., and Novick, P. (1996). The Exocyst is a multiprotein complex required for exocytosis in Saccharomyces cerevisiae. EMBO J *15*, 6483-6494.

TerBush, D.R., and Novick, P. (1995). Sec6, Sec8, and Sec15 are components of a multisubunit complex which localizes to small bud tips in Saccharomyces cerevisiae. J Cell Biol *130*, 299-312.

Togneri, J., Cheng, Y.S., Munson, M., Hughson, F.M., and Carr, C.M. (2006). Specific SNARE complex binding mode of the Sec1/Munc-18 protein, Sec1p. Proc Natl Acad Sci U S A *103*, 17730-17735.

Wagner, W., Bielli, P., Wacha, S., and Ragnini-Wilson, A. (2002). Mlc1p promotes septum closure during cytokinesis via the IQ motifs of the vesicle motor Myo2p. EMBO J *21*, 6397-6408.

Walch-Solimena, C., Collins, R.N., and Novick, P.J. (1997). Sec2p mediates nucleotide exchange on Sec4p and is involved in polarized delivery of post-Golgi vesicles. J Cell Biol *137*, 1495-1509.

Wu, S., Mehta, S.Q., Pichaud, F., Bellen, H.J., and Quiocho, F.A. (2005). Sec15 interacts with Rab11 via a novel domain and affects Rab11 localization in vivo. Nat Struct Mol Biol *12*, 879-885.

Zhang, X., Orlando, K., He, B., Xi, F., Zhang, J., Zajac, A., and Guo, W. (2008). Membrane association and functional regulation of Sec3 by phospholipids and Cdc42. J Cell Biol *180*, 145-158.

Zhang, X., Zajac, A., Zhang, J., Wang, P., Li, M., Murray, J., TerBush, D., and Guo, W. (2005). The critical role of Exo84p in the organization and polarized localization of the exocyst complex. J Biol Chem *280*, 20356-20364.

CHAPTER TWO

The Ghost in the Machine: Small GTPases as Spatial Regulators of Exocytosis

Reproduced with permission from: Wu H, Rossi G, Brennwald P. Trends Cell Biol. 2008 Sep; 18(9):397-404.The ghost in the machine: small GTPases as spatial regulators of exocytosis

ABSTRACT

Temporal and spatial regulation of membrane trafficking events is critical to both membrane identity and overall cell polarity. Small GTPases of the Rab, Ral, and Rho families have been implicated as important regulators of vesicle docking and fusion events. In this review we focus on how these GTPases interact with the exocyst complex, a multisubunit tethering complex involved in the regulation of cell surface transport and cell polarity. The Rab and Ral GTPases are thought to function in exocyst assembly and vesicle tethering processes whereas the Rho family GTPases appear to function in the local activation of the exocyst complex to facilitate downstream vesicle fusion events. The localized activation of the exocyst by Rho GTPases is likely to play an important role in spatial regulation of exocytosis.

INTRODUCTION

The ability of cells to direct specific membrane and protein components to defined places on the cell surface is fundamental to the establishment and maintenance of cell polarity. The mechanism by which proteins and lipids are delivered to the cell surface is through transport, docking and fusion of secretory vesicles with the plasma membrane. In polarized cells, the location of these transport events is highly regulated but the precise mechanism of regulation is still poorly understood. A protein complex, whose function appears to be closely linked to polarized cell surface delivery events in a number of cell types, is known as the exocyst complex. This complex has been reported to be involved in the tethering, docking and fusion of post-Golgi vesicles with the plasma membrane. It is composed of eight subunits, which are conserved from yeast to mammalian cells: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. Recent structural studies have suggested that these proteins are primarily composed of structurally similar helical bundles which appear to associate through an extensive network of interactions within the complex (Hamburger *et al.*, 2006; Munson and Novick, 2006). The exocyst complex also appears to be distantly related to vesicle tethering complexes that function at other stages of membrane trafficking such as the COG and GARP complexes (Whyte and Munro, 2001; Conibear *et al.*, 2003). Although it is clear that the exocyst complex plays an important role in regulating exocytosis, little is known about the mechanism by which it functions in promoting exocytosis or cell polarity. Information from a number of model systems has demonstrated that the exocyst complex is regulated by a number of small GTPases. In this review, we will focus on how Rab, Ral, and

Rho small GTPases regulate exocytosis through regulation of both the localization and function of the exocyst complex on the plasma membrane.

RabGTPases: Conserved Regulators of Vesicle Tethering to Target Membranes

Rab proteins are one of the most abundant families within the Ras superfamily of small GTPases. There are 11 Rab proteins in yeast and more than 60 in mammalian cells(Barrowman and Novick, 2003). Rab proteins have been reported to regulate different membrane trafficking and signaling pathways through their interaction with various effectors. Like other small GTPases in the Ras superfamily, Rab proteins cycle between a GTP bound active form and a GDP bound inactive form(Pfeffer, 1994) and interact with downstream effectors through their active conformation. Sec4 is a Rab family small GTPase in the yeast *Saccharomyces cerevisiae,* that was first identified in a screen for mutants with secretory defects (Novick *et al.*, 1980). The *sec4-8* mutant, which contains a substitution of glycine to aspartic acid at position 147, was shown to accumulate post Golgi vesicles by electron microscopy and by invertase secretion assay(Salminen and Novick, 1987). Immunofluorescence and subcellular fractionation experiments demonstrated that Sec4 resides on secretory vesicles as well as on the plasma membrane(Goud *et al.*, 1988). Genetic and cell biological evidence demonstrated that duplication of Sec4 suppressed the loss of Sec15 function and that the polarized localization pattern of the Sec15 protein was lost in *sec4-8* mutants(Salminen and Novick, 1989), suggesting that Sec15 might represent an effector of Sec4. However, the first evidence that Sec15 might encode a direct downstream target of Sec4, came in the late 90s when researchers(Guo *et al.*, 1999b) found that Sec4

interacted with the Sec15 component of the exocyst component using a yeast two-hybrid assay. Importantly, this interaction appeared to be GTP-dependent in that mutant alleles of Sec4 predicted to be in the GDP form failed to interact, while GTP-locked mutants show a significant increase in interaction as measured in the two-hybrid system. This interaction was supported by immunoprecipitation experiments which demonstrated that, following chemical cross-linking, Sec4 could be co-immunoprecipitated with Sec15. Further analysis using mutant forms of Sec4 suggested that this interaction was specific to Sec4 but not to other closely related yeast Rab proteins, such as Ypt1 and Ypt51. Making chimeric proteins between the effector domain of Sec4 and Ypt1 suggested that the effector domain is responsible for interacting with Sec15, as Sec4 with the effector domain of Ypt1 failed to interact with Sec15(Guo *et al.*, 1999b).

Recently it was reported that the yeast lethal giant larvae (lgl) family protein Sro7 may represent a second direct effector for Sec4 (Grosshans *et al.*, 2006)Sro7 was previously identified as a binding partner for the plasma membrane t-SNARE, Sec9, and loss of this protein and its paralog, Sro77, resulted in severe post-Golgi secretory defects similar to those seen in *sec4* and *sec9* mutant cells(Lehman *et al.*, 1999). Consistent with the idea that Sro7 may act in parallel to the exocyst as an effector downstream of Sec4 [12], overexpression of Sro7 suppresses defects associated both with mutations in the exocyst components (Lehman *et al.*, 1999)and in Sec4(Lehman *et al.*, 1999; Zhang *et al.*, 2005a; Grosshans *et al.*, 2006). Biochemical experiments support the notion that Sro7 may be a direct effector of Sec4 in that purified Sro7 was found to bind specifically to Sec4 preloaded with GTP, but not to Sec4 preloaded with GDP. Further characterization of the interaction of the t-SNARE, Sec9, with

Sro7 suggested that this was likely to be a highly regulated and transient event *in vivo*. In particular the interaction with the essential SNAP-25 domain of Sec9 was found to be stimulated by release of an autoinhibitory interaction within the Sro7 protein(Hattendorf *et al.*, 2007). This led to the model that Sro7 may regulate SNARE assembly events in response to an upstream binding event, which would trigger the localized presentation of the Sec9 SNARE domain to its cognate t- and v-SNAREs. If, in fact, Sec4 is part of this "trigger" then it would help to coordinate the timing of the SNARE assembly with the arrival of the Sec4-bound vesicle. Two other proteins involved in polarized exocytosis in yeast, the Exo84 component of the exocyst, and the type V myosin, Myo2, have also been shown to interact with Sro7 and may contribute to the final "triggering" of Sro7-dependent SNARE assembly(Gangar *et al.*, 2005; Zhang *et al.*, 2005a).

More recently a homologous interaction between Rab11 and mammalian Sec15 has been described (Zhang *et al.*, 2004; Wu *et al.*, 2005). This supports the idea that Rab GTPase regulation of exocyst function during tethering is likely to represent an ancestral and therefore central regulatory interaction (**Figure 2**).

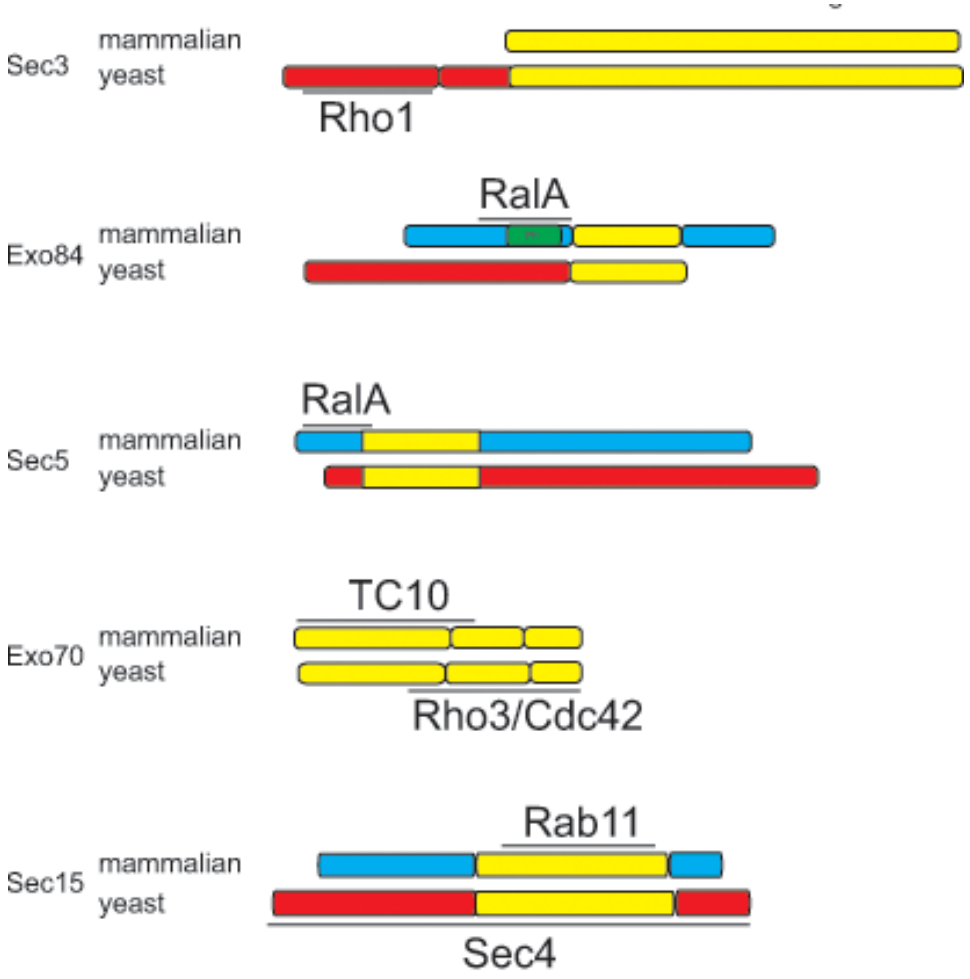


Figure 2. Alignment of Mammalian and Yeast Exocyst Subunits which Interact with Rho, Ral, and Rab small GTPases
Regions of each subunit conserved between yeast and mammals are colored in yellow. Regions lacking obvious sequence similarity are labeled as blue in mammalian cells and red in yeast.

Ral GTPases: Metazoan Regulators of Exocyst Assembly

Ral GTPases are evolutionarily relatively recent additions to the small GTPase family found only in animal cells(Camonis and White, 2005). They have been implicated in the regulation of a diverse array of cellular processes, including oncogenic transformation, endocytosis(Jullien-Flores *et al.*, 2000) and actin-cytoskeleton dynamics(Ohta *et al.*, 1999; Moskalenko *et al.*, 2002). Ral proteins have also been shown to associate with secretory

granules and synaptic vesicles(Vitale *et al.*, 2005). Recently, it was reported that Ral small GTPases directly interact with components of the exocyst complex, Exo84 and Sec5, which, in mammalian cells, have been implicated in the targeting of Golgi-derived vesicles to the basolateral membrane of polarized epithelial cells and to the growth cones of differentiating PC12 cells(Jin *et al.*, 2005).

Sec5 as an effector for Ral small GTPases was first identified in a yeast two hybrid screen searching for novel downstream targets using an activated form of RalA as bait. An effector domain mutation of RalA, which fails to interact with Sec5, results in mis-sorting of basolateral membrane proteins (for example the EGF Receptor) to the apical surface of polarized epithelial cells(Moskalenko *et al.*, 2002), indicating that RalA is required for appropriate basolateral membrane protein targeting. RalA interacts with Sec5 in a GTP dependent manner, and truncation studies indicated that the N terminal domain (1-120 amino acids) of Sec5 is necessary for interacting with RalA. The effector domain mutant of RalA72L49E, which does not interact with Sec5 and Exo84, fails to promote delivery of E cadherin to the basolateral surface of MDCK cells. This suggests that exocyst binding is critical for the RalA GTPase to promote exocytic function. Interestingly, another Ral effector mutant RalA72L49N, which retains the ability to bind to Sec5 and Exo84, also fails to enhance basolateral membrane delivery, suggesting that exocyst binding is necessary but not sufficient for RalA to enhance secretion (Shipitsin and Feig, 2004). Knockdown of RalA by siRNA, resulted in disassembly or destabilization of the exocyst complex, suggesting that Ral might regulate exocytosis by facilitating the proper assembly of the exocyst complex (**Figure 3**). Overexpression of the constitutively active Ral (Ral23V) results in mislocalization of

basolateral membrane proteins, suggesting that Ral function also requires the cycling between the GTP-bound state and the GDP-bound state. However, studies using another form Ral, RalA72L, predicted to be "locked" in the active GTP-bound state, found this mutant had enhanced trafficking to the basolateral membrane. It is not clear if these different results are due to differences in the RalA mutants used or differences in the basolateral trafficking assays. Exo84 is another effector of RalA which was identified through a similar two hybrid screen with the activated form of RalA as bait. Structural analysis of the RalA-Exo84 interaction indicated that the binding site on Exo84 was located at residues 228-234, which represents a conserved motif AxxNx(K/R)D, retained in all metazoan members of the Exo84 family(Jin *et al.*, 2005).

RalB shares 88% identity to RalA in its first 162 amino acids. Although both proteins contain binding sequences for the exocyst components within this region, activated RalB binds to the exocyst components much less efficiently than active RalA. In addition to the difference in binding to the exocyst components, RalA and RalB also display distinct localization pattern due to the C-terminal variable domain. Immunofluorescence studies in MDCK cells suggested that RalA is predominantly localized on the plasma membrane at the cell-cell junctions with diffuse punctuate staining throughout the cytoplasm. Antibody staining of RalA72L induced MDCK cells not only have increased staining pattern of on the plasma membrane, but also reveal intense perinuclear staining. This perinuclear staining disappeared when the effector domain mutants (RalA72L49N, or RalA72L49E) were introduced to the cell, suggesting that this staining pattern is likely to be functionally relevant to the role of RalA in basolateral trafficking. RalB, on the other hand, has a denser punctuate

intracellular staining pattern and little RalB is observed on the plasma membrane. This difference in the localization pattern is consistent with the observation that RalA but not RalB is important for basolateral membrane targeting in polarized epithelial cells (Shipitsin and Feig, 2004).

In addition to its role in regulating polarized membrane trafficking in epithelial cells, a more recent study discovered a role for the interaction between RalA and the exocyst complex in insulin-dependent Glut4 translocation in adipocytes. To identify proteins that might be involved in vesicle: exocyst recognition, researchers screened for vesicle localized GTPases in adipocytes by pull-down experiments. RalA, but not RalB, Arf6 or Rab11 specifically precipitated the exocyst components, including Sec5, Sec8, Exo84, and Exo70 in a GTP dependent manner in both 3T3L1 adipocytes and primary mouse adipocytes(Chen *et al.*, 2007). RalA is activated upon insulin stimulation in a dose dependent manner. Overexpression of the dominant negative (GDP-mutant) form of RalA blocked the insulin stimulated Glut4 translocation as well as its subsequent fusion with the plasma membrane, suggesting that RalA plays an important role in Glut4 trafficking. In adipocytes, RalA was also found to be associated with the unconventional myosin, Myo1c, suggesting Ral may play a role in recruiting a vesicle motor as well as docking the Glut4-containing vesicles to the membrane by allowing formation of a stable tethering complex(Chen *et al.*, 2007).

Rho GTPases: Yeast as a Model for Polarity

The Rho family of small GTPases are regulators of many biological processes including cell polarization, morphogenesis, cell growth and development(Symons and Rusk, 2003). The

function of the Rho family small GTPases in spatial regulation of exocytosis has been most extensively examined in the yeast, *Saccharomyces cerevisae.* Yeast is an excellent model for studying polarized secretion due to the highly polarized nature of its pattern of growth and the extensive genetic and cell biological tools available to analyze membrane trafficking and cytoskeletal structures within these cells(Brennwald and Rossi, 2007). Yeast has six Rho proteins, Rho1-5 and Cdc42. Among these six proteins, Rho1, Rho3 and Cdc42 have been most carefully studied and have each been implicated in regulation of polarized exocytosis.

Rho1: Important for the Localization of the Sec3 Component of the Exocyst Complex

The Rho1GTPaseis essential to many biological processes in yeast and has been suggested to regulate a variety of downstream effectors, including protein kinase C, PKC1(Nonaka *et al.*, 1995; Helliwell *et al.*, 1998), the formin family protein, Bni1(Tolliday *et al.*, 2002), and the cell wall beta glucan synthases, Fks1 and Fks2(Mazur and Baginsky, 1996). Studies in different *rho1* mutants revealed that Rho1 has an important role in regulating the localization of the Sec3 component of the exocyst complex(Guo *et al.*, 2001). This was found to be due to a GTP dependent interaction between Rho1 and the non-essential N terminal domain of Sec3 (**Figure 2**). However, in strains where the sole source of Sec3 lacked the N-terminal Rho1 interaction domain, the remaining exocyst subunits were found to be polarized normally and secretion was also normal(Guo *et al.*, 2001; Roumanie *et al.*, 2005). This demonstrates that the remaining components of the exocyst complex must be polarized by a distinct pathway which is independent of both the N-terminal domain of Sec3 and Rho1. While this interaction is not essential, Sec3 mutants lacking this domain exhibit synthetic genetic interactions with a secretory deficient allele of Cdc42 and other late acting

secretory mutants (Roumanie *et al.*, 2005). This is consistent with the notion that this domain of Sec3 functions to increase the local concentration of Sec3 at sites of growth, although significantly lower amounts are sufficient to promote full secretory function under most circumstances.

Rho3: A Direct Regulator of Exocytosis

The first evidence for the participation of a Rho GTPase in exocytic function came from two genetic screens. The first screen focused on genes which, when overexpressed, rescued the extremely slow growth phenotype associated with loss of Rho3 (Matsui and Toh-e, 1992; Matsui and Toh, 1992). This screen isolated a number of genes including *BEM1*, *CDC42*, and two genes later identified as coding for the yeast Rab GTPase, *SEC4* and its effector, *SRO7*(Matsui and Toh-e, 1992; Matsui and Toh, 1992; Imai *et al.*, 1996). A second screen identified *RHO3* itself as a potent suppressor of a cold-sensitive allele in *SEC4* (Brennwald *et al.*, 1994; Adamo *et al.*, 1999). Further characterization demonstrated that Rho3 was the only one of the five *RHO* genes in yeast that could function as a suppressor for the *sec4-P48* mutant (Adamo *et al.*, 1999). Rho3 also showed significant level of suppression of both the *sec15-1* and the *sec8-9* mutants, both of which are components of the exocyst, suggesting that Rho3 plays an important role in regulating exocytosis through the exocyst complex.

Analysis of a cold-sensitive effector domain mutant of Rho3, *rho3-V51*, was particularly informative (Adamo *et al.*, 1999). This mutant demonstrated a profound secretory defect and accumulation of post-Golgi vesicles following a shift to the restrictive temperature. However, unlike other *rho3* mutants examined, the polarization of the actin cytoskeleton was found to be normal at both permissive and non-permissive conditions. This

was the first evidence for a direct role for Rho3 in exocytosis independent of the cytoskeleton. Biochemical and yeast-two hybrid analysis demonstrated that the *rho3-V51* mutation blocked the ability of otherwise activated forms of Rho3 to bind to the Exo70 subunit of the exocyst. This suggests that Exo70 may be the immediate target of Rho3 regulation of exocytic function. However, unlike the effect of Rho1 on Sec3 localization, this mutation was found to exert its effects on exocytosis independent of any detectable effects on Exo70 or exocyst localization (Roumanie *et al.*, 2005).

Cdc42: A Cell Cycle-Specific Regulator of Exocytosis

Cdc42 is a member of the Rho GTPase family that that plays an important role in coordinating a number of events necessary for polarized growth in yeast cells (Park and Bi, 2007).The identification of a novel temperature sensitive mutant, *cdc42-6*, led to the characterization of a new role for Cdc42 function in exocytosis (Adamo *et al.*, 2001). This mutant displays properties that are distinct from previously described alleles of Cdc42 in that both actin polarity and budding appeared relatively normal. Genetic analysis demonstrated that *cdc42-6* was likely to be defective for a pathway closely linked to that of *rho3-V51*asboth mutants were suppressed by a common set of genes including *SEC4*, *SRO7*, and *SEC9*. In addition, high copy *CDC42* was found to suppress *rho3* mutant growth defects, as *RHO3* was found to suppress *cdc42-6* growth defects suggesting these two Rho GTPases likely function to regulate a common effector pathway. Furthermore, the synthetic lethality observed in crosses of the *cdc42-6* and *rho3-V51* mutants provided strong evidence that the effector pathways of these two GTPases functionally overlap.

Analysis of the secretory capacity of the*cdc42-6* mutant revealed a severe defect in the secretion of Bgl2, an abundant periplasmic enzyme involved in cell wall remodeling, as well as the accumulation of 80-100 nm post-Golgi vesicles by electronic microscopy. Interestingly, this mutant showed no defect in secretion of invertase—which is thought to be carried by a separate class of vesicles from that used to transport Bgl2 to the cell surface (Harsay and Bretscher, 1995). Surprisingly, electron microscopy studies on *cdc42-6* cells demonstrated that only cells with small buds were found to accumulate post-Golgi vesicles, while larger budded cells showed no abnormal numbers of vesicles. Consistent with the idea that the exocytic defect in the *cdc42-6* mutant is specifically associated with early bud emergence, Bgl2 secretory defects were found to mirror the time of appearance of small buds when secretion assays were conducted on synchronized populations of cells. Similarly to *rho3-V51*, the *cdc42-6* mutation was found to exert its effects on exocytosis independent of any detectable effects on Exo70 or exocyst localization (Adamo *et al.*, 2001; Roumanie *et al.*, 2005).

TC10: A Cdc42 Family GTPase Involved in Glucose Transporter Trafficking

Insulin stimulation results in a dramatic translocation of the GLUT4 protein to the plasma membrane via a dynamic membrane trafficking system, including vesicle sorting, budding, trafficking, tethering, docking and fusion of the GLUT4 containing post-Golgi vesicles. Extensive efforts have been made to identify the mechanism by which plasma membrane translocation of GLUT4 occurs upon insulin stimulation. Recently, it has been reported that the Rho family small GTPase TC10 plays a critical role in regulating this signaling pathway. To search for potential effectors of TC10 that have a role in insulin-

stimulated glucose transport, researchers screened a yeast two hybrid cDNA library derived from 3T3L1 adipocytes with a constitutively active (GTP) form of human TC10alpha(Inoue *et al.*, 2003). From this screen, they identified Exo70 as a potential downstream target for TC10. This interaction is specific to the GTP-bound form of TC10 and it is not observed with GTP-bound forms of other small GTPases such as Rac and Cdc42. A dominant negative form of Exo70 blocked the effects of insulin on Glut4 transport to the surface in 3T3 L1 adipocytes (Inoue *et al.*, 2003). Interestingly, dominant negative TC10 in the presence of insulin results in Glut4-containing vesicles appearing close to the cell surface. This suggests that the function of TC10 and the exocyst on Glut4 surface transport is at the level of Glut4 vesicle fusion rather than delivery or docking events (see **Figure 3**) (Inoue *et al.*, 2003).

Rho/Cdc42 Regulation of the Exocyst is Distinct from Rab Regulation.

While many GTPases appear to work as signal transduction agents, other GTPases are thought to act to control the specificity and timing of macromolecular recognition events. Examples of the latter include Elongation Factor Tu (Bourne, 1988; Kaziro *et al.*, 1991; Rodnina *et al.*, 1995) and the SRP/SRP receptor complexes(Shan *et al.*, 2007). In the latter two examples, GTP hydrolysis and cycling through the GDP-bound, nucleotide-free, and GTP-bound states are critical for these GTPases to carry out their biological function (Rodnina *et al.*, 1995; Shan *et al.*, 2007). In contrast, GTPases which function as signal-transducers are able to do so without any need for GTP hydrolysis per se. A simple test of this distinction is to examine the effect of GTP-hydrolysis deficient mutants on the biological activity of the protein. Such mutations are predicted to lead to heightened activity or gain-of-

function effects on signaling GTPases but are expected to lead to loss-of-function effects on cycling or non-signaling GTPases.

Extension of this analysis to Rab and Rho GTPase function in exocytosis leads to some interesting differences in behavior—even in situations where the target "effector" complex is shared by these different GTPases. Hydrolysis mutant forms of the yeast Rab, Sec4, are known to enhance the interaction with the Sec15 component of the exocyst. However, when introduced as the sole source of Sec4, the mutant behaves as a recessive loss-of-function allele, which is cold-sensitive and lethal when combined with other late-acting secretory mutants (Walworth *et al.*, 1992). Similar recessive loss-of-function phenotypes were observed with a GTP-hydrolysis mutant in Ypt1, a Rab involved in ER-to-Golgi transport (Richardson *et al.*, 1998). In contrast GTP hydrolysis deficient forms of Rho3 which stimulate the interaction with the Exo70 component of the exocyst are fully functional as the only source of Rho3 and behave as gain-of-function alleles strongly suppressing a number of late-acting secretory mutants(Roumanie *et al.*, 2005). Similarly, GTP hydrolysis-deficient forms of Cdc42 also appear to be functional in promoting secretory function when expressed at low levels—although they are toxic to other pathways when expressed at higher levels (Roumanie *et al.*, 2005). Taken together these data implicate Rho3/Cdc42 regulation of the exocyst as a pathway similar to other signaling GTPases such as Ras, while the function of Sec4 in regulating the exocyst is similar to recognition/cycling GTPases such as the SRP or EFTu.

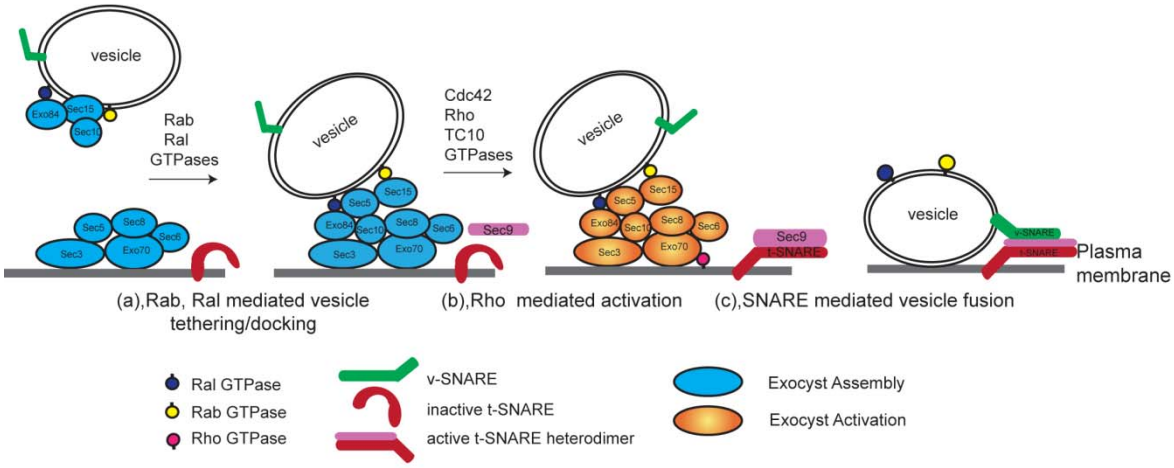


Figure 3:A three step model for vesicle docking, exocyst activation and vesicle fusion regulated by small GTPases.

(A) The initial vesicle docking or tethering event is regulated by Rab and Ral GTPases perhaps by promoting exocyst assembly. The association of particular exocyst subunits with the vesicle or plasma membrane in this cartoon is speculative. There is evidence that exocyst assembly is regulated by Ral and this function, like that of Rab GTPases, is first required for vesicle tethering rather than fusion(Moskalenko *et al.*, 2003). FRAP studies in yeast have suggested that all of the exocyst subunits except Sec3 are likely to be delivered to sites of polarized growth through vesicle-mediated events(Boyd *et al.*, 2004)**(B)** This is followed by local activation of the exocyst complex by Rho3/Cdc42/TC10 family GTPases in their active GTP-bound state. Exocyst activation results in a stimulation of downstream fusion activity, very likely by promoting assembly of active t-SNARE heterodimers. **(C)** The presence of active t-SNARE dimers would result in SNARE-mediated fusion of the secretory vesicles at the site of exocyst activation.

The Exocyst as a Landmark or an Activated Machine? Local Activation vs. Local Recruitment Models

Signaling GTPases regulate their effectors by one of two general mechanisms. The first mechanism involves regulation of the subcellular location of the downstream effector. In this mode the binding of the GTPase to its effector helps to localize and concentrate the effector at a particular place within the cell. This may then stimulate a signaling event by placing the effector within close proximity to its downstream signaling partner. A good

example of this mode of GTPase function is the Ras GTPase helps to promote oncogenic transformation through GTP-dependent interaction with the Raf kinase. The initial activation event in the Ras-Raf pathway involves recruitment of Raf kinase from the cytosol to the plasma membrane by membrane-bound Ras (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). This initial recruitment event then propagates other subsequent activation and signaling events (Mineo *et al.*, 1997) by placing activated Raf kinase in close proximity to its both its activator Ras, and downstream targets in the MAP kinase cascade to promote sustained activation and signaling (Morrison and Davis, 2003; Raman *et al.*, 2007).

A second mechanism of regulation by signaling GTPases involves regulation of activity rather than the location of the downstream effector. In this mode, the binding of the GTPase to the effector induces a conformational change which either directly or indirectly stimulates an associated enzymatic activity. A good example of direct regulation is the Formin family of actin nucleating enzymes which normally resides in an inactive "autoinhibited" conformation due to the association of the DID and DAD domains within the protein (Goode and Eck, 2007; Lu *et al.*, 2007) (see Figure 4). The closed conformation is inactive due to the inaccessibility of the catalytic domain to its substrate (actin monomers) in this structure. The binding of the Cdc42/Rho to the RBD domain adjacent to the DID domain disrupts the inhibitory interaction resulting in an opening of the structure and allowing its associated catalytic activity to be "active".

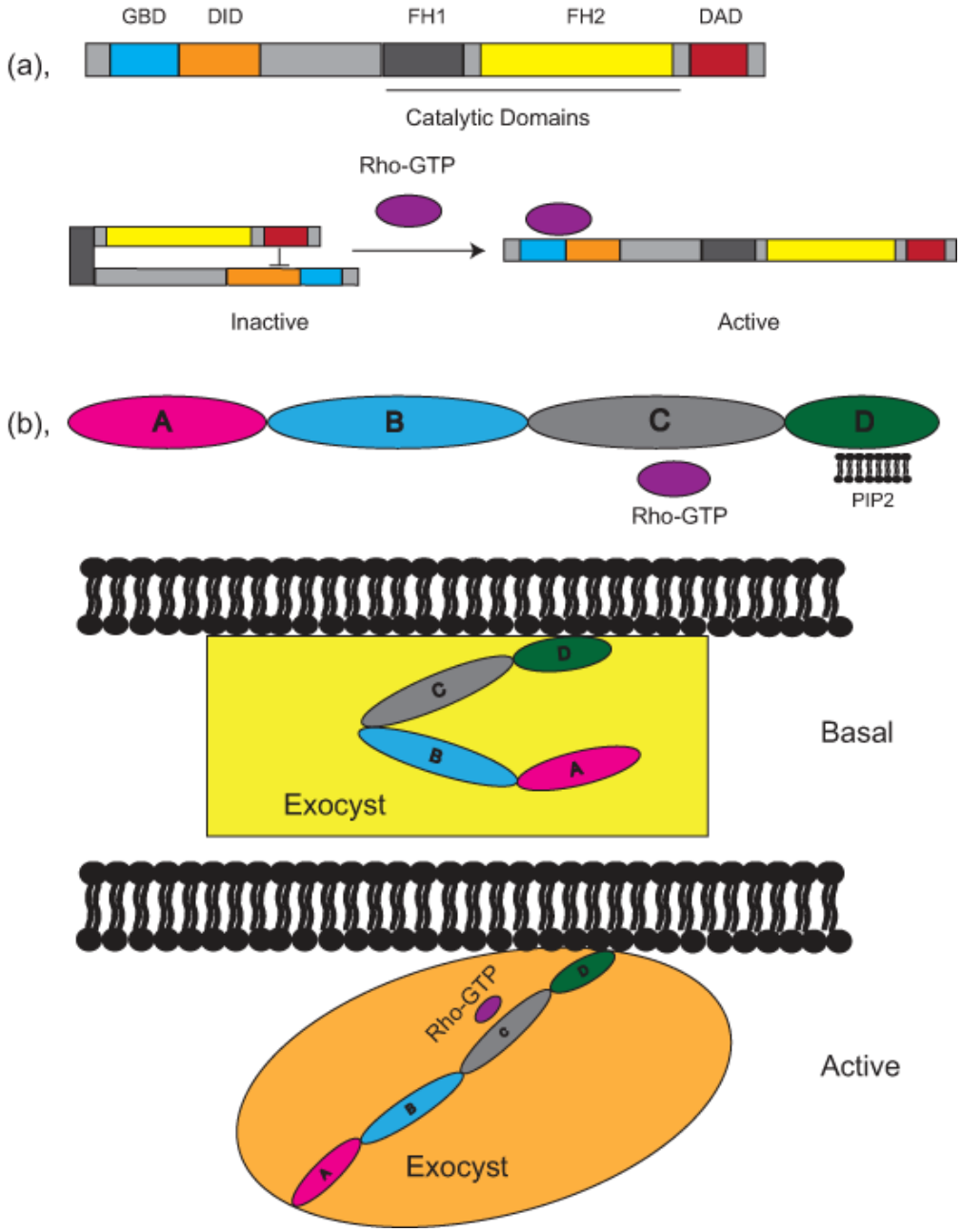


Figure 4:A model for activation of the exocyst complex by Rho family GTPases.

(A). Domain organization and molecular regulation of formins. In the absence of Rho GTP, formins are maintained in an inactive state by an autoinhibitory interaction between the DAD and DID domains, which is relieved by association of an active, GTP-bound Rho GTPases with the GBD domain. This interaction allows DID to adopt a structural conformation that induces release of the DAD domain, and leads to the activation of the formin protein.**(B)** Domain organization and model for molecular regulation of Exo70 and the exocyst complex

by Rho GTPase. The D domain of Exo70 interacts with phospholipids containing $PI(4,5)P_2$ and the C domain is necessary for the interaction with Rho family small GTPases. In the absence of Rho GTP, Exo70 along with other components of the exocyst complex, remain in the inactive or basal activity state. Upon interaction of Exo70 with Rho GTP, Exo70 adopts an alternate conformation which leads to the activation of the exocyst complex. The activation in this case could be the result of disrupting an inhibitor interaction between Exo70 and another subunit of the exocyst complex or a direct change in the conformation of Exo70 itself, which then leads to a change in the overall structure of the complex.

There are aspects of Rho regulation of the exocyst in yeast which fit each of these models. An example of the first would be the recruitment of the yeast Sec3 by binding of its N-terminus to Rho1(Guo *et al.*, 2001) (and to a lesser degree Cdc42(Zhang *et al.*, 2001)). While this interaction is not necessary for the function of Sec3 in promoting efficient exocytosis and growth (Guo *et al.*, 2001; Roumanie *et al.*, 2005), it is important for allowing Sec3 to efficiently localize to sites of polarized growth. Thus the interaction of the N-terminus of Sec3 with Rho1 is an example of Rho regulation by recruitment, similar to the Ras/Raf example.

In contrast to the Rho1/Sec3 model described above, regulation of exocytosis by Rho3 and Cdc42 appears to be independent of any effect on the localization of the exocytic machinery. The analysis of the loss of function mutants*rho3-V51* and *cdc42-6*clearly demonstrate that these GTPases have a direct and critical regulatory function on this process (Adamo *et al.*, 1999; Adamo *et al.*, 2001; Roumanie *et al.*, 2005). A simple explanation for these results is that Rho3 and Cdc42 regulation is through localized activation of the exocytic apparatus (likely through the Exo70 component of the exocyst—see below). This activation can be imagined to be a slight variation from the "relief of autoinihibition" mechanism used by Rho GTPases to modulate effector function of the formins, ROCK/Ste20 kinases, and

WASP (Prehoda *et al.*, 2000; Higgs and Pollard, 2001). The major difference is that Exo70 appears to function primarily as part of a larger multiprotein complex. In this way, the inhibitory interactions disrupted by Rho GTPase binding might disrupt a protein: protein interaction within the exocyst complex rather than within the Exo70 protein itself (see **Figure 4**). The result of the binding, however, would be quite similar: the exocyst would go from being in a form that has basal function to an "activated" complex which would support increased rates of docking and fusion events while in this state. In this view the exocyst complex functions not as a static scaffold for allowing vesicles to dock with the plasma membrane, but rather as a quite dynamic machine with real catalytic function that can be modulated to control the rate at which vesicle docking and fusion with a specific site in the membrane can occur.

Consistent with the model that the Rho proteins are mostly responsible for activating the exocyst at the site of polarized growth but not localizing the exocyst complex, there are new results showing that the interaction between the exocyst components and phospholipids might be important for mediating the targeting of the exocyst to the plasma membrane. Both Exo70 and Sec3 have been shown to interact with phospholipids (He *et al.*, 2007b; Zhang *et al.*, 2008). However, the interactions are mediated through two different domains. Sec3 interacts with PI4,5P through its N terminus (Zhang *et al.*, 2008), whereasExo70 interacts with PI4,5P through the C terminal domain (He *et al.*, 2007b), which is the most conservative domain on Exo70 in different species (Liu *et al.*, 2007). The interactions with Rho family small GTPases and with phospholipids are both required for proper localization and final activation of the exocyst complex(He *et al.*, 2007b; Liu *et al.*, 2007; Zhang *et al.*, 2008).

What more precisely might this machine be catalyzing? There are many possibilities for this but an attractive target for this catalysis may be the formation of active t-SNARE complexes on the plasma membrane. It is likely that the vast majority of t-SNAREs are present on the plasma membrane in an uncomplexed form (Brennwald *et al.*, 1994; Grote *et al.*, 2000). Biochemical and kinetic analyses have also made it clear that the formation of the t-SNARE dimers of Sec9 and Sso1/2 is likely to be an extremely slow and inefficient process (Rossi *et al.*, 1997; Nicholson *et al.*, 1998). However, evidence for stable interactions between intact exocyst complex and SNARE proteins has not been detected (Brennwald *et al.*, 1994). Recently, it was found that recombinant forms of the Sec6 protein, in the absence of the other exocyst subunits, show high affinity interactions with the t-SNARE, Sec9(Sivaram *et al.*, 2005). This suggests the possibility that within the exocyst complex, Sec6 may transiently interact with the Sec9 as a means of regulating t-SNARE assembly and vesicle fusion. This transient interaction with Sec9 would be regulated by the functional state of the exocyst complex which may involve both Rho/Cdc42 "throttling" as well as a requirement for Sec4-GTP binding and release as the final triggering event. Clearly an important area for future work will be to clarify the molecular mechanism of how exocyst activation is transmitted onto the downstream SNARE-dependent fusion events.

Concluding Remarks

Work over the last decade has shown small GTPases to be critical regulators of both cell polarity and membrane trafficking events in the cell. The multisubunit protein complex known as the exocyst complex has turned out to be an important target for coordination of trafficking and cell polarization decisions. A number of different subunits of the exocyst

have evolved mechanisms by which regulatory signals from small GTPases act on specific aspects of exocyst function. These signals appear to directed at one of two stages of exocyst activity. The first point of regulation is in the vesicle tethering or docking stage where the exocyst helps to proof-read the correct vesicle target membrane combination. The second stage is the vesicle fusion reaction where the exocyst appears likely to regulate localized SNARE assembly. By acting at these two steps, members of the Rab, Ral, and Rho GTPase families are able to regulate the fidelity of these events at the same time they modulate the temporal and spatial nature of cell surface delivery. Future work will help to unravel the details of how these regulatory interactions act mechanistically to spatially and temporally regulate exocytosis is both and unpolarized cells.

ACKNOWLEDGEMENT

We thank James Gardner for helpful comments on the manuscript. This work was supported by grants from the National Institutes of Health (GM54712) and The G. Harold and Leila Y. Mathers Charitable Foundation.

REFERENCE

Adamo, J.E., Moskow, J.J., Gladfelter, A.S., Viterbo, D., Lew, D.J., and Brennwald, P.J. (2001). Yeast Cdc42 functions at a late step in exocytosis, specifically during polarized growth of the emerging bud. J Cell Biol *155*, 581-592.

Adamo, J.E., Rossi, G., and Brennwald, P. (1999). The Rho GTPase Rho3 has a direct role in exocytosis that is distinct from its role in actin polarity. Mol Biol Cell *10*, 4121-4133.

Barrowman, J., and Novick, P. (2003). Three Yips for Rab recruitment. Nat Cell Biol *5*, 955-956.

Bourne, H.R. (1988). Do GTPases direct membrane traffic in secretion? Cell *53*, 669-671.

Brennwald, P., Kearns, B., Champion, K., Keranen, S., Bankaitis, V., and Novick, P. (1994). Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. Cell *79*, 245-258.

Brennwald, P., and Rossi, G. (2007). Spatial regulation of exocytosis and cell polarity: yeast as a model for animal cells. FEBS Lett *581*, 2119-2124.

Camonis, J.H., and White, M.A. (2005). Ral GTPases: corrupting the exocyst in cancer cells. Trends Cell Biol *15*, 327-332.

Chen, X.W., Leto, D., Chiang, S.H., Wang, Q., and Saltiel, A.R. (2007). Activation of RalA is required for insulin-stimulated Glut4 trafficking to the plasma membrane via the exocyst and the motor protein Myo1c. Dev Cell *13*, 391-404.

Conibear, E., Cleck, J.N., and Stevens, T.H. (2003). Vps51p mediates the association of the GARP (Vps52/53/54) complex with the late Golgi t-SNARE Tlg1p. Mol Biol Cell *14*, 1610-1623.

Gangar, A., Rossi, G., Andreeva, A., Hales, R., and Brennwald, P. (2005). Structurally conserved interaction of Lgl family with SNAREs is critical to their cellular function. Curr Biol *15*, 1136-1142.

Goode, B.L., and Eck, M.J. (2007). Mechanism and function of formins in the control of actin assembly. Annu Rev Biochem *76*, 593-627.

Goud, B., Salminen, A., Walworth, N.C., and Novick, P.J. (1988). A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. Cell *53*, 753-768.

Grosshans, B.L., Andreeva, A., Gangar, A., Niessen, S., Yates, J.R., 3rd, Brennwald, P., and Novick, P. (2006). The yeast lgl family member Sro7p is an effector of the secretory Rab GTPase Sec4p. J Cell Biol *172*, 55-66.

Grote, E., Carr, C.M., and Novick, P.J. (2000). Ordering the final events in yeast exocytosis. J Cell Biol *151*, 439-452.

Guo, W., Roth, D., Walch-Solimena, C., and Novick, P. (1999). The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. EMBO J *18*, 1071-1080.

Guo, W., Tamanoi, F., and Novick, P. (2001). Spatial regulation of the exocyst complex by Rho1 GTPase. Nat Cell Biol *3*, 353-360.

Hamburger, Z.A., Hamburger, A.E., West, A.P., Jr., and Weis, W.I. (2006). Crystal structure of the S.cerevisiae exocyst component Exo70p. J Mol Biol *356*, 9-21.

Harsay, E., and Bretscher, A. (1995). Parallel secretory pathways to the cell surface in yeast. J Cell Biol *131*, 297-310.

Hattendorf, D.A., Andreeva, A., Gangar, A., Brennwald, P.J., and Weis, W.I. (2007). Structure of the yeast polarity protein Sro7 reveals a SNARE regulatory mechanism. Nature *446*, 567-571.

He, B., Xi, F., Zhang, X., Zhang, J., and Guo, W. (2007). Exo70 interacts with phospholipids and mediates the targeting of the exocyst to the plasma membrane. EMBO J *26*, 4053-4065.

Helliwell, S.B., Schmidt, A., Ohya, Y., and Hall, M.N. (1998). The Rho1 effector Pkc1, but not Bni1, mediates signalling from Tor2 to the actin cytoskeleton. Curr Biol *8*, 1211-1214.

Higgs, H.N., and Pollard, T.D. (2001). Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. Annu Rev Biochem *70*, 649-676.

Imai, J., Toh-e, A., and Matsui, Y. (1996). Genetic analysis of the Saccharomyces cerevisiae RHO3 gene, encoding a rho-type small GTPase, provides evidence for a role in bud formation. Genetics *142*, 359-369.

Inoue, M., Chang, L., Hwang, J., Chiang, S.H., and Saltiel, A.R. (2003). The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin. Nature *422*, 629-633.

Jin, R., Junutula, J.R., Matern, H.T., Ervin, K.E., Scheller, R.H., and Brunger, A.T. (2005). Exo84 and Sec5 are competitive regulatory Sec6/8 effectors to the RalA GTPase. EMBO J *24*, 2064-2074.

Jullien-Flores, V., Mahe, Y., Mirey, G., Leprince, C., Meunier-Bisceuil, B., Sorkin, A., and Camonis, J.H. (2000). RLIP76, an effector of the GTPase Ral, interacts with the AP2 complex: involvement of the Ral pathway in receptor endocytosis. J Cell Sci *113 (Pt 16)*, 2837-2844.

Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M., and Satoh, T. (1991). Structure and function of signal-transducing GTP-binding proteins. Annu Rev Biochem *60*, 349-400.

Leevers, S.J., Paterson, H.F., and Marshall, C.J. (1994). Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature *369*, 411-414.

Lehman, K., Rossi, G., Adamo, J.E., and Brennwald, P. (1999). Yeast homologues of tomosyn and lethal giant larvae function in exocytosis and are associated with the plasma membrane SNARE, Sec9. J Cell Biol *146*, 125-140.

Liu, J., Zuo, X., Yue, P., and Guo, W. (2007). Phosphatidylinositol 4,5-bisphosphate mediates the targeting of the exocyst to the plasma membrane for exocytosis in mammalian cells. Mol Biol Cell *18*, 4483-4492.

Lu, J., Meng, W., Poy, F., Maiti, S., Goode, B.L., and Eck, M.J. (2007). Structure of the FH2 domain of Daam1: implications for formin regulation of actin assembly. J Mol Biol *369*, 1258-1269.

Matsui, Y., and Toh-e, A. (1992). Isolation and characterization of two novel ras superfamily genes in Saccharomyces cerevisiae. Gene *114*, 43-49.

Matsui, Y., and Toh, E.A. (1992). Yeast RHO3 and RHO4 ras superfamily genes are necessary for bud growth, and their defect is suppressed by a high dose of bud formation genes CDC42 and BEM1. Mol Cell Biol *12*, 5690-5699.

Mazur, P., and Baginsky, W. (1996). In vitro activity of 1,3-beta-D-glucan synthase requires the GTP-binding protein Rho1. J Biol Chem *271*, 14604-14609.

Mineo, C., Anderson, R.G., and White, M.A. (1997). Physical association with ras enhances activation of membrane-bound raf (RafCAAX). J Biol Chem *272*, 10345-10348.

Morrison, D.K., and Davis, R.J. (2003). Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annu Rev Cell Dev Biol *19*, 91-118.

Moskalenko, S., Henry, D.O., Rosse, C., Mirey, G., Camonis, J.H., and White, M.A. (2002). The exocyst is a Ral effector complex. Nat Cell Biol *4*, 66-72.

Munson, M., and Novick, P. (2006). The exocyst defrocked, a framework of rods revealed. Nat Struct Mol Biol *13*, 577-581.

Nicholson, K.L., Munson, M., Miller, R.B., Filip, T.J., Fairman, R., and Hughson, F.M. (1998). Regulation of SNARE complex assembly by an N-terminal domain of the t-SNARE Sso1p. Nat Struct Biol *5*, 793-802.

Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A., and Takai, Y. (1995). A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in Saccharomyces cerevisiae. EMBO J *14*, 5931-5938.

Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell *21*, 205-215.

Ohta, Y., Suzuki, N., Nakamura, S., Hartwig, J.H., and Stossel, T.P. (1999). The small GTPase RalA targets filamin to induce filopodia. Proc Natl Acad Sci U S A *96*, 2122-2128.

Park, H.O., and Bi, E. (2007). Central roles of small GTPases in the development of cell polarity in yeast and beyond. Microbiol Mol Biol Rev *71*, 48-96.

Pfeffer, S.R. (1994). Rab GTPases: master regulators of membrane trafficking. Curr Opin Cell Biol *6*, 522-526.

Prehoda, K.E., Scott, J.A., Mullins, R.D., and Lim, W.A. (2000). Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. Science *290*, 801-806.

Raman, M., Chen, W., and Cobb, M.H. (2007). Differential regulation and properties of MAPKs. Oncogene *26*, 3100-3112.

Richardson, C.J., Jones, S., Litt, R.J., and Segev, N. (1998). GTP hydrolysis is not important for Ypt1 GTPase function in vesicular transport. Mol Cell Biol *18*, 827-838.

Rodnina, M.V., Pape, T., Fricke, R., and Wintermeyer, W. (1995). Elongation factor Tu, a GTPase triggered by codon recognition on the ribosome: mechanism and GTP consumption. Biochem Cell Biol *73*, 1221-1227.

Rossi, G., Salminen, A., Rice, L.M., Brunger, A.T., and Brennwald, P. (1997). Analysis of a yeast SNARE complex reveals remarkable similarity to the neuronal SNARE complex and a novel function for the C terminus of the SNAP-25 homolog, Sec9. J Biol Chem *272*, 16610-16617.

Roumanie, O., Wu, H., Molk, J.N., Rossi, G., Bloom, K., and Brennwald, P. (2005). Rho GTPase regulation of exocytosis in yeast is independent of GTP hydrolysis and polarization of the exocyst complex. J Cell Biol *170*, 583-594.

Salminen, A., and Novick, P.J. (1987). A ras-like protein is required for a post-Golgi event in yeast secretion. Cell *49*, 527-538.

Salminen, A., and Novick, P.J. (1989). The Sec15 protein responds to the function of the GTP binding protein, Sec4, to control vesicular traffic in yeast. J Cell Biol *109*, 1023-1036.

Shan, S.O., Chandrasekar, S., and Walter, P. (2007). Conformational changes in the GTPase modules of the signal reception particle and its receptor drive initiation of protein translocation. J Cell Biol *178*, 611-620.

Shipitsin, M., and Feig, L.A. (2004). RalA but not RalB enhances polarized delivery of membrane proteins to the basolateral surface of epithelial cells. Mol Cell Biol *24*, 5746-5756.

Sivaram, M.V., Saporita, J.A., Furgason, M.L., Boettcher, A.J., and Munson, M. (2005). Dimerization of the exocyst protein Sec6p and its interaction with the t-SNARE Sec9p. Biochemistry *44*, 6302-6311.

Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M., and Hancock, J.F. (1994). Activation of Raf as a result of recruitment to the plasma membrane. Science *264*, 1463-1467.

Symons, M., and Rusk, N. (2003). Control of vesicular trafficking by Rho GTPases. Curr Biol *13*, R409-418.

Tolliday, N., VerPlank, L., and Li, R. (2002). Rho1 directs formin-mediated actin ring assembly during budding yeast cytokinesis. Curr Biol *12*, 1864-1870.

Vitale, N., Mawet, J., Camonis, J., Regazzi, R., Bader, M.F., and Chasserot-Golaz, S. (2005). The Small GTPase RalA controls exocytosis of large dense core secretory granules by interacting with ARF6-dependent phospholipase D1. J Biol Chem *280*, 29921-29928.

Walworth, N.C., Brennwald, P., Kabcenell, A.K., Garrett, M., and Novick, P. (1992). Hydrolysis of GTP by Sec4 protein plays an important role in vesicular transport and is stimulated by a GTPase-activating protein in Saccharomyces cerevisiae. Mol Cell Biol *12*, 2017-2028.

Whyte, J.R., and Munro, S. (2001). The Sec34/35 Golgi transport complex is related to the exocyst, defining a family of complexes involved in multiple steps of membrane traffic. Dev Cell *1*, 527-537.

Wu, S., Mehta, S.Q., Pichaud, F., Bellen, H.J., and Quiocho, F.A. (2005). Sec15 interacts with Rab11 via a novel domain and affects Rab11 localization in vivo. Nat Struct Mol Biol *12*, 879-885.

Zhang, X., Bi, E., Novick, P., Du, L., Kozminski, K.G., Lipschutz, J.H., and Guo, W. (2001). Cdc42 interacts with the exocyst and regulates polarized secretion. J Biol Chem *276*, 46745-46750.

Zhang, X., Orlando, K., He, B., Xi, F., Zhang, J., Zajac, A., and Guo, W. (2008). Membrane association and functional regulation of Sec3 by phospholipids and Cdc42. J Cell Biol *180*, 145-158.

Zhang, X., Wang, P., Gangar, A., Zhang, J., Brennwald, P., TerBush, D., and Guo, W. (2005). Lethal giant larvae proteins interact with the exocyst complex and are involved in polarized exocytosis. J Cell Biol *170*, 273-283.

Zhang, X.M., Ellis, S., Sriratana, A., Mitchell, C.A., and Rowe, T. (2004). Sec15 is an effector for the Rab11 GTPase in mammalian cells. J Biol Chem *279*, 43027-43034.

CHAPTER THREE

The Exo70 Subunit of the Exocyst is an Effector for Both Cdc42 and Rho3 Function in Polarized Exocytosis

ABSTRACT

The Rho3 and Cdc42 members of the Rho GTPase family are important regulators of exocytosis in yeast. However, the precise mechanism by which they regulate this process is controversial. Here we present evidence that the Exo70 component of the exocyst complex is a direct effector of both Rho3 and Cdc42. We identify gain-of-function mutants in *EXO70* that potently suppress mutants in *RHO3* and *CDC42* defective for exocytic function. We show that Exo70 has the biochemical properties expected of a direct effector for both Rho3 and Cdc42. Surprisingly, we find that C-terminal prenylation of these GTPases both promotes the interaction and influences the sites of binding within Exo70. Finally, we demonstrate that the phenotypes associated with novel loss-of-function mutants in *EXO70*, are entirely consistent with Exo70 as an effector for both Rho3 and Cdc42 function in secretion. These data suggests that interaction with the Exo70 component of the exocyst is a key event in spatial regulation of exocytosis by Rho GTPases.

INTRODUCTION

The ability of eukaryotic cells to grow asymmetrically relies on the delivery of lipids and proteins to specific sites on the cell surface. This delivery is largely mediated by the docking and fusion of secretory vesicles with a precisely demarcated region of the plasma membrane. The budding yeast *Saccharomyces cerevisiae* undergoes polarized growth throughout its cell cycle, which makes it an attractive model for studying the regulation of polarized exocytosis and its role in overall cell polarity. An important factor in targeting of secretory vesicles to the plasma membrane is an evolutionary conserved eight-subunit protein complex, known as the exocyst complex, which is composed of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 (for review, see (Hsu *et al.*, 2004; Munson and Novick, 2006; Wu *et al.*, 2008).

Both Rab and Rho family small GTPases have been shown to be the upstream regulators of exocyst function in vesicle docking and fusion. The Rab family small GTPase, Sec4, directly interacts with the Sec15 subunit of the exocyst and it is this interaction that is thought to help link the exocyst complex to the vesicle during docking (Guo *et al.*, 1999b). The Rho family small GTPases are also important regulators of polarized exocytosis in yeast. Previous studies have shown that specific conditional mutations in Rho3 or Cdc42 results in actin-independent defects in exocytosis (Adamo *et al.*, 1999; Adamo *et al.*, 2001). While Rho3 and Cdc42 appear to have partially overlapping functions, they are also individually required for polarized exocytosis; loss of function in each GTPase shows a specific set of phenotypes when their secretory function is impaired. The secretory defective mutant form of Cdc42, *cdc42-6,* accumulates post-Golgi vesicles only during bud emergence (i.e. small

budded cells) and displays a pronounced defect in secretion of Bgl2 but not in secretion of invertase. In contrast, the secretory defective form of Rho3, *rho3-V51*, accumulates post-Golgi vesicles throughout the cell cycle and has pronounced defects in secretion of both Bgl2 and invertase pathways.

Exo70 has been implicated as a possible effector for Rho3 based on its ability to interact with Rho3 in a GTP-dependent manner (Robinson *et al.*, 1999), and the fact that the interaction of Rho3 with Exo70 is blocked by the presence of the *rho3-V51* effector domain mutation (Adamo *et al.*, 1999). Interestingly, recent studies have identified *exo70* mutant alleles, *exo70-35* and *exo70-38*, that demonstrate a small-bud specific and Bgl2-specific secretion defect, phenotypes that are remarkably similar to that of *cdc42-6*. These results could suggest a role for Exo70 as a target for Cdc42 during exocytosis, however a physical interaction between activated recombinant Cdc42 and Exo70 was not detected (He *et al.*, 2007a).

The recent determination of the crystal structure of Exo70 proteins from yeast and mice revealed a conserved rod structure with four domains arranged sequentially from the N to the C terminus (Dong *et al.*, 2005; Hamburger *et al.*, 2006; Moore *et al.*, 2007). Structural similarity also appears to exist between the C-terminal domain of Exo70 and other members of the exocyst complex (Dong *et al.*, 2005; Munson and Novick, 2006; Sivaram *et al.*, 2006). However, despite the conservation in overall structure of yeast and mammalian Exo70, the site of interaction with Rho family GTPases appeared to be quite distinct. While recombinant Rho3 was found to bind primarily through interactions within domain C of yeast

Exo70, the site of interaction between the mouse Cdc42 homolog TC10 and mouse Exo70 was mapped to the N-terminal AB domains (Chiang *et al.*, 2001).

Recent work has called into question the role of Exo70 as an effector of Rho3 function in exocytosis. Examination of two alleles in *EXO70* suggested that Exo70 function was required only in small budded cells (He *et al.*, 2007a), in contrast to studies on Rho3 demonstrating exocytic defects in both small and large budded yeast (Adamo *et al.*, 1999; Adamo *et al.*, 2001). More importantly, mutants or deletions in Exo70 predicted to block the interaction with Rho3 *in vitro*, showed little to no effect on growth or secretory function of *EXO70* (He et al., 2007b; Hutagalung et al., 2009). Taken together these results suggested that Exo70 was unlikely to be the effector for Rho3 function in exocytosis.

In the current study we report a combination of genetic and biochemical examinations to determine the possible role of Exo70 as a target of Rho3 and Cdc42 function in exocytosis. First, we describe the isolation of dominant gain-of-function alleles of *EXO70* which strongly suppress the loss of either Rho3 or Cdc42 function in the exocytic pathway. Second, we describe a biochemical assay utilizing post-translationally modified forms of Rho3 and Cdc42 to demonstrate that Exo70 has the biochemical properties of a direct effector for both of these GTPases. Interestingly, we found that C-terminally prenylated forms of Rho3 and Cdc42 are able to interact with Exo70 in a manner that is structurally distinct from that of unmodified recombinant Rho3. Finally, we describe the isolation of novel recessive alleles of *EXO70* which demonstrates that the spectrum of phenotypes associated with Exo70 loss of function is in strong agreement with its role as an effector for both Cdc42 and Rho3 function in polarized exocytosis.

MATERIALS AND METHODS

Yeast Strains, Reagents, and Genetic Techniques

Cells were grown in YPD media containing 1% bacto-yeast extract, 2% bacto-peptone, and 2% glucose. The components of the media were from Fisher Scientific. For all assays performed, 25°C was the permissive temperature, whereas 14°C and 37°C were used as the restrictive temperatures. Sorbital, sodium azide (NaN3), *N*-ethylmaleimide (NEM), β-mercaptoethanol, *o*-dianasidine, glucose oxidase, peroxidase, Triton X-100, NP40, and HIS-Select® Nickel Affinity Gel were obtained from Sigma Chemical (St. Louis, MO). Zymolyase (100T) was from Seikagaku (Tokyo, Japan). BSA, yeast nitrogen base, raffinose galactose and 5-Fluoroorotic Acid were from US Biologicals (Swampscott, MA). Glutathione sepharose beads and protein A beads are from Amershan Biosciences. GTPγS and GDP are from Roche-Applied-Science. Secondary antibodies for Odyssey Imaging system are from LI-COR Biosciences and Molecular Probes. Rabbit anti-mouse antibody is from Jackson ImmunoResearch. Formaldehyde (37%), gluteraldehyde, and Spurr's resin were from Electron Microscopy Sciences (Ft. Washington, PA). The TNT *in vitro* translation system for PCR products are from Promega. L-[^{35}S] methione is from Perkin Elmer. Mevalonic acid is from Sigma. The bead beater for making yeast lysate is from Biospec Products. Transformations for suppression analysis were performed using the lithium acetate method described in Guthrie and Fink (1991). Strain crossing, tetrad dissection, diploid sporulation, and mating-type determination were performed as described by Guthrie and Fink (1991).

Dominant suppressor screen for Exo70 dominant mutants:

The *EXO70* dominant mutants in this study were generated by Error-prone PCR as described below. 50 ng digested vector and 3 µl PCR reaction were transformed into the *RHO3* plasmid shuffle strain (*rho3Δ::LEU2;his3-Δ200, ura3-52;leu2-3,112; pRS316-RHO3,URA3,CEN,*) and the *cdc42-6* strain (BY648 α, *cdc42-6*, *leu2,3-112; ura3-52*). *CEN HIS3* vector was used for *RHO3* plasmid shuffle strain, and *CEN URA3* vector was used for *cdc42-6* strain. Transformants were first grown on selective media for two days, and then replica plated onto 5FOA plates at 30^{o}C for the *RHO3* plasmid shuffle strain and YPD plates at 32^{o}C for *cdc42-6* mutant strain. Colonies from the restrictive conditions were grown in liquid media for plasmid recovery. The plasmids were retransformed into *RHO3* plasmid shuffle strain and *cdc42-6* mutant strain to confirm that the plasmids were responsible for the gain of function phenotype. From 6030 transformants from the *RHO3* plasmid shuffle strain, we isolated three plasmids that suppressed the *rho3Δ* at 30^{o}C. From 5660 transformants in the *cdc42-6* strain, we isolated 38 plasmids that had the ability to suppress the temperature sensitivity of the *cdc42-6* mutant at 32^{o}C.

Isolation of novel Exo70 mutants by random mutagenesis

The *EXO70* mutants isolated in this study were generated by GeneMorph II Random Mutagenesis Kit (Stratagene). 50ng plasmid pRS315 Exo70 was used as a template for PCR reaction performed under conditions that produce 0-4.5 mutations/kb (low frequency of mutagenesis) according to the manufacturers protocol. Oligonucleotide primers were designed to generate mutagenized PCR products containing the *EXO70* open reading frame flanked by 629 bp of 5' and 295bp of 3' sequence. The *EXO70* gap repair vector (BB1673)

was digested with *BamH*I and *Xba*I. 50 ng digested vector were co transformed with 3 µl PCR product into *EXO70* plasmid shuffle strain(BY861: *exo70Δ::HIS3;his3-Δ200, ura3-52;leu2-3,112; pRS316-EXO70,URA3,CEN*). Transformants that repaired the gapped plasmid by homologous recombination were selected at 30^{o}C on SD plates for two days. Transformants that could lose the wild type plasmid pRS316 Exo70 were selected by replica plating onto synthetic minimal media containing 5FOA while simultaneously selecting for the temperature sensitivity by incubation of replica plates at 37^{o}C, 25^{o}C, 17^{o}C, and 14^{o}C.

From 10396 colonies, 40 colonies exhibited temperature sensitivity and 57 colonies exhibited cold sensitivity. Plasmids from these colonies were isolated and transformed back into the original *EXO70* plasmid shuffle strain (BY861) to confirm that the plasmids were responsible for the temperature sensitivity and cold sensitivity. One plasmid was found to give rise to a cold sensitive phenotype (*exo70-188*) and one plasmid was found to have a temperature sensitive phenotype (*exo70-113*). Sequence analysis demonstrated that the cold-sensitive mutant, *exo70-188,* contained three mutations S305G, T523P, and L621I. Analysis of the temperature-sensitive allele, *exo70-113*, contained two mutations: N79K and incorporation of a stop codon at Y327 leading to truncation of the C-terminal half of the predicted protein.

Genetic analysis of mutants

To study the phenotypes of *exo70-188 and exo70-113* alleles as the only copy of *Exo70*, the constructs were integrated into the chromosome of the diploid strain BY841 (*a/α; exo70Δ::HIS3/EXO70; his3Δ200/his3Δ200; ura3-52/ura3-52; leu2-3,112/leu2-3,112*) at the *EXO70* locus. Transformants were sporulated and tetrads were dissected with the use of a

micromanipulator on YPD plates. The plates were grown at 25°C and the haploid progeny were analyzed for the presence of the mutants (scored as Kan^R), the absence of the wild-type *EXO70* (scored as *his3Δ200*), viability, and conditional growth defects.

Electron Microscopy

The exo70-188 and exo70-113 mutants were grown overnight to mid-log phase in YPD media. Half of the cultures were shifted to restrictive temperature for various time periods. The *exo70-188* mutant was shifted to a 14°C water bath shaker for nine hours, while the *exo70-113* mutant was shift to 37°C water bath shaker for 1.5 hours. Shifted and unshifted cells were processed as previously described (Adamo *et al.*, 1999).

Invertase assay and Bgl2 assay

The EXO70, exo70-188, exo70-113, rho3-V51, and sec6-4 cells were grown overnight to mid-log phase at 25°C in YPD liquid media. For the cold sensitive strains *exo70-188* and *rho3-v51*, the cells were pre-shifted to the restrictive temperature of 14°C for 1 hour. After the pre-shift, one sample was taken as T_0 and the rest of the cells were shifted to pre-chilled YP media containing 0.1% glucose for 9 hours. Samples after the temperature shift were collected as T_{final}. For temperature sensitive alleles *exo70-113 and sec6-4*, the cells were directly shifted to warm YP media containing 0.1% glucose for 1.5 hours at 37°C. Samples collected before and after the temperature shift were collected as T_0 and T_{final} respectively.

After the temperature shift, the cells were spheroplasted and the internal and external fractions were separated. Samples were processed as described previously (Adamo *et al.*, 1999; Lehman *et al.*, 1999). The percentage of internal invertase accumulation is calculated by: Δinternal / (Δ internal + Δ external).

To analyze the accumulation of Bgl2 enzyme, cells were grown in YPD media overnight to mid-log phase. The *exo70-188* mutant cells were then shifted to a 14^{o}C water bath shaker for 10 hours. The *exo70-113* mutant cells were shifted to 37^{o}C water bath shaker for 2 hours. The cells were spheroplasted, and the internal and external fractions were separated. Samples were boiled with 6x sample buffer for 5 minutes at 95^{o}C. Internal and external samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to nitrocellulose, and blotted with affinity purified anti-Bgl2 antibody at a dilution of 1:100. Quantification of the western blots was analyzed by Odyssey Infrared Imaging system.

Construction of pGEX-6His vector and protein expression, purification, and quantification

The pGEX6p1 vector was digested with *Not*I and *Sal*I. An additional PreScission enzyme site and a 6-histidine tag were generated by fusion PCR reaction and introduced into the NotI and SalI sites of pGEX6p1 to create pGEX-6P6H (pB1579). The resulting plasmid contained an N terminal GST tag, and a C terminal 6xHis tag flanking the polylinker and was used to create the following GST-fusion constructs: GST-Sec9 (aa 402-651), GST-Ste20 (aa 314-432), GST-Exo70 (aa 1-623), GST-Exo70-1521, GST-Exo70-DC. GST-Sec4 (1-211), GST-Rho1 (aa1-205), GST-Rho2 (aa1-188), GST-Rho3 (aa1-227), GST-Rho4 (aa1-287), and GST-Cdc42 (aa1-187) were subcloned into pGEX-6p-1 vector. All constructs were confirmed by sequencing and protein expression was performed in *Escherichia coli* BL21 cells. Cells were grown at 37°C in Terrific broth medium to an OD_{599} 2.0-2.5. Cells were shifted to 25°C and protein expression was induced with 0.1 μM IPTG for 3 hours at 25^{o}C.

To maximize protein solubility, GST-Exo70-ΔC was induced at 22^{o}C for 4 hours. Cells were pelleted and frozen at -80 °C until lysis.

6xHis-tag purification was performed by binding the bacteria lysate to HIS-Select® Nickel Affinity Gel (Sigma), and then eluting with 500 mM imidazole. The 6xHistidine eluates were then incubated with glutathione Sepharose beads at 4^{o}C for one hour, and the beads were washed with wash buffer (20 mM Tris pH7.5, 120 mM NaCl, and 1% Tween 20) to remove unbound proteins. Protein concentration was determined by comparison to purified protein standards after SDS-PAGE and Coomassie Blue staining. Quantification of Coomassie Blue stained gels was performed by Odyssey Infrared Imaging System (LiCor).

***In vitro* binding assays**

Glutathione beads carrying GST,GST-Rho1-4, GST-Cdc42 were washed with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT and were incubated in 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1 mM DTT in the presence of 100 μM GTPγS for 30minutes at 25^{o}C. MgCl2 was added to a final concentration of 25 mM and incubated 30 min at 25^{o}C. Binding assays were performed in binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.5% Triton X-100). The final concentrations of GST, GST-Rho1-4, and GST-Cdc42 in the binding reactions were 2 μM. Samples were incubated at 4^{o}C for 2 hours. The beads were washed five times with binding buffer, and boiled with 85μl sample buffer at 95^{o}C for 5 min. Samples were subjected to SDS PAGE and autoradiography.

Generation of yeast cell lysate

RHO3L74, *RHO3N30*, *CDC42L61*, *CDC42N17*, *RHO1L68* were amplified by PCR reaction and subcloned behind the GAL1 promoter in a *LEU2* integrating vector (BB24, pRS305 with GAL1 promoter). The vector was linearized by digesting with *BstX*I restriction enzyme and transformed into a Gal^+ strain BY17 (*a; GAL*1*; leu2-3,112; ura3-52*). Yeast strains were first grown in YP 3% raffinose overnight at 30°C to mid-log phase of OD 1-1.5. The small GTPases were induced by adding final concentration of 1% galactose for two more doublings (four hours) at 30°C. Cells were pelleted by centrifugation at 5000 rpm for 5 minutes and washed with ddH_2O. Pellets were immediately frozen on dry ice. Frozen pellets were lysed with a bead beater from Biospec products. The optimal wet weight for the small chamber was 5-6 grams. The pellet was beaten for 1 minute followed by a 3 minutes pause for five cycles. Lysate was then subjected to centrifugation for 10 min at 17,000xg, followed by ultracentrifugation at 100,000xg for 30minutes. The protein concentration of the lysate was measured by a Bradford assay. Each lysate was normalized to about 25 mg/ml total protein concentration and frozen on dry ice.

GST pull-down from yeast cell lysate

All recombinant proteins were present at a final concentration of 3 μM. Cell lysates were prepared for GST pulldown experiments as described above and incubated with each fusion protein bound to glutathione sepharose beads for 1.5 hours at 4°C. The beads were washed five times with lysis buffer (20 mM tris-HCl pH 7.5, 120 mM NaCl, 10 mM $MgCl_2$, 1% Tween 20, 1 mM DTT), and boiled at 95°C for 5 minutes. Samples were subjected to SDS-PAGE gel, and analyzed by immunoblotting with Rho1, Rho3, and Cdc42 monoclonal

antibody. Quantification of western blots was performed with Odyssey infrared imaging system.

In vitro prenylation assay

The coding sequence of Rho3 was amplified by PCR reaction using standard PCR conditions. A common TNT-T7 primer was designed especially for *in vitro* transcription/translation, which contains a promoter and a Kozak consensus sequence (GCATGGATCCTAATACGACTC ACTATA GGA CAA CAG CCA CCA TGG GA). 10 µl rabbit reticulocyte lysate, 0.5µl [^{S}35] methionine, and approximately 200 ng amplified DNA (7µl PCR reaction) were first incubated at 30^{o}C for 1.5 hours, and 5 mM mevalonic acid was added to the reaction for 6 hours. The mevalonic acid was prepared as described previously (Hancock, 1995). At the end of the *in vitro* translation and prenylation reaction, 75µl binding buffer (20 mM Tris-HCl pH7.5, 120mM NaCl, 10mM $MgCl_2$, 1% Tween 20, 1mM DTT) was added to each translation mix and incubated with 15µl GST-Sec9, GST-Exo70 or GST-Ste20 for 2 hours at 4^{o}C. The beads were washed five times with binding buffer and boiled with 85µl sample buffer at 95^{o}C for 5 min. Samples were subjected to SDS-PAGE and the gels were quantitated with Storm Phosphorimager with ImageQuant softerware (Molecular Dynamics).

Immunoprecipitation of the exocyst complex

Strains containing dominant mutants of Exo70 as the only source of Exo70 were grown to mid log phase in SC media at 30^{o}C and shift to YPD rich media for 1.5 hours. 300 OD units of cells were pelleted and washed with cold 10:20:20 buffer (10mM Tris, pH7.5,

20mMNaN3 and 20mMNaF). Pellets were resuspended in lysis buffer (20mM Pipes pH6.8, 120mM NaCl, 1mM EDTA, and 1mM DTT). Cells were lysed by bead beating in lysis buffer and extracted by adding 5X extraction buffer (20mM Pipes pH6.8, 120mM NaCl, 1mM EDTA, 1%NP40, and 1mM DTT). Lysates were cleared by centrifugation at 14000rpm for 9 minutes and normalized for total protein concentration by Bradford Assay. The supernatants were added to prewashed protein beads for 30 minutes on ice to reduce non specific binding and then spun for 5 minutes at 14000rpm. Input samples were collected, and the remaining lysate was placed in fresh eppendorf tubes in the presence of 9E10 monoclonal anti-myc antibody. Samples were incubated on ice for 45 minutes, and then add 2µl rabbit anti-mouse antibody followed by incubation on ice for another 30 minutes. Lysate-antibody mix was then added to fresh, prewashed protein A beads for 60 minutes on ice. Samples were washed five times with wash buffer (20mM Pipes pH6.8, 120mM NaCl, 1mM EDTA, and 1% NP40) and boiled at 95^{o}C for five minutes with 2X sample buffer. Input and bound fractions were analyzed by SDS-PAGE gels and western blotting. Quantitation of western blots was performed by Odyssey Infrared Imaging System.

RESULTS

Isolation of Dominant Gain-of-Function Alleles of *EXO70*

We have previously suggested a model for localized stimulation of exocytosis by the GTP-bound forms of Rho3 and Cdc42. For Rho3, we proposed this activation would involve interaction with the Exo70 subunit of the exocyst complex, while the effector for Cdc42 was unknown (Roumanie *et al.*, 2005; Wu *et al.*, 2008). Using other Rho-effectors such as formins, WASP, or PAK kinases, as a model, one might expect that downstream activation would involve "relief of autoinhibition"—i.e. that binding of Rho3-GTP to the Exo70 subunit of the exocyst would activate this complex by blocking inhibitory interactions between Exo70 and other components of the complex—thus re-organizing the complex into a more active form (Wu *et al.*, 2008). A prediction of this model is that mutations in Exo70 that block or perturb inhibitory interactions of Exo70 within the exocyst should act as "activated" or gain-of-function alleles and would behave genetically as dominant-suppressors of loss of Rho3 function. Furthermore, if Rho3 and Cdc42 share a common pathway in regulating exocyst function then mutants that suppress loss of Rho3 function in exocytosis should also suppress loss of Cdc42 function in exocytosis.

To test these predictions, we performed two independent screens to look for dominant suppressing forms of *EXO70.* One screen made use of the *rho3Δ* mutant, which is sensitive to increased gene dosage of several components of the exocytic apparatus (Matsui and Toh, 1992; Imai *et al.*, 1996; Lehman *et al.*, 1999). The other screen made use of the *cdc42-6* mutant that has a highly allele-specific defect in exocytosis which genetically overlaps with

that of Rho3 (Adamo *et al.*, 2001). *EXO70* was randomly mutagenized by PCR amplification and introduced into the mutant yeast strains by co-transformation with a gap-repair plasmid containing the flanking sequences of the *EXO70* gene (see Materials and Methods). Approximately 6000 transformants were assayed from each strain and approximately 45 plasmids containing dominant suppressing forms of *EXO70* were isolated from both screens. Our initial work focused on the 41 strongest suppressors. When we examined the strongest 16 *EXO70* dominant suppressors isolated from the *cdc42-6* screen in the *rho3Δ* strain, we found that they also strongly suppressed (Figure 5). When we examined the ability of the 3 strongest *EXO70* dominant suppressors isolated from the *rho3Δ* screen to suppress *cdc42-6* we found that all but one of these also strongly suppressed *cdc42-6* (Figure 5). We also examined all 16 mutants in *rho3Δ*, *rho4Δ* and found they all strongly suppressed (Figure 5). Therefore each of these two genetic screens appeared to be selecting for mutants in *EXO70* with similar properties. The ability of these suppressors to potently suppress growth defects associated with both *rho3* and *cdc42* loss-of-function strongly supports the idea that Exo70 is a functionally important downstream target of regulation by both of these Rho GTPases.

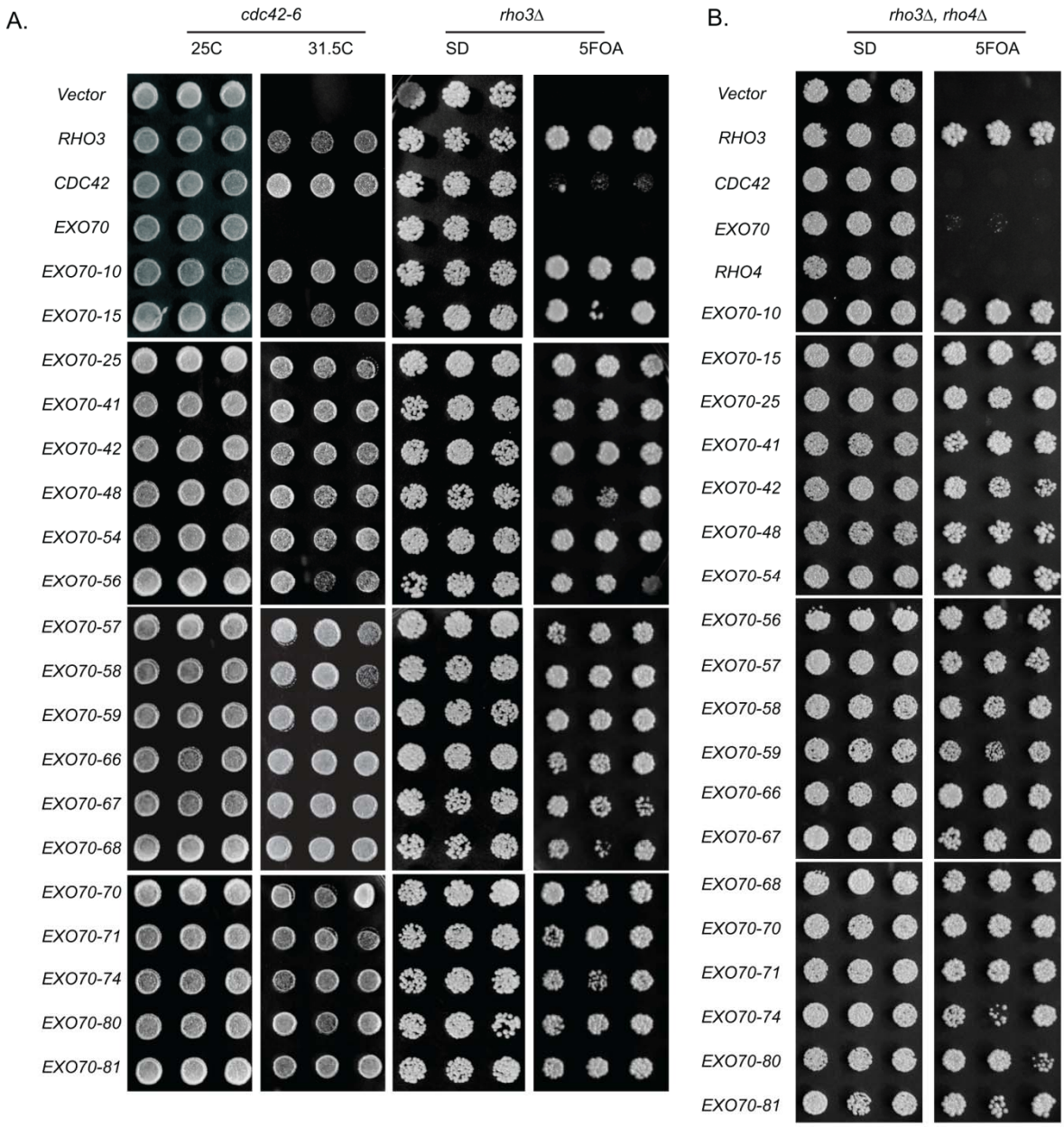


Figure 5:Cross suppression of *EXO70* dominant suppressors isolated from *cdc42-6* or *rho3Δ* screens.

(A) The dominant suppressors of *EXO70* isolated from *rho3Δ* screen (*EXO70-10, EXO70-15, EXO70-25*) and *cdc42-6* screen *(EXO70-41, EXO70-42, EXO70-48, EXO70-54, EXO70-56, EXO70-57, EXO70-58, EXO70-59, EXO70-66, EXO70-67, EXO70-68,EXO70-70, EXO70-71, EXO70-74, EXO70-80 and EXO70-81)* were transformed into a *RHO3* plasmid shuffle strain and a *cdc42-6* strain on *CEN* plasmids. The growth of three independent transformants is shown for each *EXO70* mutant following transformation into each Rho mutant strain as seen in Figure 6B. **(B)** The dominant suppressors of *EXO70* from *rho3Δ* screen and *cdc42-6*

screen (see Figure 5A) were transformed into *rho3Δ, rho4Δ* strain. The growth of three independent transformants is shown for each *EXO70* mutant as described in Figure 5B.

Sequence analysis of 41 of the strongest suppressors identified an average of 5-8 coding sequence changes per dominant suppressing allele. This was not unexpected, as the predicted frequency of mutations for the amplifying conditions used was 1-2 bp changes/kb. To identify specific amino acid substitutions linked to the suppression activity, we scanned the mutant sequences for missense mutations that appeared in two or more of the suppressing plasmids. This identified 10 single residue mutations as candidate residues to confer dominant suppression on Exo70. These mutations were then prepared as single residue changes and tested for dominant suppression in *rho3Δ* or *cdc42-6* (Figure 6).

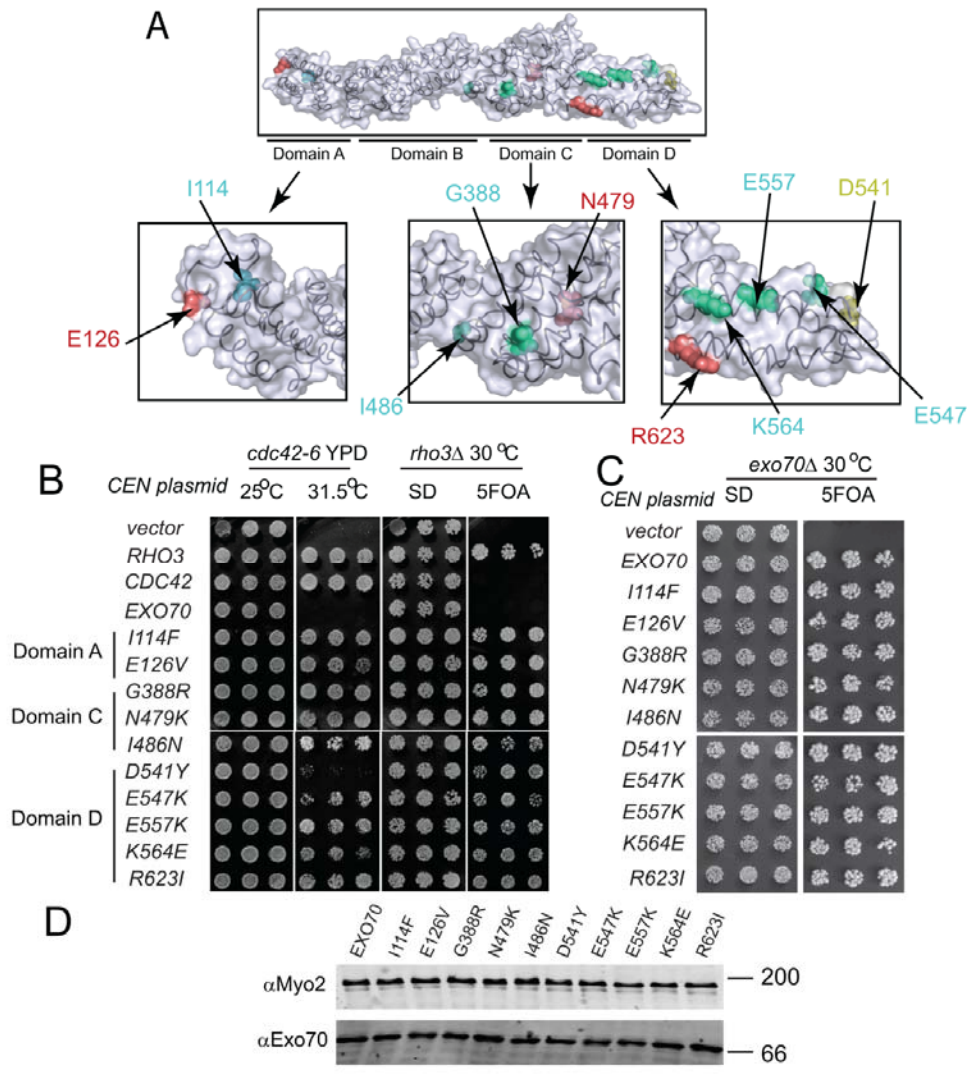


Figure 6:Dominant mutations of *EXO70* are able to suppress *rho3Δ* and *cdc42-6* mutant

(**A**) The structure of *S. cerevisiae* Exo70 (residues 62-623) is in a transparent surface representation with mutant residues in a space-filing model. The domains are labeled A through D from the N terminus to the C terminus. The arrows point to the residues involved in Exo70 gain of function mutations. Residues colored in blue represent highly conserved residues, residues colored in yellow represent residues conserved only within fungi, while residues colored in pink represent variable residues. Three residues, I114, G388, and N479 are buried within the structure, while the remaining seven mutations are surface exposed residues. The image was generated using the PDB file 2B1E. (**B**) The gain-of-function mutations of *EXO70* are capable of suppressing a *rho3*Δ and the *cdc42-6* mutant at the restrictive temperature**.** Dominant mutants of *EXO70* were transformed into a *RHO3* plasmid

shuffle strain and a *cdc42-6* strain on *CEN* plasmids. The growth of three independent transformants is shown for each *EXO70* mutant in both mutant strains under restrictive conditions. (**C**) The *EXO70* dominant mutations can function as the sole copy of Exo70 in the cell. The dominant mutations were introduced into the *EXO70* plasmid shuffle strain on a *CEN-LEU2* plasmid. Three individual colonies were picked to show the growth of each *EXO70* mutant under restrictive conditions when the plasmid containing wild type *EXO70-URA3* was lost by counter-selection on 5FOA plates. (**D**) *EXO70* dominant mutants express similar amounts of Exo70 compared to wild type cells. Whole cell lysate were made from the strains containing Exo70 gain of function mutants as the only source of *EXO70* in the cell. The lysates were subjected to SDS-PAGE analysis, and blotted with anti-Myo2 and anti-Exo70 antibodies.

The placement of the dominant gain-of-function mutations in the crystal structure of yeast Exo70 revealed a number of possible sites of regulation. Three residues, I114, G388, and N479 are buried within the structure. The other seven mutations are all surface exposed residues. Charged residues at the D domain form two patches, D541 and E547 form one patch, and E557, K564, R623 form another patch. N479 and G388 are located at the interface of domain B and domain C, which potentially cause rearrangements of the two domains relative to each other within the structure of Exo70. An alignment of Exo70 protein sequences from yeast, mouse, worm, and fly is shown in the Figure 7. The site of each dominant suppressing mutation is indicated on the alignment along with the overall conservation of each residue. As can be seen in Figure 7, six of the dominant suppressing mutations occur in highly conserved residues (I114, N479, D541, E547, E557, and K564) — four of which reside in a single helix (H17) within the Exo70 crystal structure—suggesting this may be a "hot spot" for activation of the exocyst by Exo70. Taken together, however, the results of this screen overall suggest that there are likely to be multiple, structurally distinct mechanisms by which *EXO70* gain of function can be achieved.

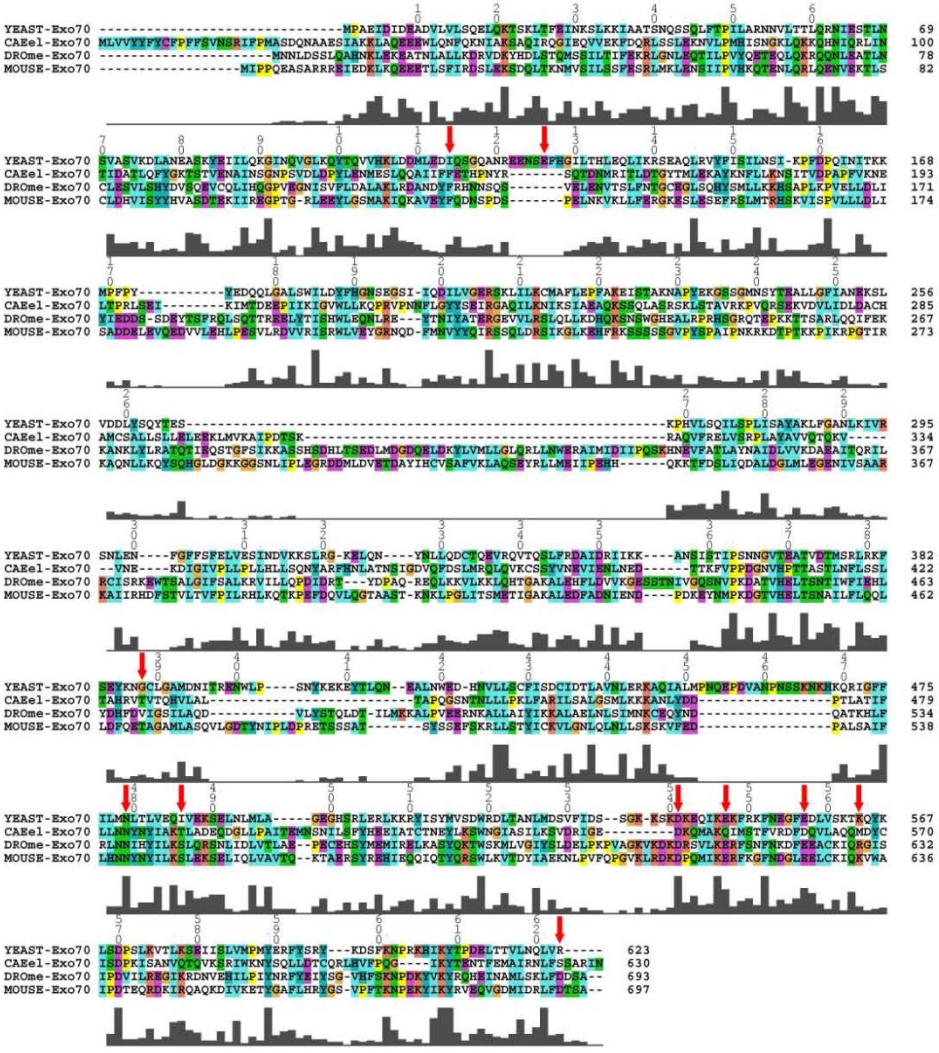


Figure 7: Conservation of Dominant gain-of Function Mutations in Exo70

An alignment of *EXO70* in four species, *M. musculus* (MOUSE), *D. melanogaster* (DROme), *C.elegans* (CAEel) and *S. cerevisiae* (YEAST), was constructed according to the structural alignment of Moore et al. 2007. Residue numbers above the alignment point to the yeast *EXO70* and are indicated at the end of each line of the sequence alignment. Arrows point to the sites of the dominant mutations in *EXO70*. The bar graph indicates the degree of conservation within the four species. Sequence coloring is based on amino acid type: hydrophobic (cyan), aromatic (blue), basic (orange), acidic (purple), polar (green), proline (yellow), and glycine (tan).

One possible mechanism for activation of the exocyst complex by the gain-of-function mutants is by effecting the assembly or disassembly of individual subunits of the complex. To determine if this was the case, we examined the assembly state of the exocyst complex in the presence of the *EXO70* gain-of-function alleles as the sole source of Exo70 in the cell. We immunoprecipated wild-type and mutant exocyst complexes using strains containing a C-terminally myc-tagged form of *SEC8* (Sec8-myc) and anti-myc antibody coated beads (TerBush and Novick, 1995), and assayed for the presence of the other exocyst subunits by quantitative western blot analysis. As can be seen in Figure 8, all six of the gain-of-function alleles of *EXO70* examined resulted in nearly identical amounts of exocyst subunits being co-precipitated with Sec8-myc. An exception was the *EXO70-E557K* allele which had a small (~25%) reduction in the mutant Exo70 protein co-precipitated with the complex. However, this appears to mirror a similar small reduction in the overall steady state amounts of this protein in the *EXO70-E557K* cells rather than an effect on assembly or disassembly of the complex. Since the activation of the exocyst complex with these gain-of-function mutants appears to mimic the effect of Rho3/Cdc42 activation, this data suggests that assembly and/or disassembly of the complex is unlikely to be the central means of regulation by Rho3 and Cdc42 GTPases.

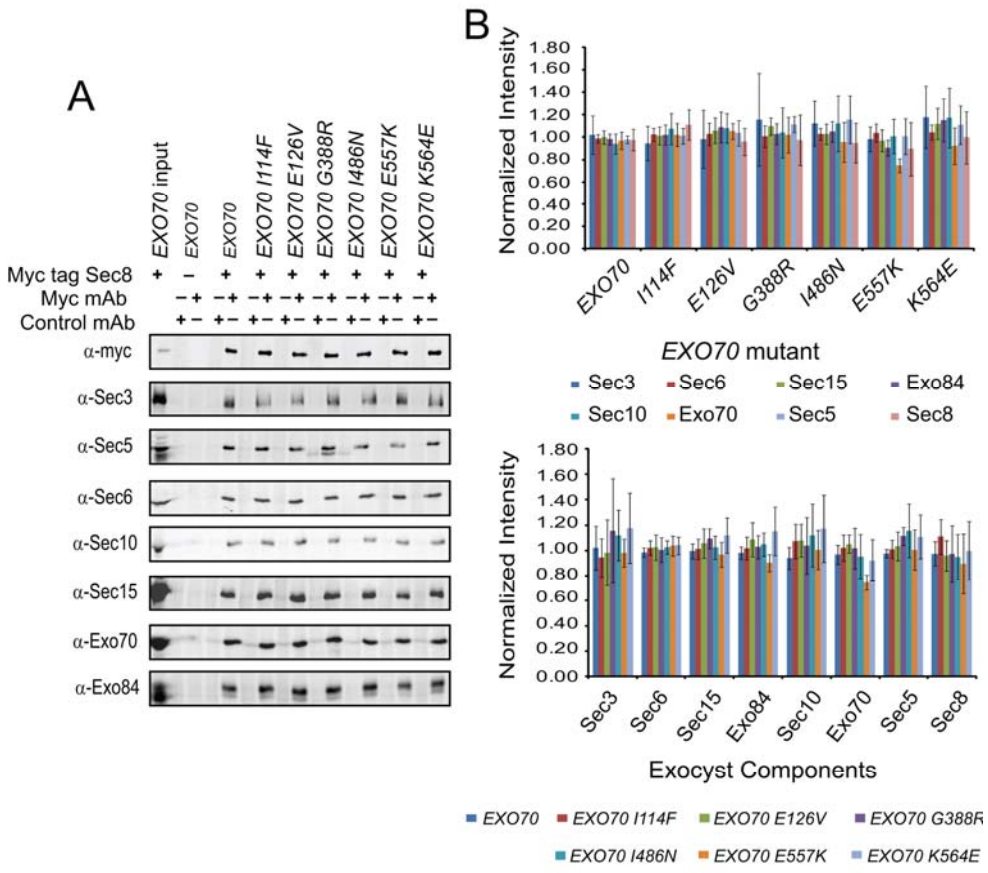


Figure 8: Dominant Mutations in Exo70 do not Affect the Assembly of the Exocyst Complex

(**A**) Analysis of coimmunoprecipitation of exocyst subunits in *EXO70* dominant mutant strains. A single copy of C-terminal myc–tagged Sec8 (*SEC8-MYC*) was introduced by integrative transformation into strains containing dominant mutants of *EXO70* (on CEN plasmid) as the only source of Exo70 in the cell. Cells were grown, lysed, and subjected to native immunoprecipations with myc antibody to analyze the integrity of the exocyst complex (see Materials and Methods for details). 12 % of total lysate and 10% of myc immunoprecipitate were loaded on SDS-PAGE gel for western blot analysis with antibodies against each exocyst components. (**B**) Quantitation of western blots described in A performed by Odyssey Infrared Imaging system. The top panel shows quantitation of each individual subunit in the Sec8 Myc immunoprecipitation organized by strain. The bottom panel shows comparison of wild type and each gain of function strain organized by individual subunit. Error bars represent standard deviation of four independent experiments.

The GTP-Bound Forms of Rho3 and Cdc42, Produced in Yeast, Interact with Exo70

The dominant suppressor analysis described above suggests that Exo70 is likely to be a critical downstream target of both Rho3 and Cdc42 regulation of exocytosis. However, work using recombinant forms of these GTPases produced in *E.coli* is inconsistent with this notion. First, recombinant Cdc42-GTP fails to show any detectable interaction with Exo70 (Figure 9A) (He *et al.*, 2007a). Recombinant Rho3 does interact with Exo70 in a GTP-dependent and effector domain-dependent fashion (Figure 9A and Figure 9B) but with relatively low affinity (Hamburger *et al.*, 2006). Second, mutations or deletions in Exo70 which severely diminish this interaction *in vitro* fail to recapitulate growth or secretory defects associated with either the *rho3-V51* or *cdc42-6* cells (He *et al.*, 2007b; Hutagalung *et al.*, 2009). Since nearly all small GTPases are subjected to post-translational modifications which do not occur following expression in *E.coli*, we sought to determine if we could detect an interaction following their overexpression in yeast. To determine the nucleotide dependence of this interaction, we made use of mutant forms of Rho3 and Cdc42 which are predicted to "lock" the GTPases in the either the GTP-bound conformation (Rho3-L74, Cdc42-L61) or GDP-bound conformation (Rho3-N30, or Cdc42-N17). The nucleotide-locked forms of each GTPase were overexpressed (approximately 20-fold) using an inducible *GAL1/10* promoter and the induced cells were used to make detergent lysates (see materials and methods). These lysates were then used in binding experiments with purified full-length Exo70 fused to GST or various control GST fusion proteins. A GST-fusion containing the C-terminal CRIB domain of the Ste20 protein was included as a positive control for Cdc42. As can be seen in Figure 9C, both the GTP-locked forms of Rho3 and Cdc42, Rho3-L74 and

Cdc42-L61, are specifically pulled down by GST-Exo70 and this binding is nearly completely lost in lysates overexpressing the GDP-mutant forms of Rho3 and Cdc42, Rho3-N30 and Cdc42-N17. In contrast to Rho3 and Cdc42, no binding to Exo70 was detected with lysates overexpressing GTP-locked Rho1, indicating that the interaction with Exo70 is specific to Rho3 and Cdc42.

Since specific mutations in the effector domain of Rho3 and Cdc42 have been described (*rho3-V51* and *cdc42-6*) which strongly interfere with the function of these GTPases in promoting exocytosis (Adamo *et al.*, 1999; Adamo *et al.*, 2001), we examined the effects of these mutations on binding to Exo70. The effector mutations were incorporated in the context of a GTP-locked form of the GTPases and overexpressed as described above. As can be seen in Figure 9 D, the presence of the V51 mutation causes a severe defect in binding to Exo70. The *cdc42-6* mutant contains a cluster of three mutations (L29, V31, H32) in the effector domain which are critical to the temperature-sensitive defect in exocytosis (Adamo *et al.*, 2001). The inclusion of these mutations in the context of a GTP-locked form of Cdc42, causes a significant loss of binding to GST-Exo70 (Figure 9D). Therefore the Exo70 binding with Rho3 and Cdc42 from yeast lysates presented here shows clear specificity, nucleotide-dependence, and effector domain sensitivity expected for a bona fide assay for GTPase: effector interaction.

Prenylation of Rho3 and Cdc42 is an Important Determinant for Binding to Exo70

One possible explanation for the results described above is that a eukaryotic-specific post-translational modification such as prenylation of the C-terminal CAAX might be required for binding with Cdc42 and Exo70. We therefore produced lysates of GTP-locked

forms of Cdc42 and Rho3 with a mutation at the C-terminal Cysteine (in the CAAX motifs) and monitored the effect on binding to recombinant effector proteins. The results are shown in Figure 10. As expected the CAAX (C188A) mutation had no effect on binding of Cdc42-L61 to GST-Ste20. In contrast, a dramatic loss of binding to Exo70 was observed for Cdc42-L61, A188 suggesting that C-terminal prenylation of Cdc42 is a pre-requisite for binding. Interestingly, we found that mutation of the CAAX motif of activated Rho3 (L74,A228) also resulted in a dramatic loss of binding to GST-Exo70, suggesting that the binding detected with recombinant Rho3 reflects only a residual level of interaction with the modified protein.

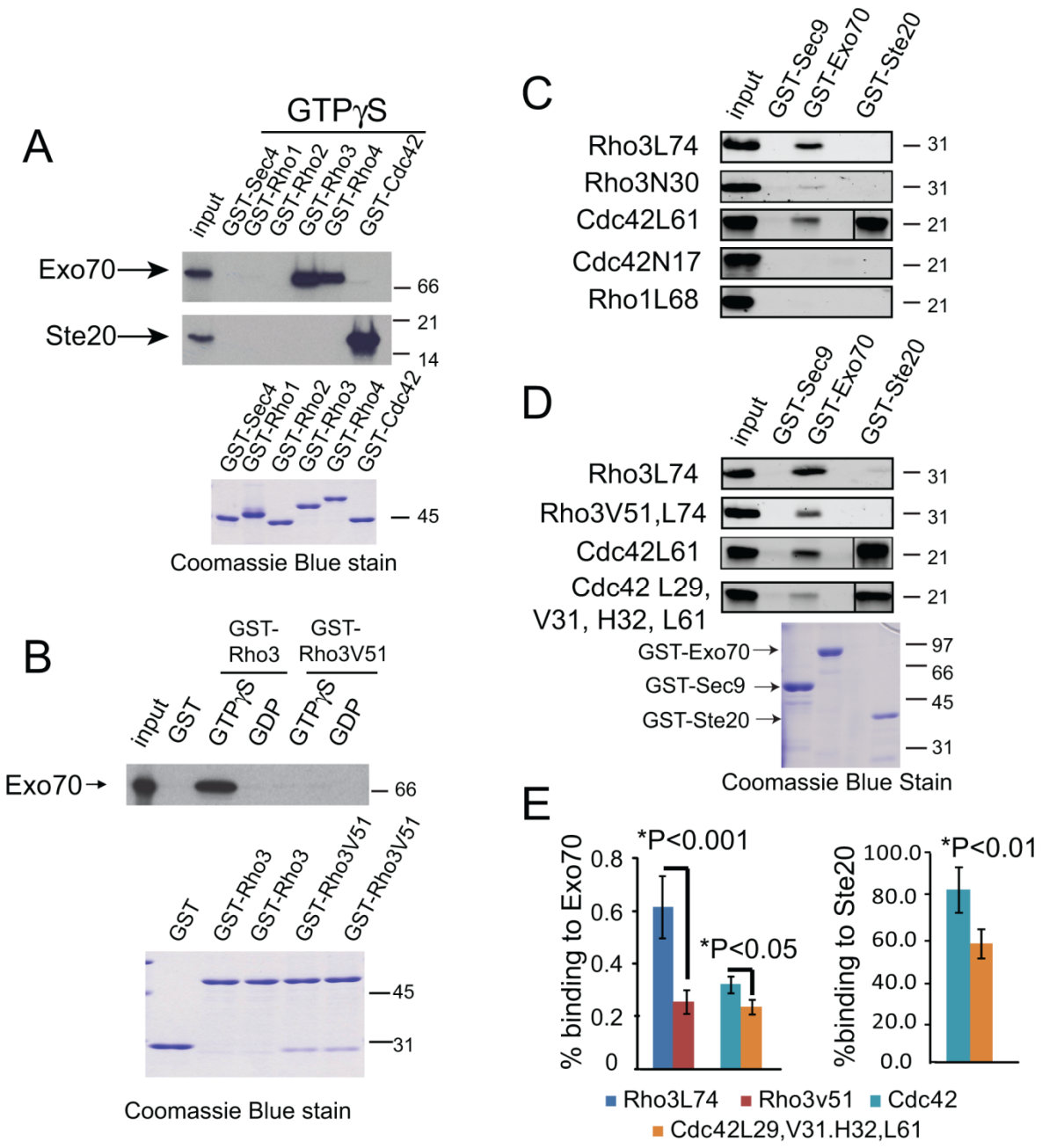


Figure 9:Exo70 binding to Rho GTPases in E.coli or Yeast

(**A**) Yeast Rho GTPases (Rho1-4, and Cdc42) were expressed, purified from bacteria as GST fusion proteins, and immobilized on glutathione sepharose beads. The beads were preloaded with GTP, and bound to radiolabeled *in vitro* translated full length Exo70 and the CRIB domain of Ste20. Input represents 1% binding. (**B**) The V51 mutation in the effector domain of Rho3 (Rho3V51) abolishes the interaction between Rho3 and Exo70 *in vitro*. Recombinant GST-Rho3 and GST-Rho3V 51 were purified from bacteria and the

immobilized GST fusion protein was preloaded with GTPγS or GDP. Input represents 1% binding. (**C**) Exo70 interacts with GTP-locked Rho3 and Cdc42 obtained from a yeast lysate. GST fusion proteins were purified from bacteria and immobilized on glutathione sepharose beads. GTP or GDP locked forms of small GTPases were expressed behind a galactose inducible promoter in yeast. Lysates obtained from these strains were used in the binding experiments (See Material and Methods). The input represents 2.5% binding. Bound material was analyzed by western blot using monoclonal anti-Rho3, anti-Rho1 and anti-Cdc42 antibodies. Because of the extremely strong binding to Ste20 binding reactions with Cdc42-L61 (but not Rho3, Rho1) were diluted 20 fold prior to loading to improve quantitation and avoid obscuring the adjacent lanes. (**D**) The interaction between Exo70 and Rho3/Cdc42 obtained from yeast is sensitive to mutations in the effector domain of the two GTPases. The *rho3V51, L74* and *cdc42-6* effector domain mutations (*cdc42-L29, V31, H32, L61*) were expressed behind a galactose inducible promoter in yeast. Lysates generated from the overexpressing yeast strains were subjected to binding experiments as described above. (**E**) Bar graphs represent quantitation of the blots using the Odyssey Infrared Imaging system. Data were analyzed by two-tail student's t test with error bars representing standard deviation from four independent experiments.

To further examine the apparent requirement for CAAX prenylation in the interaction between these Rho GTPases and Exo70, we made use of rabbit reticulocyte lysates, which have been shown to be active in prenylating CAAX motifs *in vitro* (Hancock, 1995). We examined whether we could detect prenylation of either yeast GTPase following *in vitro* translation. When Rho3-L61 (GTP) or Rho3-N30 (GDP) were translated in reticulocyte lysates and further incubated for 6 hours, a faster migrating form of the protein appeared over time. When activated Rho3 containing a mutation of the critical Cysteine (C228) in the CAAX motif was used, the faster migrating form failed to appear (Figure 10B). The addition of mevalonic acid--a metabolic precursor of both farnesyl and geranylgeranyl moieties used to modify CAAX motifs--has been shown to stimulate both types of prenylation in reticulocyte lysates. As can be seen from Figure 10B, the addition of mevalonic acid to the reaction following translation results in a dramatic increase in the conversion of Rho3-L74

and Rho3-N30 proteins to the faster migrating form but has no effect on the Rho3-L74, A226 mutant lacking the prenylation acceptor cysteine.

We examined the ability of *in vitro* prenylated Rho3 from reticulocyte lysates to bind to GST-Exo70 under conditions similar to that used for the yeast lysate binding studies described above. The results show that *in vitro* prenylated Rho3-L61 (GTP-locked) binds well to the GST-Exo70, whereas prenylated Rho3-N30 (GDP form) fails to bind (Figure 10 C). Importantly, only very weak binding is seen when unprenylated but activated Rho3-L61,A226 is present. Unlike Rho3, we were unable to detect a cysteine-dependent mobility shift in a parallel set of translations with Cdc42 translated in the reticulocyte system. However when we examined the binding of translations of activated Cdc42 (Cdc42L61) in the presence or absence of mevalonate and the presence or absence of the C-terminal cysteine we find that, binding to Exo70 is dependent on GTP, the addition of mevalonate, and the CAAX box. This provides strong evidence that, like Rho3, prenylation of Cdc42 CAAX motif is required for interaction with Exo70 in this system. Taken together with the binding assays using GTPases from yeast lysates, these data clearly demonstrate that prenylation of Rho3 and Cdc42 is an important determinate in promoting the binding between these Rho GTPase and Exo70.

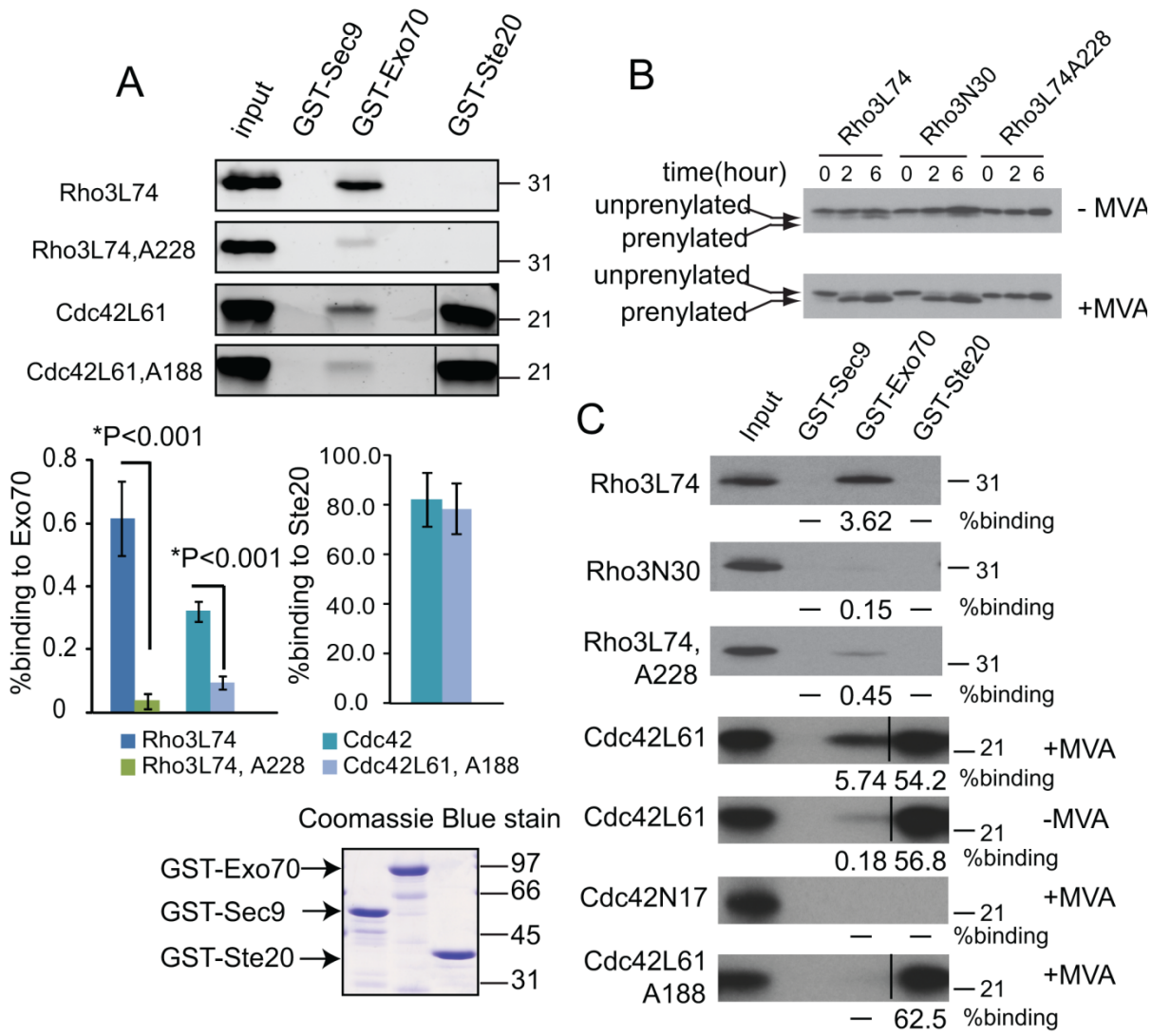


Figure 10:Prenylation of Rho3 and Cdc42 Promotes the GTP-dependent interaction with Exo70.

(**A**) The interaction between Exo70 and the two Rho GTPases, Rho3 and Cdc42, from yeast lysate is dependent on C-terminal prenylation. Activated forms of Rho3 and Cdc42 (Rho3L74, Cdc42L61) containing a cysteine-to-alanine mutation in the CAAX box (Rho3-L74, A228, and Cdc42-L61, A188) were overexpressed in yeast. Lysates were prepared in parallel for these mutants and for the activated GTPases containing the intact CAAX motif (Rho3-L74 and Cdc42-L61) and used in binding experiments (see Materials and Methods). Ste20 binding assays with Cdc42 (but not Rho3) were diluted 20 fold prior to loading. As expected Ste20 binding was unaffected by the prenylation site mutation. Bar graphs represent quantitation of binding experiment with error bars representing standard deviation of three independent experiments. Coomassie Blue staining of the GST-fusion proteins used

in the experiment is shown below the quantitation. (**B**) *In vitro* prenylation of Rho3 results in a change in mobility and is stimulated by addition of mevalonic acid to reticulocyte lysate. Rho3 was *in vitro* translated in rabbit reticulocyte lysates (see Materials and Methods) and then subjected to extended incubation (for the time shown) in the presence or absence of mevalonic acid (+/-MVA). The appearance of a faster migrating species appears during the extended incubation and is strongly stimulated by the addition of mevalonic acid and is abolished by mutation of the CAAX box cysteine 228 to alanine. (**C**) The interaction between Exo70 and Rho3 and Cdc42 produced in reticulocyte lysastes is dependent on C-terminal prenylation. Immoblized GST fusion proteins were incubated with prenylated Rho3 or Cdc42 obtained from the *in vitro* translation described in Materials and Methods. Input lanes for Rho3 binding experiments represent signal equivalent to 2.5% binding. Input lane for Cdc42 binding experiments represent signal equivalent to 10% binding. The Cdc42-N17 binding experiment was exposed to film twice as long as the other experiments due to lower efficiency of translation for this mutant.

Binding of Prenylated Rho3 and Cdc42 to Exo70 is Not Dependent on the C-Domain

Previous studies to map the site of Rho3 interaction within Exo70 depended largely on the use of recombinant (i.e. unprenylated) forms of Rho3. These studies identified an important role for domain C of Exo70 in mediating the interaction with recombinant Rho3. Either mutations in, or deletion of domain C of Exo70 result in dramatic loss of binding to Rho3 (Dong *et al.*, 2005; He *et al.*, 2007b; Hutagalung *et al.*, 2009). To determine if prenylated Rho3 and Cdc42 were similarly dependent on the Exo70 C-domain for their interaction, we made use of two mutant forms of Exo70, Exo70-1521 and Exo70-ΔC. Exo70-1521 has a combination of 6 alanine substitutions in surface exposed residues within the C-domain which block binding to recombinant Rho3 (He *et al.*, 2007b) and Exo70-ΔC mutant contains a replacement of the entire C domain with an eight residue flexible linker (Hutagalung *et al.*, 2009). We compared the binding of these two forms of Exo70 fused to GST to equivalent amounts of wild-type full-length Exo70 using the Rho GTPases produced

in yeast lysates. Remarkably, we found that both GST-Exo70-1521 and GST-Exo70-ΔC mutant proteins showed binding activity to yeast Rho3-L74 and Cdc42-L61 that was indistinguishable from that of wild-type Exo70 (Figure 11 B). As before this binding was highly dependent on the activation status of the Rho3 and Cdc42. We repeated these binding assays using the *in vitro* translated/*in vitro* prenylated Rho3 protein. Using the *in vitro* modified Rho3 we see a small loss of binding activity to the GST-Exo70-1521 and GST-Exo70-ΔC mutant proteins, but both mutants demonstrate very clear binding which is highly dependent on both the activation (i.e. GTP) and prenylation status of Rho3. Therefore both of these assays reveal that Exo70 is able to interact with Rho GTPases in a manner which does not depend on the C-domain and that the prenylation state of these GTPases appears to be critical in revealing this mode of interaction. It is worth noting that mammalian TC10 GTPase (produced in NIH 3T3 cells and hence likely prenylated) also has been shown to interact with mammalian Exo70 in a fashion that does not depend of the C-domain (Chiang *et al.*, 2001).

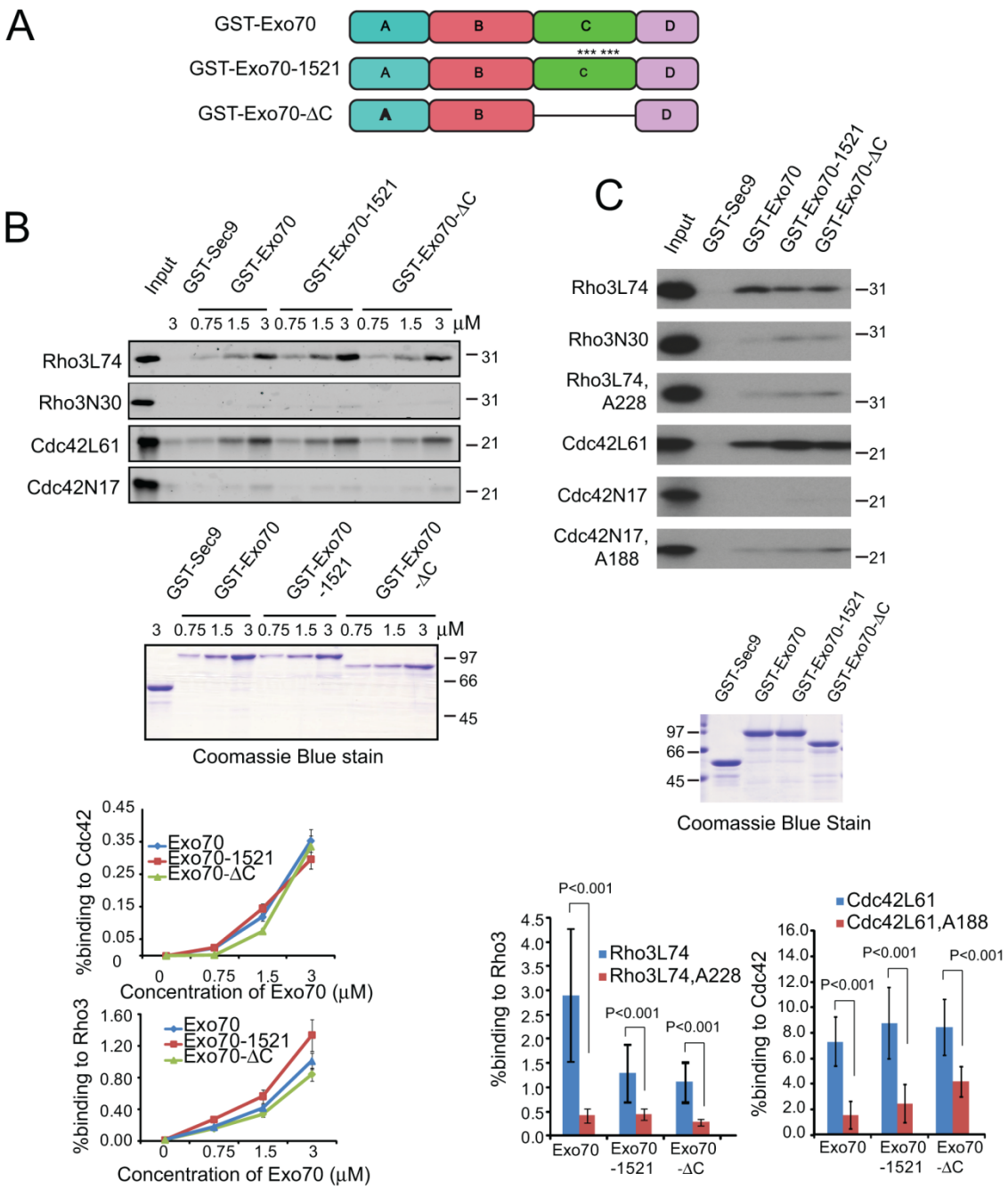


Figure 11:Binding of Prenylated Rho3 and Cdc42 to Exo70 Does Not Depend on Domain C.

(**A**) Schematics of the Exo70-1521 mutation and a domain C deletion. (**B**) Mutations in or a deletion of Exo70 domain C does not significantly affect the interaction between Exo70 and Rho3 or Cdc42 produced in yeast. Yeast lysates from cells overexpressing GTP or GDP locked Rho3 and Cdc42 were incubated with immobilized GST fusion proteins at different concentrations. Binding experiments were performed as described in Materials and Methods.

Graphs represent quantitation of the binding with error bars representing standard deviation of three independent experiments. (**C**) Mutations in or a deletion of Exo70 domain C does not significantly affect the interaction between Exo70 and Rho3 or Cdc42 prenylated *in vitro* following translation in reticulocyte lysates. Immobilized GST proteins were incubated with prenylated Rho3 and Cdc42 obtained from the *in vitro* translation as described in Materials and Methods. Input lanes for Rho3 binding experiments represent signal equivalent to 2.5% binding. Input lane for Cdc42 binding experiments represent signal equivalent to 10% binding. The Cdc42-N17 binding experiment was exposed to film twice as long as the other experiments due to lower efficiency of translation for this mutant. Shown below is Coomassie blue staining of the GST fusion proteins used for binding and bar graphs representing quantitation of the binding with error bars representing standard deviation of three independent experiments.

Isolation of Novel Conditional-Lethal Alleles of *EXO70*

A previous screen for mutants in *EXO70* resulted in the identification of two alleles, *exo70-38* and *exo70-35*, with unusual phenotypes nearly identical to those described for *cdc42-6* (He et al., 2007a). Like *cdc42-6* mutants, *exo70-38* and *exo70-35*, exhibit defects in secretion of Bgl2 but not invertase and show accumulation of post-Golgi vesicles in small, but not large budded cells. Invertase and Bgl2 are thought to be delivered to the cell surface through two distinct populations of post-Golgi vesicles, and both populations are blocked by all of the original 10 late *sec* mutants (Harsay and Bretscher, 1995). The remarkable similarity of the unusual phenotypes of the *exo70-38* and *exo70-35* mutants with the *cdc42-6* mutant gives strong *in vivo* support for the notion that Exo70 is a direct effector of Cdc42 function in exocytosis, as suggested by the dominant suppressor and biochemical interaction studies described above. However, the phenotypes associated with the *exo70-38* and *exo70-35* mutants are not consistent with the model that Exo70 is a downstream effector of the Rho3 GTPase. The *rho3-V51* mutant has significantly more expansive secretory defects than

seen in these two mutants. Specifically, the *rho3-V51* mutant shows pronounced defects in both invertase and Bgl2 secretion and accumulates post-Golgi vesicles in both small and large budded cells. Since these phenotypes are at odds with the genetic and biochemical studies described above linking Exo70 as a downstream effector of both GTPases, we postulated that the *exo70-38* and *exo70-35* mutants might represent hypomorphic alleles of *EXO70*, defective in transmitting Cdc42 function but still permissive for promoting Rho3 function. To test this idea we set out to identify and characterize novel conditional-lethal alleles in *EXO70* and describe the loss-of-function phentoypes associated with them.

To identify additional alleles of *EXO70* with recessive conditional growth defects, we screened a library of random PCR-generated *exo70* mutants using a plasmid shuffle strategy (see materials and methods). Following growth on 5-FOA media at 25^{o}C (to evict the *EXO70* plasmid and reveal possible recessive phenotypes), 57 independent colonies were identified that exhibited low-temperature sensitive growth at 14^{o}C (cs-) and 40 colonies were identified that exhibited high temperature sensitive (ts-) growth. Following recovery of the mutant plasmids from these strains and re-transformation into the *exo70Δ/CEN-URA3-EXO70* plasmid shuffle strain, we identified one plasmid containing a cold-sensitive allele (*exo70-188*), and one plasmid containing a temperature-sensitive allele (*exo70-113*). These alleles (as well as wild-type *EXO70* control) were then integrated at the *EXO70* locus by replacing a disruption in a heterozygous diploid. Following sporulation and tetrad dissection, the pattern of spores showing conditional growth was seen to be tightly linked to the newly introduced allele. Thus the phenotypes of the integrated alleles were similar to that of the original plasmid-borne mutants. These integrated mutant strains were then used for all the

subsequent analyses of the growth and secretion phenotypes associated with these new alleles of *exo70*.

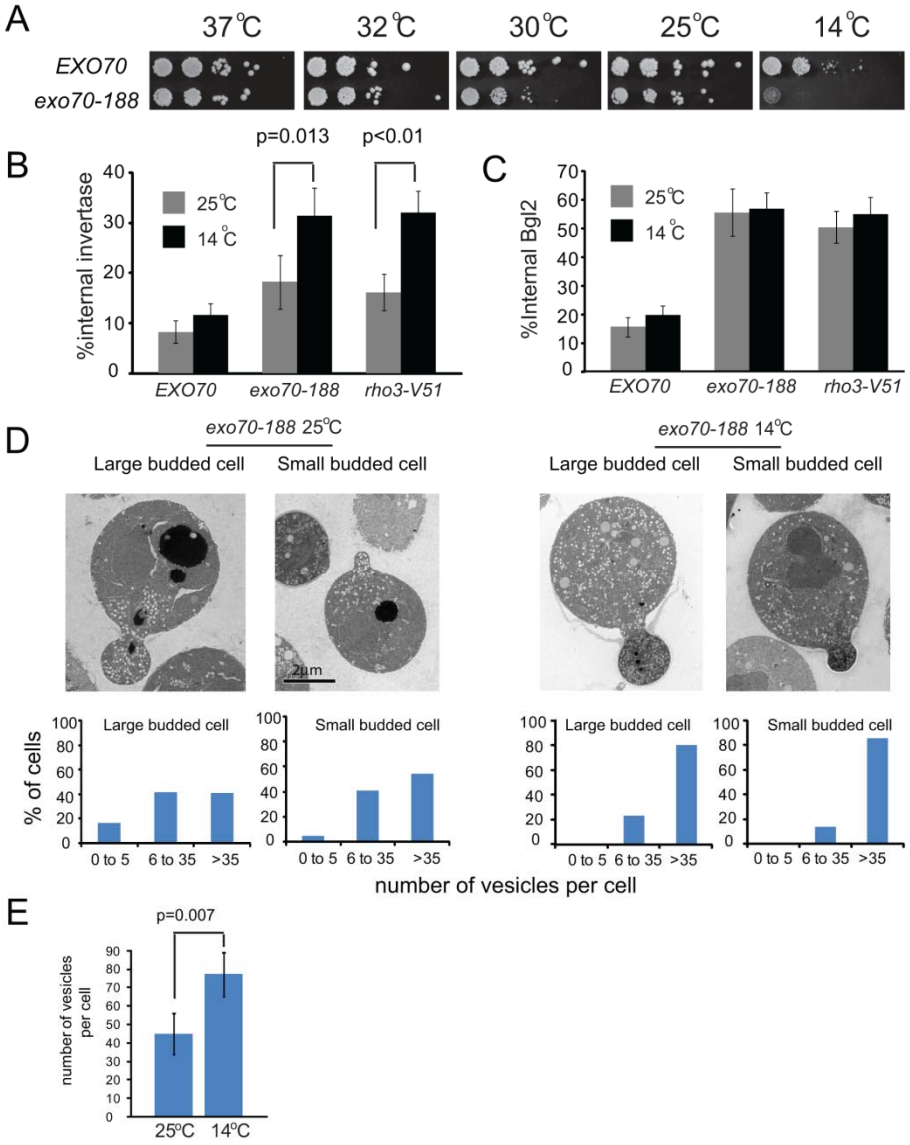


Figure 12: Characterization of a novel cold-sensitive mutant *exo70-188*.

(**A**) Growth properties of the cold sensitive mutant, *exo70-188* at permissive and non-permissive temperatures. Wild type *EXO70* and *exo70-188* mutant alleles were integrated at the *EXO70* locus. Ten fold serial dilutions of yeast cells were grown on YPD plates at the indicated temperatures. (**B**) The *exo70-188* mutant is defective in secretion of the periplasmic enzyme invertase. Invertase assays were conducted on cells shifted to low glucose media at the permissive temperature (25°C) for 2 hours or the restrictive temperature (14°C) for 9 hours. The graph shows the percentage of internal invertase at 25°C and 14°C in each strain

analyzed. The error bars represent standard deviation of five independent experiments. (**C**)The *exo70-188* is defective in Bgl2 secretion. Bgl2 assays were performed on cells kept at permissive temperature or shifted to restrictive temperature for 9 hours. The percent distribution of Bgl2 was determined by immunoblot analysis using affinity-purified Bgl2 antibody. The graph depicts the percentage of Bgl2 protein that remains internal in *EXO70*, *exo70-188*, and *rho3-V51* strains. Quantitations were performed by Odyssey Infrared Imaging system. Error bars represent standard deviation of four independent experiments. (**D**) The *exo70-188* mutant accumulates post-Golgi vesicles in both large budded cells and small budded cells. The *exo70-188* mutant cells kept at the permissive temperature or shifted to restrictive temperature for 9 hours were fixed and processed for electron microscopy. Examples of both small (<1 μm) and large-budded (1 μm or greater) cells are shown. Cells were scored for number of vesicles accumulated throughout the cell and bar graphs show the percentage of small budded or large budded cells containing different number of vesicles. Scale bar,2 μm. (**E**) Overall post-Golgi vesicle accumulation was determined for EM sections by scoring cells greater than 3 um in diameter (irrespective of bud or bud size) for 80-100 nm vesicles. For each condition micrographs were divided into three groups representing 20-25 cells each to determine the standard deviation in this assay.

We examined the *exo70-188*$^{cs-}$ allele for its ability to secrete invertase and Bgl2 at permissive temperature (25^{o}C) and following a 9 hr shift to the non-permissive conditions (14^{o}C). A wild-type *EXO70* and cold-sensitive *rho3-V51* strain were assayed in parallel as negative and positive controls for cold-sensitive secretory defects. As can be seen in Figure 12B and C, the *exo70-188* mutant shows pronounced invertase and Bgl2 secretion defects at 14^{o}C (relative to *EXO70*) and both the magnitude and temperature dependence of these secretory effects are remarkably similar to that seen in *rho3-V51* cells (Roumanie *et al*., 2005). When *exo70-188* cells were shifted to 14^{o}C and then examined by thin section electron microscopy, a highly penetrant (>80% of cells) and dramatic (>75 vesicles/cell on average) accumulation of 80-100 nm post-Golgi vesicles was apparent (Figure 12D and Figure 12E). As we had seen previously with *rho3-V51*, and consistent with the secretion data, a lower—and less penetrant—level of vesicle accumulation was observed in *exo70-188*

mutants grown at the permissive temperatures (Figure 12D and Figure 12E). To determine if there was an effect of the stage of the cell cycle on vesicle accumulation in this mutant we compared the vesicle accumulation in cells with small (<1 uM diameter) buds compared to larger (>1 uM diameter) buds, as we have previously with *cdc42-6* and *exo70-38*. The results, shown in Figure 12D clearly demonstrate that both large and small budded *exo70-188* cells have a similar high-degree of penetrance and vesicle accumulation at both permissive and non-permissive temperatures.

The temperature-sensitive *exo70-113* mutant was characterized in a similar fashion except that cells were shifted to 37^{o}C for 2 hr to examine the effect of temperature-shift on exocytic function. An isogenic wild-type *EXO70* and *sec6-4* mutant were examined in parallel as controls. As can be seen in Figure 13B and Figure 13C, the *exo70-113*$^{ts-}$ mutant shows pronounced invertase and Bgl2 secretion defects following a shift to 37^{o}C and when examined by thin section electron microscopy, a highly penetrant (>80% of cells) and strong (>80 vesicles/cell on average) accumulation of 80-100 nm post-Golgi vesicles was apparent in both large and small budded cells (Figure 13 D and Figure 13 E). Taken together our analysis of these two new alleles demonstrates that Exo70 function is required for all the secretory functions seen with other late-acting *sec* genes including secretion of both the Bgl2 and the invertase class of vesicles, and exocytosis in early bud emergence as well as in later stages of bud growth. These observations are important to the present study because they demonstrate that the spectrum of phenoytpes associated with loss-of Exo70 function is entirely consistent with Exo70 being a downstream effector of both Cdc42 and Rho3 function in exocytosis.

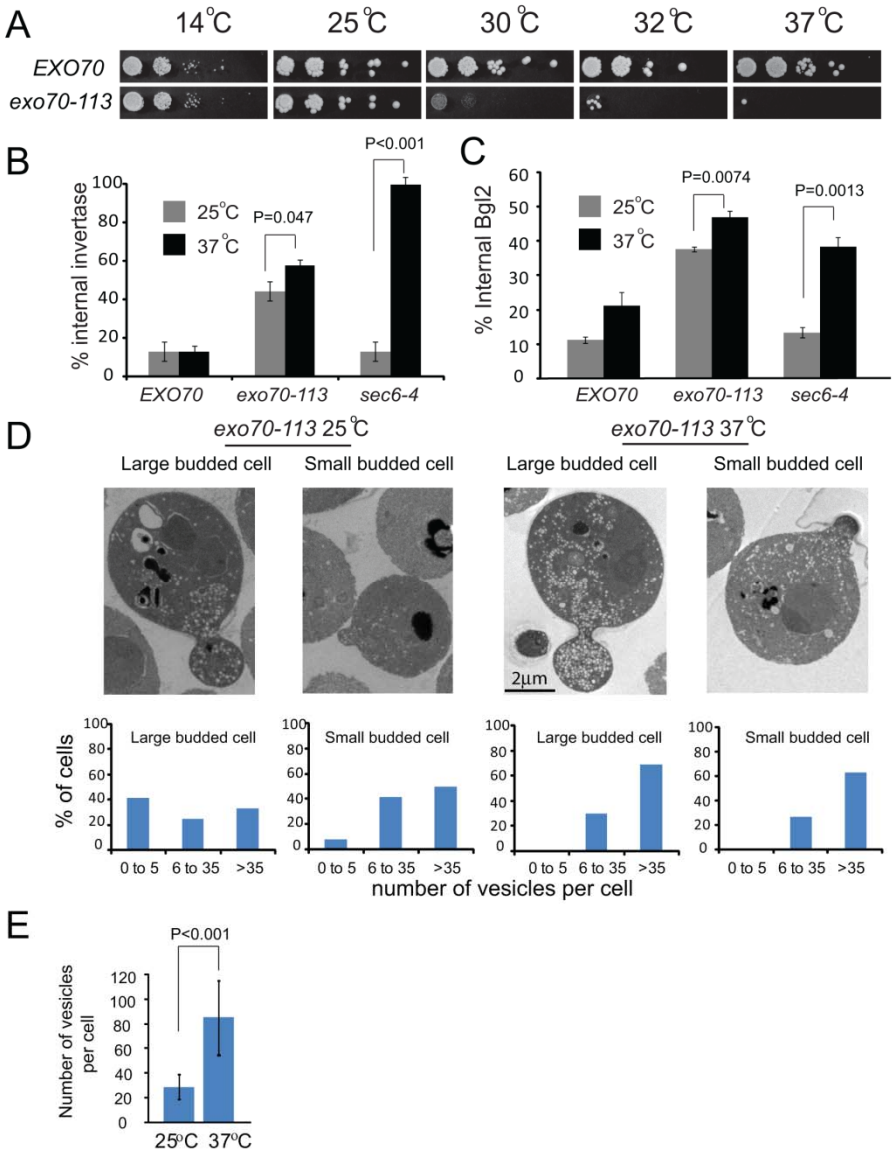


Figure 13:Characterization of a novel temperature sensitive mutant *exo70-113*.

(**A**) Growth defect of a novel temperature sensitive mutant *exo70-113*. Wild type *EXO70* and *exo70-113* alleles were integrated at the *EXO70* locus. Ten fold serial dilutions of yeast cells were grown on YPD plates at the indicated temperatures. (**B**) The *exo70-113* mutant is defective in secretion of the periplasmic enzyme invertase. Invertase assays were performed on cells after a 2 hour shift to low glucose media at permissive (25°C) or a 1.5 hour shift to restrictive temperature (37°C). The graph shows the percentage of internal invertase in the strains analyzed. The error bars represent standard deviation of four independent experiments. (**C**) *exo70-113* is defective in Bgl2 secretion. Bgl2 assays were performed on cells kept at permissive temperature or shifted to the restrictive temperature of 37°C for 2 hours. The percent distribution of Bgl2 was determined by immunoblot analysis using affinity-purified

Bgl2 antibody. The graph depicts the percentage of Bgl2 protein that remains internal in *EXO70*, *exo70-113*, and *sec6-4* strains. Quantitations were performed by Odyssey Infrared Imaging system. Error bars represent standard deviation of four independent experiments. (**D**) *exo70-113* mutant accumulates post-Golgi vesicles in both large budded cells and small budded cells. *exo70-113* mutant cells kept at the permissive temperature or shifted to restrictive temperature for 2 hours were fixed and processed for electron microscopy. Examples of both small (<1 μm) and large-budded (1 μm or greater) cells are shown. Cells were scored for number of vesicles accumulated throughout the cell and bar graphs show the percentage of small budded or large budded cells containing different number of vesicles. Scale bar, 2 μm. (**E**) Overall post-Golgi vesicle accumulation was determined for EM sections by scoring cells greater than 3 um in diameter (irrespective of bud or bud size) for 80-100 nm vesicles.

DISCUSSION

We have previously characterized a role for Cdc42 and Rho3 as positive regulators of exocytosis and found that this role was independent of polarization of both the actin cytoskeleton and of the exocyst complex (Roumanie, *et al*. 2005). We suggested a model where these GTPases would work by locally increasing the activity (or throughput) of the exocytic apparatus at sites occupied by GTP-bound Rho/Cdc42 proteins (Roumanie *et al.*, 2005; Wu *et al.*, 2008). However, the precise effector pathway by which such a mechanism would take place was either unknown—in the case for Cdc42—or highly controversial—in the case of Rho3. In this report we provide three independent lines of evidence which strongly supports this model of regulation, and demonstrates that the Exo70 component of the exocyst complex represents a critical and direct effector for both Rho3 and Cdc42 function in spatial regulation of exocytosis.

A number of studies have been carried out to investigate the interaction of Exo70 and Rho family GTPases in yeast and mammalian cells. However, despite the fact that both yeast

and mammalian Exo70 have been reported to bind Rho family GTPases and the overall structural similarity between yeast and mammalian Exo70 proteins is very high throughout their length, the sites of interaction reported for the GTPases appeared to be quite distinct. Studies using tagged-TC10 in transfected mammalian cells identified a region within domains A and B in mouse Exo70 as the major site of binding, while studies using recombinant Rho3 have identified a binding site within domain C as the major binding site for the yeast Exo70 protein (Dong *et al.*, 2005; He *et al.*, 2007b; Hutagalung *et al.*, 2009). To make matters more difficult, mutations or deletions in Exo70 which block recombinant Rho3 binding had little or no effect on growth or secretion of yeast cells containing these mutant *exo70* alleles as the sole source of Exo70 in the cell (He *et al.*, 2007b; Hutagalung *et al.*, 2009). Finally, phenotypic analysis of two conditional alleles of *exo70*, showed exocytic phenotypes that were remarkably similar to that of *cdc42-6*, but very distinct from that of *rho3-V51* (He et al., 2007a). However, despite the similarity in phenotypes with the Cdc42 mutant, biochemical analyses failed to show any detectable interaction between Exo70 and recombinant GTP-bound forms of Cdc42 (He *et al.*, 2007a). Collectively these results appeared to be entirely incompatible with the notion that Exo70 was a direct effector of Cdc42 or Rho3 in yeast. Moreover, the ability of Exo70 family members to interact with Rho GTPases is highly conserved in yeast and mammals, suggesting that structurally divergent mechanisms had evolved into interactions with completely distinct regions of the yeast and mammalian Exo70 proteins.

A key finding to unraveling these apparently contradictory observations was the use of post-translationally modified forms of Rho3 and Cdc42 in our Exo70 binding studies, a

strategy similar to that used to map the TC10 interaction with mammalian Exo70. In particular, we found that mutation of the cysteine present in the C-terminal CAAX motif of either Cdc42 or Rho3 resulted in a dramatic loss of interaction with Exo70. Since this modification does not occur in bacterially-produced forms of these GTPases, the absence of this modification is likely responsible for the dramatic differences seen in the binding assays described here from those described previously. Furthermore, since the mapping of the binding of the Cdc42 homolog TC10 to mouse Exo70 made use of a mammalian expression system (hence prenylated TC10), this may explain the apparent distinction in the site of binding between the yeast and mammalian forms of Exo70. Further mapping of the Rho GTP binding site in both yeast and mammalian Exo70 will be necessary to determine how structurally similar these binding events are. Since Rho3 and Cdc42 are predicted to be prenylated with different prenyl moities—Rho3 with farnesyl and Cdc42 with geranlygeranyl—we think it unlikely that the prenyl groups themselves are part of the effector interaction. Rather we suggest that the presence of the prenyl group is likely to affect presentation of the GTP-bound Rho protein to Exo70 in the context of the detergent micelles present in the binding assays. This may reflect the nature of the interaction which likely occurs in close proximity to the membrane *in vivo*, since both Exo70 and the GTPase have C-terminal membrane targeting motifs. A role for prenylation in effector binding is unusual but not unprecedented. Ohya and colleagues (Inoue *et al.*, 1999) demonstrated that the physical interaction of Rho1 with its effector Fks1 was highly dependent on the C-terminal geranylgeranylation. It remains to be seen if other Rho effector relationships depend on prenylation, as this is it not a feature which is commonly examined.

What does "activation" of the exocyst by Rho GTPases do to regulate exocytosis? In its simplest form this activation would involve an increase in the rate of vesicle docking and fusion at a specific place on the plasma membrane demarcated by the GTP-bound form of Cdc42 and/or Rho3. Since the loss of function mutants in *cdc42* and *rho3*~~,~~ fail to show a detectable effect on polarization of Sec4 or exocyst subunits to sites of polarized growth, and the mutants are strongly suppressed by increased levels of the t-SNARE Sec9, we favor the model that these GTPases act through the exocyst to increase localized SNARE assembly leading to more rapid vesicle fusion. The details of how this activation mechanism works remain to be elucidated. However since dominant alleles of Exo70 described here~~,~~ act as though they are "constitutively active" forms of Exo70—they will be valuable tools in helping to understand the nature of this activation. For example, all of the gain-of-function mutants appear to have similarly fully assembled exocyst complex, suggesting that assembly and/or disassembly of the complex is unlikely to represent the major mode of regulation by these GTPases.

In addition to Exo70, Sec3--another component of the exocyst complex--has been proposed to be an effector in regulating polarized growth downstream of the Rho1 and Cdc42 GTPases (Guo *et al.*, 2001; Zhang *et al.*, 2008; Hutagalung *et al*., 2009). However, it is unlikely that Sec3 is the principal effector for Cdc42 function in exocytosis. First, deletion of the N-terminal Rho-binding domain of Sec3 is non-essential and has no detectable impact on polarized growth or secretion at any temperature, while the *cdc42-6* mutant results in a severe temperature-sensitive growth and secretion defect. Second, we have previously shown that deletion of the Sec3 N-terminal domain results in a synthetic lethal phenotype

when combined with the *cdc42-6* mutant, which strongly suggests that this domain of Sec3 has an important role that acts in parallel (perhaps through Rho1 signaling) with Cdc42 signaling through Exo70 (Roumanie *et al.*, 2005; Zhang *et al.*, 2008; Hutagalung *et al.*, 2009). In agreement with the idea that the Cdc42-Exo70 pathway functions in parallel with a Sec3-Rho pathway, several groups have shown synthetic lethality between *sec3-ΔNT* and several *EXO70* loss-of-function mutants (Zhang *et al.,* 2008; Hutagalong *et al.,* 2009). It also important to note that while the Exo70 effector activity appears to be regulated by both Rho3 and Cdc42 but not Rho1, the N-terminus of Sec3 has been reported to interact with both Rho1 and Cdc42 but not Rho3. Therefore this process appears to be regulated by a complex network of partially overlapping Rho GTPase/effector relationships which may provide increased robustness to the overall spatial regulation of polarized exocytosis.

ACKNOWLEDGEMENT

The authors are grateful to Hal Mekeel (University of North Carolina) for assistance with electron microscopy and Garret Thompson for technical assistance. We would like to thank Dr. Mary Munson (University of Massachusetts Worcester Campus) for the gift of Sec6, Sec10, and Exo84 antibodies. We thank Dr. Guendalina Rossi and Leah Watson for helpful discussions and critical reading of the manuscript. We thank Dr. Jan McColm for editorial assistance on this manuscript. This work was supported by grants from the Mathers Charitable Foundation, and the National Institutes of Health (GM54712) to P.J. Brennwald.

Table 1: Yeast Strains used in Chapter Three

Strain	Genotype	Reference
BY582	*MATa rho3Δ::LEU2 rho3-V51::URA3 his3-Δ200*	Adamo et al, 1999
BY944	*MAT a rho3Δ::LEU2 rho3-V51::URA3 his3-Δ200 ura3-200*	Adamo et al, 1999
BY1051	*MATa Gal^{+} leu2-3,112 LEU2::RHOL74*	P.Brennwald Collection
BY1052	*MATa Gal^{+} leu2-3,112 LEU2::rho3N30*	P.Brennwald Collection
BY1054	*MATa Gal^{+} leu2-3,112 LEU2::CDC42L61*	P.Brennwald Collection
BY1055	*MATa Gal^{+} leu2-3,112 LEU2::cdc42N17*	P.Brennwald Collection
BY2132	*MATa Gal^{+} leu2-3,112 LEU2::RHO1L68*	This study
BY2142	*MATa Gal^{+} leu2-3,112 LEU2::cdc42L29, V31, H32,L61*	This study
BY2227	*MATa Gal^{+} leu2-3,112 LEU2:: rho3V51QL*	This study
BY2228	*MATa Gal^{+} leu2-3,112 LEU2::cdc42QLC188A*	This study
BY2229	*MATa Gal^{+} leu2-3,112 LEU2:: rho3QLC228A*	This study
BY2198	*MATα EXO70::KanMX6 his3-Δ200 leu2-3,112 ura3-52*	This study
BY2130	*MATa exo70-188^{cs}::KanMX6 his3-Δ200 leu2-3,112 ura3-52*	This study
BY2136	*MATa exo70-113^{ts}::KanMX6 his3-Δ200 leu2-3,112 ura3-52*	This study
BY2277	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70*	This study
BY2278	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70I114F*	This study
BY2279	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70E126V*	This study
BY2280	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70G388R*	This study
BY2281	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70N479K*	This study
BY2282	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70I486N*	This study
BY2283	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70D533N*	This study
BY2284	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70D541Y*	This study
BY2285	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70E543K*	This study
BY2286	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70E557K*	This study
BY2287	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70K564E*	This study
BY2288	*MATa exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70R623I*	This study
BY2296	*MATa SEC8-3XMyc::URA3 exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70*	This study
BY2297	*MATa SEC8-3XMyc::URA3 exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70G388R*	This study
BY2299	*MATa SEC8-3XMyc::URA3 exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70E557K*	This study
BY2300	*MATa SEC8-3XMyc::URA3 exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70K564E*	This study
BY2302	*MATa SEC8-3XMyc::URA3 exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70I114F*	This study
BY2303	*MATa SEC8-3XMyc::URA3 exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70E126V*	This study
BY2304	*MATa SEC8-3XMyc::URA3 exo70Δ::HIS3 ura3-52 his3-Δ200 leu2-3,112 pRS315-EXO70I486N*	This study
NY17	*MATa ura3-52 sec6-4*	P.Novick Collection

Table 2: Plasmids used in Chapter Three

Strain	Host	Description
BB1701	DH5α	*pRS315 EXO70*
BB1622	DH5α	*pRS315 EXO70I114F*
BB1623	DH5α	*pRS315 EXO70E126V*
BB1624	DH5α	*pRS315 EXO70G388R*
BB1625	DH5α	*pRS315 EXO70N479K*
BB1627	DH5α	*pRS315 EXO70D541Y*
BB1628	DH5α	*pRS315 EXO70E547K*
BB1629	DH5α	*pRS315 EXO70E557K*
BB1801	DH5α	*pRS315 EXO70K564E*
BB1630	DH5α	*pRS315 EXO70R623I*
BB1614	DH5α	*pRS313 EXO70E126V*
BB1615	DH5α	*pRS313 EXO70G388R*
BB1616	DH5α	*pRS313 EXO70N479K*
BB1618	DH5α	*pRS313 EXO70D541Y*
BB1619	DH5α	*pRS313 EXO70E547K*
BB1620	DH5α	*pRS313 EXO70E557K*
BB1621	DH5α	*pRS313 EXO70R623I*
BB1702	DH5α	*pRS315 exo-188*
BB1704	DH5α	*pRS315 exo-113*
BB1579	DH5α	pGEX6P1PH (N terminal GST tag and C terminal 6 His tag)
BB1587	BL21	pGEX6P1PH *EXO70*
BB1709	BL21	pGEX6P1PH *EXO70-1521 (E443A,R444A,K445A,E488A,K489A,E491A)*
BB1828	BL21	pGEX6P1PH *EXO70-ΔC* (aa 346-515 is deleted)
BB1814	BL21	pGEX6P1PH *STE20* (aa314-432)
BB442	BL21	pGEX4T1 Sec9 (aa402-651)

REFERENCE

Adamo, J.E., Moskow, J.J., Gladfelter, A.S., Viterbo, D., Lew, D.J., and Brennwald, P.J. (2001). Yeast Cdc42 functions at a late step in exocytosis, specifically during polarized growth of the emerging bud. J Cell Biol *155*, 581-592.

Adamo, J.E., Rossi, G., and Brennwald, P. (1999). The Rho GTPase Rho3 has a direct role in exocytosis that is distinct from its role in actin polarity. Mol Biol Cell *10*, 4121-4133.

Barrowman, J., and Novick, P. (2003). Three Yips for Rab recruitment. Nat Cell Biol *5*, 955-956.

Bourne, H.R. (1988). Do GTPases direct membrane traffic in secretion? Cell *53*, 669-671.

Bowser, R., Muller, H., Govindan, B., and Novick, P. (1992). Sec8p and Sec15p are components of a plasma membrane-associated 19.5S particle that may function downstream of Sec4p to control exocytosis. J Cell Biol *118*, 1041-1056.

Bowser, R., and Novick, P. (1991). Sec15 protein, an essential component of the exocytotic apparatus, is associated with the plasma membrane and with a soluble 19.5S particle. J Cell Biol *112*, 1117-1131.

Boyd, C., Hughes, T., Pypaert, M., and Novick, P. (2004). Vesicles carry most exocyst subunits to exocytic sites marked by the remaining two subunits, Sec3p and Exo70p. J Cell Biol *167*, 889-901.

Brennwald, P., Kearns, B., Champion, K., Keranen, S., Bankaitis, V., and Novick, P. (1994). Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. Cell *79*, 245-258.

Brennwald, P., and Rossi, G. (2007). Spatial regulation of exocytosis and cell polarity: yeast as a model for animal cells. FEBS Lett *581*, 2119-2124.

Camonis, J.H., and White, M.A. (2005). Ral GTPases: corrupting the exocyst in cancer cells. Trends Cell Biol *15*, 327-332.

Chen, X.W., Leto, D., Chiang, S.H., Wang, Q., and Saltiel, A.R. (2007). Activation of RalA is required for insulin-stimulated Glut4 trafficking to the plasma membrane via the exocyst and the motor protein Myo1c. Dev Cell *13*, 391-404.

Chiang, S.H., Baumann, C.A., Kanzaki, M., Thurmond, D.C., Watson, R.T., Neudauer, C.L., Macara, I.G., Pessin, J.E., and Saltiel, A.R. (2001). Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. Nature *410*, 944-948.

Conibear, E., Cleck, J.N., and Stevens, T.H. (2003). Vps51p mediates the association of the GARP (Vps52/53/54) complex with the late Golgi t-SNARE Tlg1p. Mol Biol Cell *14*, 1610-1623.

Croteau, N.J., Furgason, M.L., Devos, D., and Munson, M. (2009). Conservation of helical bundle structure between the exocyst subunits. PLoS One *4*, e4443.

Dong, G., Hutagalung, A.H., Fu, C., Novick, P., and Reinisch, K.M. (2005). The structures of exocyst subunit Exo70p and the Exo84p C-terminal domains reveal a common motif. Nat Struct Mol Biol *12*, 1094-1100.

Finger, F.P., and Novick, P. (1998). Spatial regulation of exocytosis: lessons from yeast. J Cell Biol *142*, 609-612.

Gangar, A., Rossi, G., Andreeva, A., Hales, R., and Brennwald, P. (2005). Structurally conserved interaction of Lgl family with SNAREs is critical to their cellular function. Curr Biol *15*, 1136-1142.

Goode, B.L., and Eck, M.J. (2007). Mechanism and function of formins in the control of actin assembly. Annu Rev Biochem *76*, 593-627.

Goud, B., Salminen, A., Walworth, N.C., and Novick, P.J. (1988). A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. Cell *53*, 753-768.

Govindan, B., Bowser, R., and Novick, P. (1995). The role of Myo2, a yeast class V myosin, in vesicular transport. J Cell Biol *128*, 1055-1068.

Grosshans, B.L., Andreeva, A., Gangar, A., Niessen, S., Yates, J.R., 3rd, Brennwald, P., and Novick, P. (2006). The yeast lgl family member Sro7p is an effector of the secretory Rab GTPase Sec4p. J Cell Biol *172*, 55-66.

Grote, E., Carr, C.M., and Novick, P.J. (2000). Ordering the final events in yeast exocytosis. J Cell Biol *151*, 439-452.

Grote, E., and Novick, P.J. (1999). Promiscuity in Rab-SNARE interactions. Mol Biol Cell *10*, 4149-4161.

Guo, W., Grant, A., and Novick, P. (1999a). Exo84p is an exocyst protein essential for secretion. J Biol Chem *274*, 23558-23564.

Guo, W., Roth, D., Walch-Solimena, C., and Novick, P. (1999b). The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. EMBO J *18*, 1071-1080.

Guo, W., Tamanoi, F., and Novick, P. (2001). Spatial regulation of the exocyst complex by Rho1 GTPase. Nat Cell Biol *3*, 353-360.

Hamburger, Z.A., Hamburger, A.E., West, A.P., Jr., and Weis, W.I. (2006). Crystal structure of the S.cerevisiae exocyst component Exo70p. J Mol Biol *356*, 9-21.

Hancock, J.F. (1995). Reticulocyte lysate assay for in vitro translation and posttranslational modification of Ras proteins. Methods Enzymol *255*, 60-65.

Harsay, E., and Bretscher, A. (1995). Parallel secretory pathways to the cell surface in yeast. J Cell Biol *131*, 297-310.

Harsay, E., and Schekman, R. (2002). A subset of yeast vacuolar protein sorting mutants is blocked in one branch of the exocytic pathway. J Cell Biol *156*, 271-285.

Hattendorf, D.A., Andreeva, A., Gangar, A., Brennwald, P.J., and Weis, W.I. (2007). Structure of the yeast polarity protein Sro7 reveals a SNARE regulatory mechanism. Nature *446*, 567-571.

He, B., Xi, F., Zhang, J., TerBush, D., Zhang, X., and Guo, W. (2007a). Exo70p mediates the secretion of specific exocytic vesicles at early stages of the cell cycle for polarized cell growth. J Cell Biol *176*, 771-777.

He, B., Xi, F., Zhang, X., Zhang, J., and Guo, W. (2007b). Exo70 interacts with phospholipids and mediates the targeting of the exocyst to the plasma membrane. EMBO J *26*, 4053-4065.

Helliwell, S.B., Schmidt, A., Ohya, Y., and Hall, M.N. (1998). The Rho1 effector Pkc1, but not Bni1, mediates signalling from Tor2 to the actin cytoskeleton. Curr Biol *8*, 1211-1214.

Higgs, H.N., and Pollard, T.D. (2001). Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. Annu Rev Biochem *70*, 649-676.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene *77*, 61-68.

Hsu, S.C., Hazuka, C.D., Roth, R., Foletti, D.L., Heuser, J., and Scheller, R.H. (1998). Subunit composition, protein interactions, and structures of the mammalian brain sec6/8 complex and septin filaments. Neuron *20*, 1111-1122.

Hsu, S.C., TerBush, D., Abraham, M., and Guo, W. (2004). The exocyst complex in polarized exocytosis. Int Rev Cytol *233*, 243-265.

Hutagalung, A.H., Coleman, J., Pypaert, M., and Novick, P.J. (2009). An internal domain of Exo70p is required for actin-independent localization and mediates assembly of specific exocyst components. Mol Biol Cell *20*, 153-163.

Imai, J., Toh-e, A., and Matsui, Y. (1996). Genetic analysis of the Saccharomyces cerevisiae RHO3 gene, encoding a rho-type small GTPase, provides evidence for a role in bud formation. Genetics *142*, 359-369.

Inoue, M., Chang, L., Hwang, J., Chiang, S.H., and Saltiel, A.R. (2003). The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin. Nature *422*, 629-633.

Inoue, S.B., Qadota, H., Arisawa, M., Watanabe, T., and Ohya, Y. (1999). Prenylation of Rho1p is required for activation of yeast 1, 3-beta-glucan synthase. J Biol Chem *274*, 38119-38124.

Irazoqui, J.E., Gladfelter, A.S., and Lew, D.J. (2003). Scaffold-mediated symmetry breaking by Cdc42p. Nat Cell Biol *5*, 1062-1070.

Jin, R., Junutula, J.R., Matern, H.T., Ervin, K.E., Scheller, R.H., and Brunger, A.T. (2005). Exo84 and Sec5 are competitive regulatory Sec6/8 effectors to the RalA GTPase. EMBO J *24*, 2064-2074.

Jullien-Flores, V., Mahe, Y., Mirey, G., Leprince, C., Meunier-Bisceuil, B., Sorkin, A., and Camonis, J.H. (2000). RLIP76, an effector of the GTPase Ral, interacts with the AP2 complex: involvement of the Ral pathway in receptor endocytosis. J Cell Sci *113 (Pt 16)*, 2837-2844.

Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M., and Satoh, T. (1991). Structure and function of signal-transducing GTP-binding proteins. Annu Rev Biochem *60*, 349-400.

Leevers, S.J., Paterson, H.F., and Marshall, C.J. (1994). Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature *369*, 411-414.

Lehman, K., Rossi, G., Adamo, J.E., and Brennwald, P. (1999). Yeast homologues of tomosyn and lethal giant larvae function in exocytosis and are associated with the plasma membrane SNARE, Sec9. J Cell Biol *146*, 125-140.

Liu, J., Zuo, X., Yue, P., and Guo, W. (2007). Phosphatidylinositol 4,5-bisphosphate mediates the targeting of the exocyst to the plasma membrane for exocytosis in mammalian cells. Mol Biol Cell *18*, 4483-4492.

Lu, J., Meng, W., Poy, F., Maiti, S., Goode, B.L., and Eck, M.J. (2007). Structure of the FH2 domain of Daam1: implications for formin regulation of actin assembly. J Mol Biol *369*, 1258-1269.

Matsui, Y., and Toh-e, A. (1992). Isolation and characterization of two novel ras superfamily genes in Saccharomyces cerevisiae. Gene *114*, 43-49.

Matsui, Y., and Toh, E.A. (1992). Yeast RHO3 and RHO4 ras superfamily genes are necessary for bud growth, and their defect is suppressed by a high dose of bud formation genes CDC42 and BEM1. Mol Cell Biol *12*, 5690-5699.

Mazur, P., and Baginsky, W. (1996). In vitro activity of 1,3-beta-D-glucan synthase requires the GTP-binding protein Rho1. J Biol Chem *271*, 14604-14609.

Mineo, C., Anderson, R.G., and White, M.A. (1997). Physical association with ras enhances activation of membrane-bound raf (RafCAAX). J Biol Chem *272*, 10345-10348.

Moore, B.A., Robinson, H.H., and Xu, Z. (2007). The crystal structure of mouse Exo70 reveals unique features of the mammalian exocyst. J Mol Biol *371*, 410-421.

Morrison, D.K., and Davis, R.J. (2003). Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annu Rev Cell Dev Biol *19*, 91-118.

Moskalenko, S., Henry, D.O., Rosse, C., Mirey, G., Camonis, J.H., and White, M.A. (2002). The exocyst is a Ral effector complex. Nat Cell Biol *4*, 66-72.

Moskalenko, S., Tong, C., Rosse, C., Mirey, G., Formstecher, E., Daviet, L., Camonis, J., and White, M.A. (2003). Ral GTPases regulate exocyst assembly through dual subunit interactions. J Biol Chem *278*, 51743-51748.

Munson, M., and Novick, P. (2006). The exocyst defrocked, a framework of rods revealed. Nat Struct Mol Biol *13*, 577-581.

Nicholson, K.L., Munson, M., Miller, R.B., Filip, T.J., Fairman, R., and Hughson, F.M. (1998). Regulation of SNARE complex assembly by an N-terminal domain of the t-SNARE Sso1p. Nat Struct Biol *5*, 793-802.

Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A., and Takai, Y. (1995). A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in Saccharomyces cerevisiae. EMBO J *14*, 5931-5938.

Novick, P., Ferro, S., and Schekman, R. (1981). Order of events in the yeast secretory pathway. Cell *25*, 461-469.

Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell *21*, 205-215.

Novick, P., and Schekman, R. (1979). Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *76*, 1858-1862.

Ohta, Y., Suzuki, N., Nakamura, S., Hartwig, J.H., and Stossel, T.P. (1999). The small GTPase RalA targets filamin to induce filopodia. Proc Natl Acad Sci U S A *96*, 2122-2128.

Park, H.O., and Bi, E. (2007). Central roles of small GTPases in the development of cell polarity in yeast and beyond. Microbiol Mol Biol Rev *71*, 48-96.

Pfeffer, S.R. (1994). Rab GTPases: master regulators of membrane trafficking. Curr Opin Cell Biol *6*, 522-526.

Prehoda, K.E., Scott, J.A., Mullins, R.D., and Lim, W.A. (2000). Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. Science *290*, 801-806.

Raman, M., Chen, W., and Cobb, M.H. (2007). Differential regulation and properties of MAPKs. Oncogene *26*, 3100-3112.

Richardson, C.J., Jones, S., Litt, R.J., and Segev, N. (1998). GTP hydrolysis is not important for Ypt1 GTPase function in vesicular transport. Mol Cell Biol *18*, 827-838.

Robinson, N.G., Guo, L., Imai, J., Toh, E.A., Matsui, Y., and Tamanoi, F. (1999). Rho3 of Saccharomyces cerevisiae, which regulates the actin cytoskeleton and exocytosis, is a GTPase which interacts with Myo2 and Exo70. Mol Cell Biol *19*, 3580-3587.

Rodnina, M.V., Pape, T., Fricke, R., and Wintermeyer, W. (1995). Elongation factor Tu, a GTPase triggered by codon recognition on the ribosome: mechanism and GTP consumption. Biochem Cell Biol *73*, 1221-1227.

Rossi, G., Salminen, A., Rice, L.M., Brunger, A.T., and Brennwald, P. (1997). Analysis of a yeast SNARE complex reveals remarkable similarity to the neuronal SNARE complex and a

novel function for the C terminus of the SNAP-25 homolog, Sec9. J Biol Chem *272*, 16610-16617.

Roth, A.F., Wan, J., Bailey, A.O., Sun, B., Kuchar, J.A., Green, W.N., Phinney, B.S., Yates, J.R., 3rd, and Davis, N.G. (2006). Global analysis of protein palmitoylation in yeast. Cell *125*, 1003-1013.

Roumanie, O., Wu, H., Molk, J.N., Rossi, G., Bloom, K., and Brennwald, P. (2005). Rho GTPase regulation of exocytosis in yeast is independent of GTP hydrolysis and polarization of the exocyst complex. J Cell Biol *170*, 583-594.

Salminen, A., and Novick, P.J. (1987). A ras-like protein is required for a post-Golgi event in yeast secretion. Cell *49*, 527-538.

Salminen, A., and Novick, P.J. (1989). The Sec15 protein responds to the function of the GTP binding protein, Sec4, to control vesicular traffic in yeast. J Cell Biol *109*, 1023-1036.

Schott, D., Ho, J., Pruyne, D., and Bretscher, A. (1999). The COOH-terminal domain of Myo2p, a yeast myosin V, has a direct role in secretory vesicle targeting. J Cell Biol *147*, 791-808.

Scott, B.L., Van Komen, J.S., Irshad, H., Liu, S., Wilson, K.A., and McNew, J.A. (2004). Sec1p directly stimulates SNARE-mediated membrane fusion in vitro. J Cell Biol *167*, 75-85.

Shan, S.O., Chandrasekar, S., and Walter, P. (2007). Conformational changes in the GTPase modules of the signal reception particle and its receptor drive initiation of protein translocation. J Cell Biol *178*, 611-620.

Shipitsin, M., and Feig, L.A. (2004). RalA but not RalB enhances polarized delivery of membrane proteins to the basolateral surface of epithelial cells. Mol Cell Biol *24*, 5746-5756.

Sivaram, M.V., Furgason, M.L., Brewer, D.N., and Munson, M. (2006). The structure of the exocyst subunit Sec6p defines a conserved architecture with diverse roles. Nat Struct Mol Biol *13*, 555-556.

Sivaram, M.V., Saporita, J.A., Furgason, M.L., Boettcher, A.J., and Munson, M. (2005). Dimerization of the exocyst protein Sec6p and its interaction with the t-SNARE Sec9p. Biochemistry *44*, 6302-6311.

Songer, J.A., and Munson, M. (2009). Sec6p anchors the assembled exocyst complex at sites of secretion. Mol Biol Cell *20*, 973-982.

Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M., and Hancock, J.F. (1994). Activation of Raf as a result of recruitment to the plasma membrane. Science *264*, 1463-1467.

Symons, M., and Rusk, N. (2003). Control of vesicular trafficking by Rho GTPases. Curr Biol *13*, R409-418.

TerBush, D.R., Maurice, T., Roth, D., and Novick, P. (1996). The Exocyst is a multiprotein complex required for exocytosis in Saccharomyces cerevisiae. EMBO J *15*, 6483-6494.

TerBush, D.R., and Novick, P. (1995). Sec6, Sec8, and Sec15 are components of a multisubunit complex which localizes to small bud tips in Saccharomyces cerevisiae. J Cell Biol *130*, 299-312.

Togneri, J., Cheng, Y.S., Munson, M., Hughson, F.M., and Carr, C.M. (2006). Specific SNARE complex binding mode of the Sec1/Munc-18 protein, Sec1p. Proc Natl Acad Sci U S A *103*, 17730-17735.

Tolliday, N., VerPlank, L., and Li, R. (2002). Rho1 directs formin-mediated actin ring assembly during budding yeast cytokinesis. Curr Biol *12*, 1864-1870.

Vitale, N., Mawet, J., Camonis, J., Regazzi, R., Bader, M.F., and Chasserot-Golaz, S. (2005). The Small GTPase RalA controls exocytosis of large dense core secretory granules by interacting with ARF6-dependent phospholipase D1. J Biol Chem *280*, 29921-29928.

Wagner, W., Bielli, P., Wacha, S., and Ragnini-Wilson, A. (2002). Mlc1p promotes septum closure during cytokinesis via the IQ motifs of the vesicle motor Myo2p. EMBO J *21*, 6397-6408.

Walch-Solimena, C., Collins, R.N., and Novick, P.J. (1997). Sec2p mediates nucleotide exchange on Sec4p and is involved in polarized delivery of post-Golgi vesicles. J Cell Biol *137*, 1495-1509.

Walworth, N.C., Brennwald, P., Kabcenell, A.K., Garrett, M., and Novick, P. (1992). Hydrolysis of GTP by Sec4 protein plays an important role in vesicular transport and is stimulated by a GTPase-activating protein in Saccharomyces cerevisiae. Mol Cell Biol *12*, 2017-2028.

Wedlich-Soldner, R., Altschuler, S., Wu, L., and Li, R. (2003). Spontaneous cell polarization through actomyosin-based delivery of the Cdc42 GTPase. Science *299*, 1231-1235.

Wennerberg, K., and Der, C.J. (2004). Rho-family GTPases: it's not only Rac and Rho (and I like it). J Cell Sci *117*, 1301-1312.

Whyte, J.R., and Munro, S. (2001). The Sec34/35 Golgi transport complex is related to the exocyst, defining a family of complexes involved in multiple steps of membrane traffic. Dev Cell *1*, 527-537.

Wu, H., Rossi, G., and Brennwald, P. (2008). The ghost in the machine: small GTPases as spatial regulators of exocytosis. Trends Cell Biol *18*, 397-404.

Wu, S., Mehta, S.Q., Pichaud, F., Bellen, H.J., and Quiocho, F.A. (2005). Sec15 interacts with Rab11 via a novel domain and affects Rab11 localization in vivo. Nat Struct Mol Biol *12*, 879-885.

Yon, J., and Fried, M. (1989). Precise gene fusion by PCR. Nucleic Acids Res *17*, 4895.

Zhang, X., Bi, E., Novick, P., Du, L., Kozminski, K.G., Lipschutz, J.H., and Guo, W. (2001). Cdc42 interacts with the exocyst and regulates polarized secretion. J Biol Chem *276*, 46745-46750.

Zhang, X., Orlando, K., He, B., Xi, F., Zhang, J., Zajac, A., and Guo, W. (2008). Membrane association and functional regulation of Sec3 by phospholipids and Cdc42. J Cell Biol *180*, 145-158.

Zhang, X., Wang, P., Gangar, A., Zhang, J., Brennwald, P., TerBush, D., and Guo, W. (2005a). Lethal giant larvae proteins interact with the exocyst complex and are involved in polarized exocytosis. J Cell Biol *170*, 273-283.

Zhang, X., Zajac, A., Zhang, J., Wang, P., Li, M., Murray, J., TerBush, D., and Guo, W. (2005b). The critical role of Exo84p in the organization and polarized localization of the exocyst complex. J Biol Chem *280*, 20356-20364.

Zhang, X.M., Ellis, S., Sriratana, A., Mitchell, C.A., and Rowe, T. (2004). Sec15 is an effector for the Rab11 GTPase in mammalian cells. J Biol Chem *279*, 43027-43034.

CHAPTER FOUR

The N-terminus of Rho3 Determines its Function and Localization during Exocytosis

ABSTRACT

The Rho family small GTPases are important regulators in many cellular events such as polarized exocytosis and actin cytoskeleton. In yeast *Saccharomyces cerevisiae*, Rho3 and Cdc42 play important roles in regulating exocytosis which are independent from their roles in actin polarity. Studies on mutant alleles of Rho3 and Cdc42 suggested that while Cdc42 functions during bud emergence and early bud growth, Rho3 functions throughout the cell cycle. Consistent with its function in the early stages of the cell cycle, Cdc42 localization is concentrated at the bud tip where active secretion occurs. However, the localization of Rho3 and the functional specificity of Rho3 have not been clearly described. Using a novel monoclonal antibody for Rho3, we find that Rho3 has a distinctive localization pattern compared to that of Cdc42. While Cdc42 is concentrated at the bud tip where active secretion occurs, Rho3 localizes at the periphery of the plasma membrane. Studies on the chimeric proteins between Rho3 and Cdc42 suggested that the N terminus of Rho3 is necessary and sufficient for its function and localization in the cell. Further analysis of the Rho3 N terminus revealed two elements that are important for its function. A cysteine residue at position five is palmitoylated and dictates the Rho3 plasma membrane localization. The N terminus also contains a pair of basic residues (arginine and lysine) at positions 17, 18 that are required for interacting with the downstream target Exo70. The distinctive plasma membrane localization together with the stronger interaction with Exo70 specifies Rho3 function in exocytosis.

INTRODUCTION

In eukaryotic cells, exocytosis is a fundamental process that mediates the secretion of intracellular protein contents to the extracellular compartment. In *Saccharomyces cerevisiae*, polarized exocytosis is tightly regulated by a number of gene products to ensure proper delivery of newly synthesized membrane protein and lipids to specific domains of the plasma membrane. These gene products include the type V myosin Myo2 for vesicle transport(Schott *et al.*, 1999), the exocyst complex for vesicle tethering(TerBush and Novick, 1995), Rho family small GTPases for activation of the exocytic machinery (Roumanie *et al.*, 2005) and the SNARE complex mediating membrane fusion.

The exocyst complex is a multisubunit protein complex composed of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 (TerBush *et al.*, 1996). Early work from a number of laboratories demonstrated that components of the exocyst complex function as downstream effectors for members of the Rho family small GTPases in regulating exocytosis. There are five Rho family small GTPases in yeast, Rho1-4, and Cdc42. Studies from our laboratory demonstrated that Rho3 and Cdc42 have direct roles in regulating exocytosis. A cold sensitive mutant of Rho3, *rho3-V51,* displayed severe growth and secretion defect, but the actin polarity remained intact at restrictive conditions (Adamo *et al.*, 1999). In addition to the *rho3-V51* mutant*,* we also identified a temperature sensitive mutant in Cdc42, *cdc42-6*. Analysis of the secretory function of the *cdc42-6* revealed Bgl2 secretion defects and accumulation of post-Golgi vesicles (Adamo *et al.*, 2001). Like *rho3-V51,* the actin cytoskeleton in *cdc42-6* is normally polarized, suggesting that the function of Rho3 and Cdc42in exocytosis is separate from their roles in regulating actin polarity.

Further analysis of the *rho3-V51* and the *cdc42-6* mutants revealed several common features. First, the exocyst subunits Sec3, Sec8 and Exo70 are highly polarized in both mutants, suggesting that the function of Rho3 and Cdc42 in secretion is through allosteric activation rather than recruitment of the exocytic machinery (Roumanie *et al.*, 2005). Second, genetic analysis suggested that the pathway by which Cdc42 regulates secretion is closely linked to that of Rho3. The *rho3-V51* and the *cdc42-6* mutants can both be suppressed by *SEC4, SRO7*, and *SEC9,* all of which are essential components of the exocytic machinery (Adamo *et al.*, 2001). The shared suppressors and the synthetic lethality between *cdc42-6* and *rho3-V51* mutants provided strong genetic evidence that Rho3 and Cdc42 have overlapping functions in exocytosis. Recent work from our laboratory provided the first direct evidence that Exo70 interact with both Rho3 and Cdc42 from yeast lysate. This interaction is GTP dependent and is only detectable when both GTPases are fully prenylated (Wu *et al*, 20009).

Although Rho3 and Cdc42 share a common effector and have overlapping functions, there are different characteristics in how these two proteins regulate exocytosis in yeast. Analysis of the secretory machinery revealed that while *rho3-V51* is defective in invertase and Bgl2 secretion, *cdc42-6* mutant is only defective in Bgl2 secretion (Adamo *et al.*, 1999; Adamo *et al.*, 2001). Invertase and Bgl2 are carried by two classes of vesicles with different density and distinctive enriched cargo (Harsay and Schekman, 2002). This cargo specific phenotype suggested that Rho3 and Cdc42 might affect different branches of the exocytic pathway. Another distinctive aspect is that *rho3-V51* and *cdc42-6* mutants accumulated post-Golgi vesicles during different stages of the cell cycle. Electron microscopy showed that the *rho3-V51* mutant displayed an equivalent accumulation of post-Golgi vesicles throughout the

cell cycle, whereas the accumulation of post-Golgi vesicles in *cdc42-6* is only restricted to small budded cells. These data demonstrated that although both GTPases can function during bud emergence and early bud growth, only Rho3 is required for later bud growth.

Sequence alignment of Rho3 and Cdc42 revealed that Rho3 has a long N-terminal extension, which contains a palmitoylation motif (Roth *et al.*, 2006). This is a unique feature for Rho3 because most Rho family small GTPases are palmitoylated at their C terminus next to the CAAX domain (Wennerberg and Der, 2004). Cdc42 has a polybasic domain at its C terminus, which is a common membrane targeting signal via the electrostatic interactions with phospholipids at the plasma membrane. However, the Rho3 C terminus does not contain a polybasic domain. Furthermore, the CAAX motif of Rho3 is farnesylated instead of being geranylgeranylated as seen in Cdc42. Farnesyl chains contains 15 carbons, which does not penetrate into the membrane as deep as geranylgeranyl chains. This leads to the idea that palmitoylation might help localize Rho3 at the plasma membrane.

To understand how Rho3 and Cdc42 carry out both distinctive and shared functions, we sought to identify regions of each protein that are responsible for their functional specificity by constructing chimeric proteins between the two small GTPases based on these apparent differences. Here, we report that Rho3 has a very distinctive localization pattern compared to that of Cdc42. While Cdc42 concentrates at the bud tip where active secretion occurs, Rho3 displayed a more dispersed localization around the periphery of the cell. The function and localization of Rho3 is critically dependent on the N terminus region of this protein. Addition of this domain onto Cdc42 increased the affinity of its interaction with

Exo70, and created a chimeric protein that functions and localizes as both Rho3 and Cdc42. Dissecting the N terminus of Rho3 revealed two functionally important elements. The cysteine residue is important for its localization, and the arginine and lysine at the 17, 18 position are important for interacting with the downstream effector Exo70. The synergistic regulation of localization and downstream effector interaction determines the specificity by which Rho3 and Cdc42 regulate polarized exocytosis.

MATERIAL AND METHODS

Yeast Strains, Reagents, and Genetic Techniques

Cells were grown in YPD media containing 1% bacto-yeast extract, 2% bacto-peptone, and 2% glucose. The components of the media were from Fisher Scientific. Sorbital, sodium azide (NaN3), Sodium Flouride (NaF), Ethanolamine, β-mercaptoethanol, Triton X-100, and HIS-Select® Nickel Affinity Gel were obtained from Sigma Chemical (St. Louis, MO). Zymolyase (100T) was from Seikagaku (Tokyo, Japan). 10% Tween-20 was from BioRad. DTT, BSA, yeast nitrogen base, raffinose galactose and 5-Fluoroorotic Acid were from US Biologicals (Swampscott, MA). Glutathione sepharose beads were from Amershan Biosciences. Secondary antibodies for Odyssey Imaging system were from LI-COR Biosciences and Molecular Probes. Secondary antibodies for immunofluorescene are from Jackson ImmunoResearch. Formaldehyde (37%) was from Electron Microscopy Sciences (Ft. Washington, PA). FluorSave Reagent (mounting media) was from Calbiochem. Slides for Immunofluorescence are from Carlson Scientific, Inc. The bead beater for making yeast

lysate was from Biospec Products. Transformations for suppression analysis were performed using the lithium acetate method described in Guthrie and Fink (1991).

Construction of chimeras

Chimeric genes were constructed by overlapping PCR reaction and verified by DNA sequencing (Horton *et al.*, 1989; Yon and Fried, 1989). Primers and templates used are listed in Table 3

Protein purification and quantification

The pGEX-6P6H (pB1579) was constructed as described previously. This vector was used to create the following GST-fusion constructs: GST-Sec9 (aa 402-651) and GST-Exo70 (aa 1-623). All constructs were confirmed by sequencing and protein expression was performed in *Escherichia coli* BL21 cells. Cells were grown at 37°C in Terrific broth medium to an OD_{599} 2.0-2.5. Cells were shifted to 25°C and protein expression was induced with 0.1 μM IPTG for 3 hours at 25^{o}C.

6xHis-tag purification was performed by binding the bacteria lysate to HIS-Select® Nickel Affinity Gel (Sigma), and then eluting with 500 mM imidazole. The 6xHistidine eluates were then incubated with glutathione Sepharose beads at 4^{o}C for one hour, and the beads were washed with wash buffer (20 mM Tris pH7.5, 120 mM NaCl, and 1% Tween 20) to remove unbound proteins. Protein concentration was determined by comparison to purified protein standards after SDS-PAGE and Coomassie Blue staining. Quantification of Coomassie Blue stained gels was performed by Odyssey Infrared Imaging System (LiCor).

Generation of yeast cell lysate

RHO3L74, *rho3-NT^{C42}L61 , rho3-L74A17,18 CDC42L61*, *CDC42NT^{R3}L74* were amplified by PCR reaction and subcloned behind the *GAL1* promoter in a *LEU2* integrating vector (BB24, pRS305 with *GAL1* promoter). The vector was linearized by digesting with *BstX*I restriction enzyme and transformed into a Gal$^+$ strain BY17 (*a; GAL*1*; leu2-3,112; ura3-52*). Yeast strains were first grown in YP with 3% raffinose overnight at 30^{o}C to mid-log phase of OD 1-1.5, and incuced with a final concentration of 1% galactose for two additional doublings (four hours) at 30^{o}C. Cells were pelleted by centrifugation at 5000 rpm for 5 minutes and washed with ddH_2O. Pellets were immediately frozen on dry ice. Frozen pellets were lysed with lysis buffer using a bead beater from Biospec products. The optimal wet weight for the small chamber was 5-6 grams. The pellet was beaten for 1 minute followed by a 3 minutes pause for five cycles. Lysate was then subjected to centrifugation for 10 min at 17,000xg, followed by ultracentrifugation at 100,000xg for 30minutes. The protein concentration of the lysate was measured by a Bradford assay. Each lysate was normalized to about 25 mg/ml total protein concentration and frozen on dry ice.

GST pull-down from yeast cell lysate

All recombinant proteins were present at a final concentration of 3μM. Cell lysates were prepared for GST pulldown experiments as described above and incubated with each fusion protein bound to glutathione sepharose beads for 1.5 hours at 4^{o}C. The beads were washed five times with lysis buffer (20 mM tris-HCl pH7.5, 120 mM NaCl, 10 mM $MgCl_2$, 1% Tween 20, 1 mM DTT), and boiled at 95^{o}C for 5 minutes. Samples were subjected to SDS-

PAGE analysis and blotted with Rho3 and Cdc42 monoclonal antibodies. Quantification of western blots was performed with Odyssey infrared imaging system.

Immunofluorescence

Cells were grown overnight to mid-log phase and fixed immediately with 37% formaldehyde. Fixed cells were spheroplasted, permeabilized with 0.5% SDS, and affixed to the slides as described previously (Brennwald and Novick, 1993, Roumanie et al, 2005). Rho3 mAb at a 1:10 dilution and affinity-purified polyclonal Cdc42 at a 1:75 dilution were incubated for 90 minutes. The secondary antibodies used were FITC-conjugated goat anti–rabbit at 1:75 dilution for detection of Cdc42 and Rhodamine Red-X-AffiniPure goat anti-mouse IgG at 1:75 dilution for detection of Rho3. Stained cells were viewed on a microscope (model E600; Nikon) equipped with a 512X512 back illuminated frame-transfer charge coupled device camera (Princeton Instruments) and Metamorph software (Universal Imaging Corp.).

Subcellular fractionation

Cells containing *RHO3, rho3-NT^{C42}, RHO3-A3,4, RHO3-A5,* and *rho3-A17,18* were grown overnight in selective media, harvested, and grown in rich media for 2 hours. Approximately 200 OD_{599} were washed with 10/20/20 buffer (10 mM Tris, pH 7.5, 20mM NaN_3, and 20mM NaF) and spheroplasted in 7.2 ml of spheroplast buffer (0.1 M Tris, 10 mM azide, 1.2 M sorbitol, and 21 mM β-mercaptoethanol with 0.1mg/ml Zymolyase 100T) for 30 min at 37^{o}C. The spheroplasts were lysed in 5 ml of ice-cold triethanolamine (TEA)/sorbitol (10 mM TEA, pH 7.2, and 0.8 M sorbitol) with a protease inhibitor cocktail (1 mg/ml each of leupeptin, aprotinin, antipain, 0.5mM PMSF, 20mM Pepstatin and 2 mM 4-(2-aminoethyl)

benzenesulfonyl fluoride) and spun at 450 *g* for 3 min in a cold centrifuge to remove unbroken cells. The lysates were spun at 31,000 *g* for 20 min at 4^{o}C in a Sorvall centrifuge tube to separate supernatant and pellet fraction. All pellets were normalized to the volume of the supernatant fractions. Equal volume of total, supernatant and pellet fraction were boiled in SDS sample buffer, run on a 15% SDS–polyacrylamide gel, and blotted with polyclonal Sso1/2 antibody (1:2000), monoclonal Rho3 antibody (1:200) or Cdc42 antibody (1:250). Quantification of western blots was performed with Odyssey infrared imaging system.

RESULT

Rho3 and Cdc42 have Distinct Localization Patterns on the Plasma Membrane

The function of Cdc42 in polarized growth is thought to be closely tied to its highly polarized pattern of localization on the plasma membrane. Consistent with this notion we have previously shown that Cdc42 mutants defective in exocytic function, demonstrate this defect only during early bud emergence when the localization of Cdc42 is highly polarized and not at later stages of bud growth. In contrast secretory defective mutants in the Rho3 GTPase, demonstrate defects throughout bud growth. However the intracellular localization of Rho3 has not been described. We have isolated a new monoclonal antibody to Rho3 which we have used in double-labeling experiments to examine the pattern of localization compared to that of Cdc42. The results, shown in Figure 14, demonstrate a surprisingly distinct staining pattern. In stark contrast to the tightly polarized localization of Cdc42, Rho3 localizes along most of the plasma membrane and only slightly more abundant in the buds and bud-proximal portion of the mother cell plasma membrane. This led us to hypothesize

that the distinct plasma membrane localization patterns for these two GTPases might be linked to their distinct functions in promoting polarized growth during different stages of bud growth.

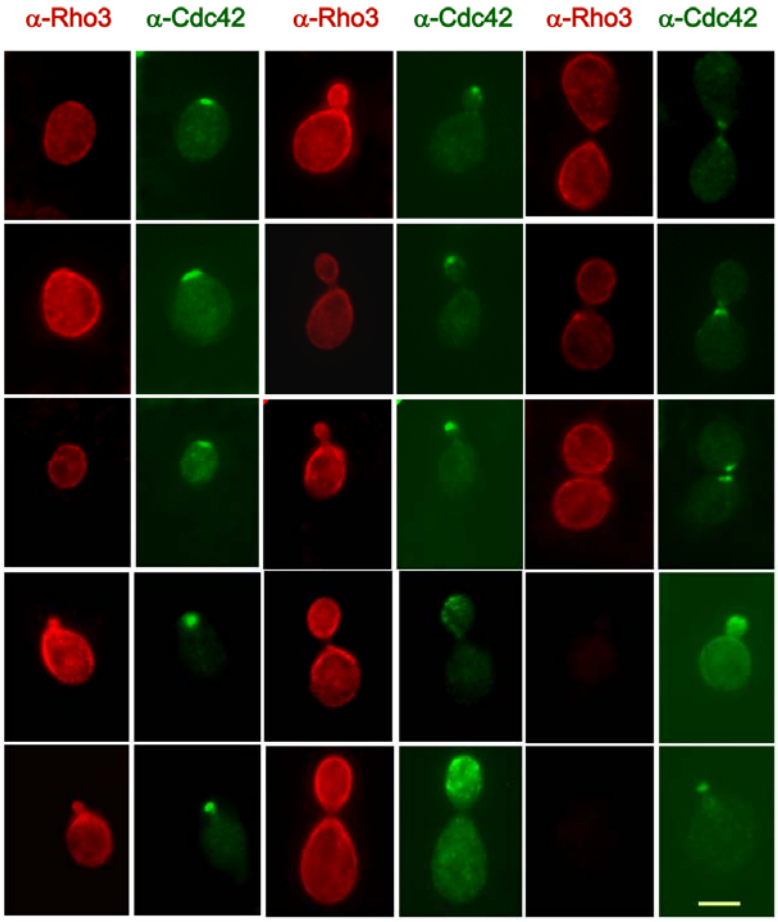


Figure 14: Localization of Rho3 and Cdc42

Localization of Rho3 and Cdc42 were examined by immunofluorescence microscopy. Each cell was double-labelled with monoclonal Rho3 antibody (red pseudo color) and affinity purified polyclonal Cdc42 antibody (green pseudo color) in a *RHO3* plasmid shuffle strain. Cells containing *rho3Δ* are used as a negative control for the specificity for Rho3 monoclonal antibody (both images in the bottom of the 5th column). Cells were grown in *SC-URA* media overnight at 25^{o}C, shifted to YPD media for 2 hours and processed for immunofluorescence microscopy as described in Material and Methods. Scale bar 2μm.

To test this model, we created chimeric forms of the two GTPases to identify the region(s) required for the distinct functions and localization in the cell. Surprisingly, we found that our initial set of chimeras that exchanged a small region (20 residues of Rho3 and 6 residues of Cdc42) at the extreme N-termini of the two proteins resulted in rather dramatic consequence to both the function and localization of these two GTPases. We examined the functional consequences of these chimeras expressed behind either the *CDC42* or *RHO3* promoter present on a low-copy *CEN* plasmid. The ability of each chimera to complement a deletion in the chromosomal copy of *RHO3* or *CDC42* was assessed using a plasmid shuffle assay (see materials and methods). The results of the complementation show that the N-terminus of Rho3 is critical for its function (Figure 15B). Rho3 lost its N terminus no longer functions as Rho3 in the cell, suggesting that the N terminus of Rho3 is required for its function. Surprisingly, addition of this region onto Cdc42 converts Cdc42 into a chimeric protein, Cdc42-NT^{R3} that completely complements the loss of Rho3 in the cell. Interestingly, the ability of the Cdc42-NT^{R3} chimera to act as the sole source of Rho3 does not result in a diminution of its ability to act as Cdc42. The Cdc42-NT^{R3} chimera shows no detectable loss-of-function as the sole source of Cdc42 as it can completely complement the *cdc42Δ*. Expression levels of the chimeras were nearly identical to their non-chimeric genes (on *CEN* plasmids), suggesting that the switch of function phenotype was not due to the elevated protein levels in the cell. Identical results were obtained for all constructs whether expressed behind the *CDC42* or *RHO3* promoter.

We then examined the effect of the N-terminal exchange on the localization of the chimeric GTPases using the same Rho3 and Cdc42 antibodies as mentioned in Figure 14. Since the Rho3 and Cdc42 antibodies react with the C terminal portion of each GTPases, this allows us to probe the localization of each chimera as the only source of Rho3 or Cdc42 in the cell. When we examined the staining pattern of Cdc42-NT^{R3} chimera as the sole source of Cdc42 in the cell, we found a striking change in the staining pattern observed with the Cdc42 antibody. The staining pattern, seen in Figure 15C, is very similar to that of Rho3, although a significant number of cells with small or incipient buds also demonstrated a "Cdc42-like" staining pattern (Figure 15C). When the Rho3-NT^{C42} chimera was analyzed, we found the normal Rho3 pattern of plasma membrane staining was lost and only diffuse cytoplasmic staining was observed. Taken together this suggested that the Rho3 N-terminus is an important determinate for localization—both in dictating the pattern of localization as well as recruitment of the Rho GTPase to the plasma membrane. Importantly the ability of the N-terminus to affect both the function and localization of the associated GTPase gives strong support to the model that localization pattern is critical to the function of these two GTPases in the cell.

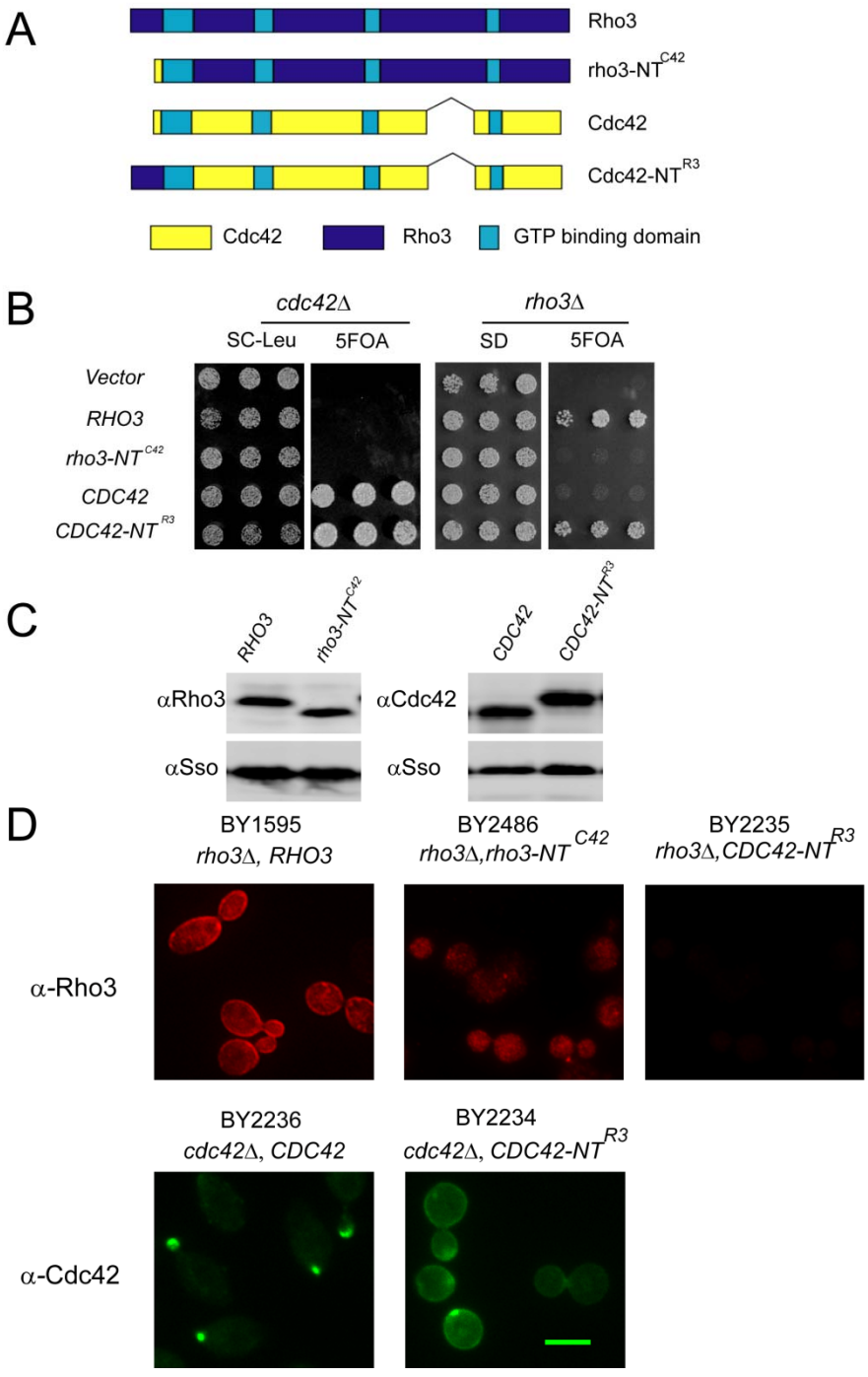


Figure 15:The N terminus of Rho3 is necessary and sufficient for its function and localization.

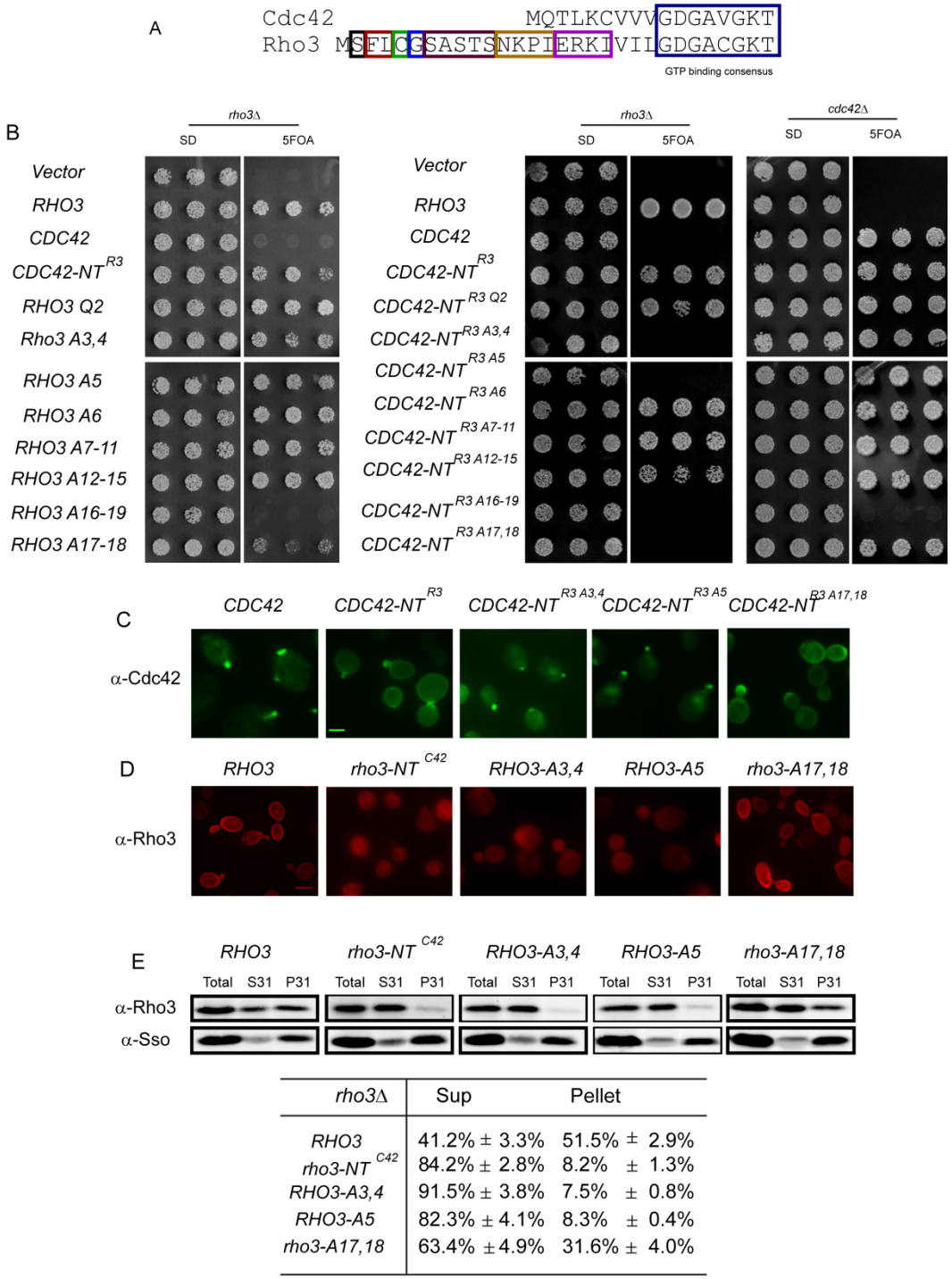
(**A**) Schematic representations of *RHO3, RHO3* with the N terminus of *CDC42* (*rho3-NT*^{C42}), *CDC42*, and *CDC42* with the N terminus of *RHO3* (*CDC42-NT*^{R3}). (**B**) *RHO3*, *rho3-NT*^{C42}*,CDC42,* and *CDC42-NT*^{R3} were transformed into a *RHO3* plasmid shuffle strain or a *CDC42* plasmid shuffle strain on a *CEN HIS3* plasmid. The growth of three independent colonies was shown under restrictive conditions where the wild type *RHO3-URA3* or the *CDC42-URA3* plasmid was lost by counter selection on 5FOA plates. (**C**) Whole cell lysates were prepared in strains with *CDC42* or *CDC42-NT*^{R3} as the only source for *CDC42* and strains with *RHO3* or *rho3-NT*^{C42} as the only source for *RHO3*. Lysates were subjected to SDS-PAGE analysis and blotted with Cdc42 or Rho3 monoclonal antibodies. Since *rho3-NT*^{C42} cannot function as the only copy of *RHO3* in the cell, the gain-of-function chimera *CDC42-NT*^{R3} was introduced into the cell to maintain its viability. (**D**) Cells containing *RHO3*, *rho3-NT*^{C42}*, CDC42*, and *CDC42-NT*^{R3} were grown at 25^{o}C, fixed and process for immunofluorescence. Monoclonal antibody against Rho3 and affinity purified polyclonal antibody against Cdc42 were used as described in materials and methods. The yeast strain (BY2235) contains *rho3Δ* complemented by *CDC42-NT*^{R3} as a control for the specificity of the Rho3 monoclonal antibody. Scale bar, 2μm.

The Rho3 N-terminus Encodes Multiple Specificity Determinants

To further dissect the mechanism by which the N-terminus of Rho3 affected the function and localization of the protein, we carried out an extensive mutagenesis of this domain and examined the effect on localization and function both in the context of the Cdc42-NT^{R3} chimera as well as in wild-type Rho3. The functional studies shown in Figure 16B, demonstrate a requirement for a cysteine at position 5, and two adjacent residues at positions 3, 4 in the ability of this domain to affect a switch of function phenotype in the context of Cdc42-NT^{R3} chimera. The cysteine residue at position five has previously been shown to be palmitoylated (Roth *et al.*, 2006). Consistent with this notion, we find that mutations at this position result in loss of plasma membrane staining and the protein is largely lost from the membrane pellet fraction following cell fractionation (Figure 16E). The effect of the A3, 4 residues appears to have a similar effect on localization and fractionation (Figure 16E) and therefore may be a component of the recognition sequence used by the palmitoyl transferase.

We next examined the effect of the palmitoylation site mutations on the ability of the N-terminus of Rho3 to affect the localization pattern of the Cdc42-NT^{R3} chimera. Interestingly, we found the cysteine to alanine mutation in this chimera resulted in a complete loss of Rho3-like staining pattern and instead revealed a staining pattern identical to seen for wild-type Cdc42. Thus palmitoylation of the N-terminus appears to be critical signal in re-directing the pattern of localization to the dispersed pattern seen for Rho3.

Surprisingly, while loss of palmitoylation was required for the switch of function phenoytpe, it was dispensible for function in the context of wild-type Rho3. Although these mutations resulted in a significantly more soluble protein (as judged by fluorescence and fractionation), presumably enough Rho3 is associated with the membrane to provide the minimal requirements for Rho3 signaling.



rho3Δ	Sup	Pellet
RHO3	41.2% ± 3.3%	51.5% ± 2.9%
rho3-NT C42	84.2% ± 2.8%	8.2% ± 1.3%
RHO3-A3,4	91.5% ± 3.8%	7.5% ± 0.8%
RHO3-A5	82.3% ± 4.1%	8.3% ± 0.4%
rho3-A17,18	63.4% ± 4.9%	31.6% ± 4.0%

Figure 16:The N terminus of Rho3 contains two elements that are important for its function and localization.

(**A)** Single residues or a group of residues are mutated to alanine at the N terminus of Rho3. Mutations were depicted with color boxes. (**B)** *CDC42,* and *RHO3, CDC42-NT*^{R3} with the

corresponding N terminal mutants were transformed into a *RHO3* plasmid shuffle strain on a *CEN HIS3* plasmid. The ability of the mutants to complement *rho3Δ*were analyzed on 5FOA plates when the original *RHO3-URA3* plasmid was lost . (**C**) *CDC42, CDC42-NT^{R3}, CDC42-NT$^{R3\ A3,4}$, CDC42-NT$^{R3\ A5}$,* and *CDC42-NT^{R3A17,18},* were first transformed in to a *CDC42* plasmid shuffle strain and then grown on 5FOA plates to evict the original *CDC42* plasmid. Immunofluorescence was performed using anti-Cdc42 polyclonal antibody as described in materials and methods. (**D**) Yeast cells containing *RHO3*, *rho3-NT^{C42} , RHO3-A3,4, RHO3-A5,* and *rho3-A17,18* on *CEN-HIS3* plasmids were grown to midlog phase at 25^{o}C, fixed and processed for immunofluorescence. Monoclonal antibody against Rho3 was used at 1: 10 dilution. Scale bar: 2μm. (**E**) Yeast cells containing *RHO3*, *rho3-NT^{C42} , RHO3-A3,4, RHO3-A5,* and *rho3-A17,18* as the only source of *RHO3* were grown at 25^{o}C, spheroplasted, lysed and spun at 31,000 *g* to separate the cytosolic and the membrane fraction. Equal volume of total cell lysates (total), supernatant (S31) and pellet (P31) were subjected to SDS-PAGE analysis and blotted with Rho3 monoclonal antibody. *rho3-NT^{C42}* (BY2486) and *rho3-A17,18* (BY2487)stains contain a *CDC42-NT^{R3}* on a *CEN-URA3* plasmid to maintain the viability of the cell.

The requirement for palmitoylation of the Rho3 N-terminus in the context of the Cdc42-NTR3 chimera, allowed us to ask which member(s) of the DHHC family of palmitoyltransferases were important for converting Cdc42-NT^{R3} chimera into a protein capable of functioning as Rho3. To test this we constructed strains containing deletions of each of the 7 DHHC family members in the context of the *RHO3* plasmid shuffle strain. We then examined the effect of loss of each palmitoyltransferase on the ability of the Cdc42-NT^{R3} chimera to complement the *rho3Δ*. Suprisingly, we found that loss of Erf2, the palmitoyltransferase previously suggested to be the major palmitoyltransferase for Rho3 (Roth *et al.*, 2006), had only a modest effect on the ability of the chimera to act as the sole source of Rho3. Another transferase, Pfa4, also showed a similar mild but detectable effect on complementation. Interestingly, the Akr1 transferase, demonstrated the strongest effect—as loss of this DHHC family member completely blocked the ability of the Cdc42-NTR3 to complement. The other four DHHC family members showed no detectable effect

on complementation in this assay (Figure 17). Taken together this data suggests the involvement of two new palmitoyltransferases, Akr1 and Pfa4, in the palmitoylation of the N-terminus of Rho3 and that multiple DHHC family palmitoyltransferases are likely to act on the Rho3 N-terminus.

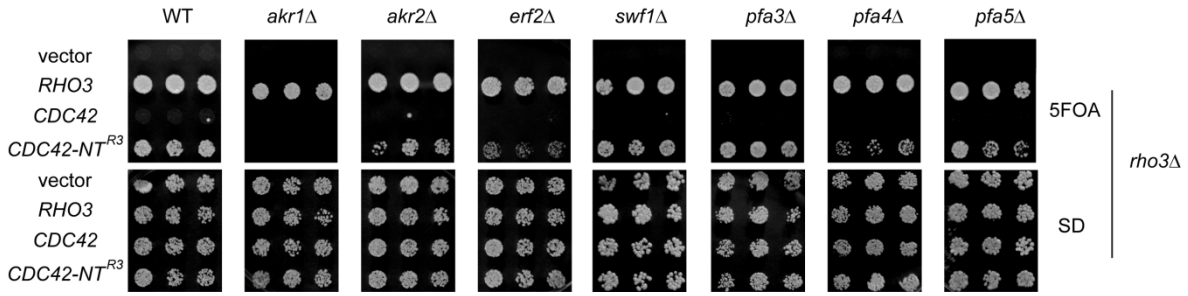


Figure 17: The effect of palmitoytransferase deletion on the ability of Cdc42-NTR3 to function as Rho3

RHO3, CDC42 and *CDC42-NT^{R3}* were constructed on CEN HIS3 plasmids and transformed into *RHO3* plasmid shuffle strains where individual palmitoytransferase was deleted. The ability of *CDC42-NT^{R3}* to function as the only source of Rho3 was analyzed on 5FOA plates where the wild type *RHO3-URA3* plasmid was lost.

The N-terminal region of Rho3 and Cdc42 determines the Strength of Binding to Exo70

A second important element in the Rho3 N-terminus was identified by mutation of a pair of basic residues at positions 17 and 18. When these residues were replaced with alanine in either the context of Rho3 or the Cdc42-NT^{R3} chimera, the ability of either gene to rescue the loss of *rho3Δ* in the cell was completely lost. When we examined the effect of this mutation on the localization of Rho3 or Cdc42-NT^{R3}, we found the mutant proteins localized similar to the un-mutated form of the proteins (Figure 16C, D), although there was a slight

reduction in the amount of Rho3 protein associated with the membrane pellets by fractionation (Figure 16E). The strong loss of function phenotypes associated with mutations at these residues suggested they may play a role in the interaction of Rho3 with an upstream or downstream component of its signaling. Work from our laboratory has demonstrated that Exo70 is a critical downstream effector for both Rho3 and Cdc42 signaling to the exocytic apparatus (Wu et al., 2009). We have shown that prenylated Rho3 and Cdc42 bind to Exo70 in a manner that is both GTP and effector domain dependent. We made use of this binding assay to examine the effects of the A17, 18 mutations on the ability of GTP-locked Rho3 (Rho3-L74) to bind to the effector protein Exo70. The results, shown in Figure 18A, demonstrate that Exo70 binding to GTP-bound Rho3 is almost completely lost in the presence of these mutations. By modeling the structure of Rho3 (based on the existing structure of Cdc42) it is apparent that Rho3 residues 17,18 are likely to be in very close proximity to the effector domain in the active GTP-bound form of the protein. Therefore these residues may represent a binding surface recognized by Exo70 in its discrimination of the GTP-bound forms of Rho3.

The results above suggested that in addition to the function of the N-terminus of Rho3 in determining its unique localization pattern, this region likely has a role in providing elements involved in binding to the downstream effector Exo70. We therefore examined the effect of exchanging the N-termini of Rho3 and Cdc42 on the binding to Exo70. The results, shown in Figure 18B, demonstrate that the Rho3 N-terminus promotes the interaction with Exo70 relative to that of the homologous region of Cdc42. In particular the binding of GTP-locked Rho3-NT^{C42} is significantly reduced relative to that of Rho3, and the binding of GTP-

locked Cdc42 is significantly improved by the presence of the Rho3 N-terminus. To examine the effect of these regions on the relative affinity of each GTPase for Exo70 we performed equilibrium binding assays over a large range of concentrations of recombinant Exo70. The extent of interaction with GTP-locked forms of Rho3, Cdc42 and each of the N-terminal chimeras was monitored by quantitative western blot analysis as shown in Figure 18C. The binding results were used to estimate the EC50—a measure of the apparent affinity observed between each GTPase and Exo70 in this assay. The analysis of EC50 demonstrates that overall Rho3 has a significantly higher affinity for Exo70 than Cdc42 (7μM vs. over 300 μM), and that in both cases the presence of the N-terminus of Rho3 significantly increases the affinity for Exo70. This suggests that stronger binding of the Rho3 GTPase to Exo70, along with the ability to attain the proper pattern of localization on the plasma membrane, are critical determinants in specifying Rho3 function in promoting polarized exocytosis.

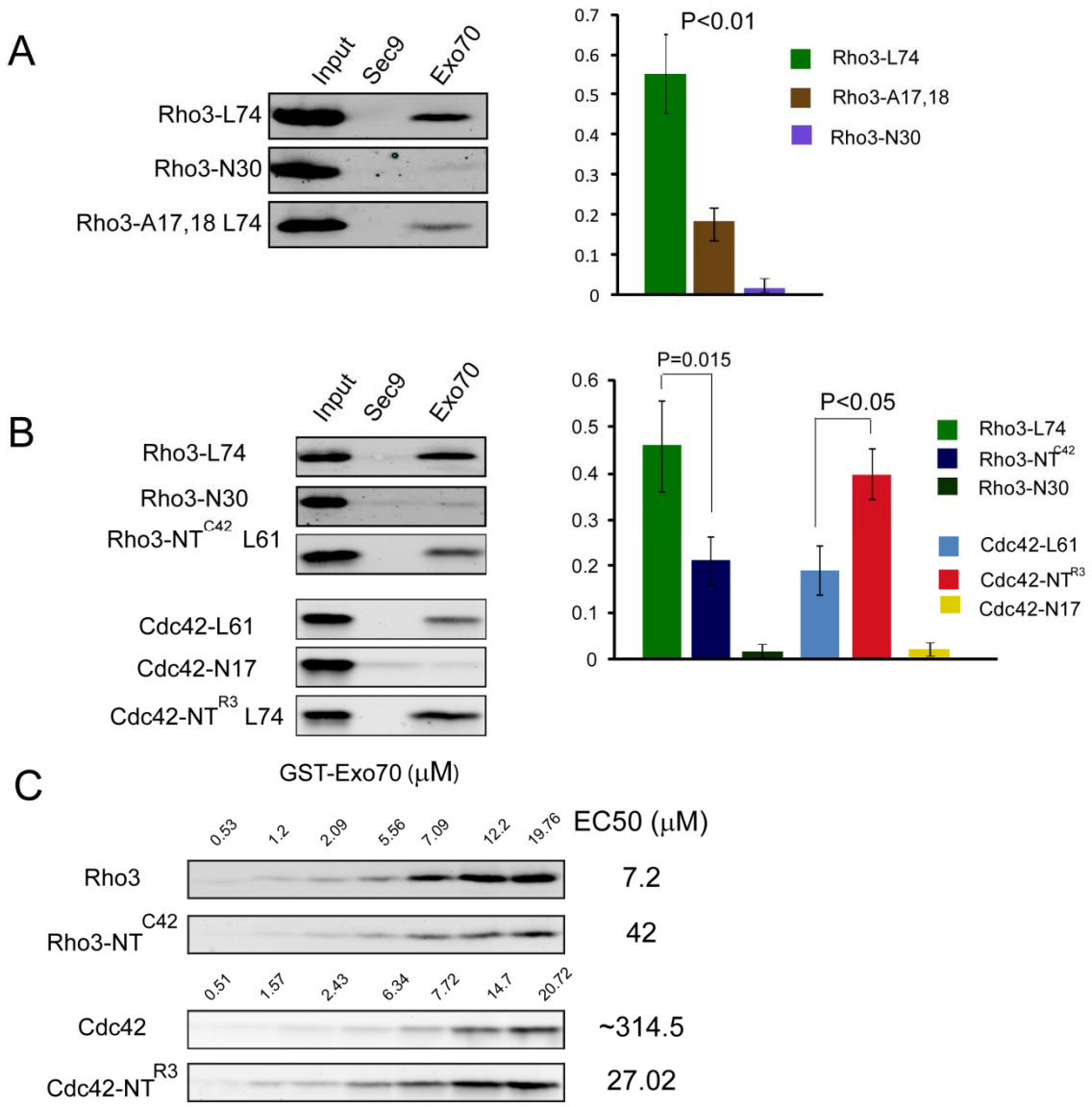


Figure 18: N terminus of Rho3 determines the affinity of the interaction between Rho3 and Exo70.

(**A**) GST-Sec9 and GST-Exo70 were purified from bacteria and immobilized on glutathione sepharose beads. GTP locked forms of Rho3, Cdc42, the gain-of function chimera *Cdc42-NT^{R3}* and the loss of function chimera *rho3-NT^{C42}* were expressed behind a galactose inducible promoter in yeast. Lysates obtained from these strains were used in the binding experiments. The input represents 1% binding. The bar graph on the right panel represents the quantitation of the western blots using the Odyssey Infrared Imaging system. Data were

analyzed by two-tail student's t test with error bars representing standard deviation from five independent experiments. (**B)** Yeast lysates overexpressing GTP bound Rho3 or Rho3-A17, 18 were expressed behind a galactose inducible promoter. The binding experiments were performed as described in above. The bar graph on the right panel represents the quantitation of the western blot. Data were analyzed by two tail student t- test with error bars representing standard deviation from four independent experiments. (**C**) Lysate was incubated with increasing concentration of GST-Exo70 as indicated above the blot. Samples were subjected to western blot analysis using monoclonal antibodies against Rho3 or Cdc42. The predicted EC50 is indicated on the right panel.

DISCUSSION

Localization of members of the Rho GTPase family is thought to play an important role in their cellular functions. Here we demonstrate that the plasma membrane localization pattern of the Rho3 GTPase is distinct from the highly polarized localization pattern observed for Cdc42. Using chimeric forms of Rho3 and Cdc42 we demonstrate that the N-terminal domain of Rho3 plays a critical role in determining this pattern of localization and that palmitoylation at a N-terminal cysteine is required for adopting this pattern. The precise mechanism by which palmitoylation of Rho3 is linked to this pattern of plasma membrane localization is presently unknown. However it is likely a DHHC-family palmitoyltransferases play a role. Previous studies implicated the Erf2 palmitoyltransferase in modification of Rho3 however significant levels of palmitoylation remained in the absence of this enzyme (Roth *et al.*, 2006). We have found that deletion of the gene encoding this enzyme has no effect on the switch-of-function property associated of the Cdc42-NT^{R3} chimera. Therefore other palmitoyltransferases may play a role in determining the dispersed plasma membrane localazation pattern seen for Rho3. Our genetic results suggest that Akr1 enzyme is likely to play an especially important role in the localization of Rho3 as this DHHC family member is essential for ability of the Cdc42-NT^{R3} chimera to function as Rho3.

In contrast to the consistently dispersed pattern of localization found for Rho3, Cdc42 is tightly polarized to bud tips during bud emergence but this polarized staining is lost as the bud enlarges. This pattern of localization appears to require a distinct set of signals from that of Rho3 that are likely to reside in the C-terminal portion of the protein. In addition to C-terminal prenylation (CAAX) signal a stretch of five basic residues, known as a poly basic region is present at the C-terminus of Cdc42. The pattern of localization observed for Cdc42 closely mirrors the pattern observed for many proteins which ride to the plasma membrane along with polarized delivery of post-Golgi secretory vesicles. This list includes Sec4, Sec2, Myo2, and most of the subunits of the exocyst complex. Consistent with this notion Cdc42 has been suggested to itself be associated with post-Golgi secretory vesicles during cell fractionation (Wedlich-Soldner *et al.*, 2003) and its polarized localization is rapidly lost with a block in polarized secretion (Irazoqui *et al.*, 2003) (Zhang *et al.*, 2001) The switch of function chimera, Cdc42-NT^{R3}, appears to have the ability to adopt both Rho3-like and Cdc42-like patterns of localization. This suggests that the pathways that mediate these two localization patterns operate independently of each other. The effects of the palmitoylation site mutation on the localization of Cdc42-NT^{R3} support this view—as we see the loss of Rho3 localization has no effect on the ability of this protein to demonstrate normal Cdc42 localization. Preliminary experiments to examine the effect of secretory blocks on the ability of Rho3 and Cdc42-NT^{R3} to adopt the Rho3-like localization pattern also support this view.

In addition to giving insight into two distinct mechanisms for localization of Rho/Cdc42 GTPases, this work demonstrates the importance of effector binding to the specific function of these two GTPases in the cell. This is particularly evident for Rho3—

which appears to have evolved a relatively high affinity means for interacting with the Exo70 component of the exocyst. Rho3 plays a critical role in secretion in larger budded cells where the exocyst complex is significantly less concentrated than its highly polarized pattern in emerging buds. Therefore the local concentrations in which Rho3 must engage its target are likely to be considerably more dilute at this point in bud growth than during the highly polarized exocytic delivery that occurs during bud emergence. In contrast Cdc42, appears to have evolved a lower affinity means of interacting with Exo70 which might have evolved to match the very high local concentrations of the both Cdc42 and the exocyst during bud emergence. This lower affinity interaction between Cdc42 and Exo70 does not appear to be required, as we find no evidence of polarity or cell shape problems in cells when the higher affinity Cdc42-NT^{R3} construct is present as the sole source of Cdc42 (unpublished observation). For Rho3 however this is not the case, as we find that the Cdc42-NT$^{R3(A17.18)}$ mutant that is able to properly localized as Rho3, but can not function –likely due to a reduced affinity of the GTPase for Exo70. Therefore localization of a functional Rho GTPase, alone, is not sufficient to provide Rho3 function.

Taken together it is clear that the N-terminus of Rho3 plays a very important role in regulating its specific function in the cell. Remarkably it does this by both specifying a dispersed unpolarized pattern of localization on the plasma membrane as well as by modulating the avidity of binding to the downstream effector. Both properties of the N-terminus appear to play a particularly important role in how Rho3 regulates vesicle docking and fusion at specific stages of bud growth. As the bud emerges, a concentrated patch of Cdc42 and Exocyst complex helps to promote a high flux of exocytosis at highly polarized

bud tips. As the bud enlarges, both Cdc42 and exocyst polarization are lost and Rho3 is left as the primary regulator of exocyst-dependent docking and fusion events.

Table 3: Primers used to Construct Chimeric Proteins

Primer	Function	Sequence (5'-3')
C42-BamHI	Cdc42 5' end BamHI site	GCATCAGGATCCTTTTTAAAAAAAGTTGCATTATTTC
C42-SaII	Cdc42 3' end SalI site	GCATCTGTC GACTGGTA G GACTC GC AAATGTC GC
C42-F3	Cdc42/Rho3 junction to amplify the N terminus of Cdc42	GAAAGAAAGATC GTTGTTGTC GGTGATGGTGC
C42-F4	Cdc42/Rho3 junction to amplify the Cdc42 without its N terminus	GTC GC C CAAAATAACACA CTTTAGC GTTTGCATTTTG
R3-BglII	Rho3 5' end BglII site	GACTCCAGATCTGGAGTAAGCGAAACTCAAATTGA
R3-SalI	Rho 3' end SalI site	GCATTCGTCGACGCACATGCTGGAGGGGAAAGAAC
R3-F3	Rho3/Cdc42 junction to amplify the N terminus of Rho3	GCAAACGCTAAAGTGTGTTATTTTGGGCGACGGTGCCT
R3-F4	Rho3/Cdc42 junction to amplify the Rho3 without its N terminus	AACAGCACCATCACCGACAACAACGATCTTTCTTTCGATC

Table 4: Yeast Strains used in Chapter Four

Strain	Genotype	Reference
BY1426	*MATa rho3Δ::LEU2;ura3-52;his3-Δ200;leu2-3,112;+pRS36RHO3*	P.Brennwald Collection
BY1595	*MATa rho3Δ::LEU2; ura3-52;his3-Δ200; leu2-3,112+pRS313 RHO3*	this study
BY1689	*MATa rho3Δ::LEU2; leu2-3,112; ura3-52; his3-Δ200; +pRS313RHO3C5A*	this study
BY1718	*MATa rho3Δ::LEU2; leu2-3,112; ura3-52; his3-Δ200; +pRS313RHO3A3,4*	this study
BY1807	*MATa cdc42Δ::HIS3; ura3-52; leu2-3,112; +pRS316CDC42*	P.Brennwald Collection
BY1846	*MATa cdc42Δ::HIS3;ura3-52; his3-Δ200;leu2-3,112;+pRS315CDC42NT^{R3A3,4}*	this study
BY2232	*MATa cdc42Δ::HIS3;ura3-52;his3-Δ200;leu2-3,112;+pRS315-CDC42-NT^{Rho3A17,18}*	this study
BY2233	*MATa cdc42Δ::HIS3;ura3-52;his3-Δ200;leu2-3,112;+pRS315-CDC42-NT^{Rho3C5A}*	this study
BY2234	*MATa cdc42Δ::HIS3;ura3-52;his3-Δ200;leu2-3,112;+pRS315-CDC42-NT^{Rho3}*	this study
BY2236	*MATa cdc42Δ::HIS3;ura3-52;his3-Δ200;leu2-3,112;+pRS315-CDC42*	this study
BY2486	*MATa rho3Δ::LEU2;ura3-52;his3-Δ200;leu2-3,112;+pRS313 rho3-NT^{C42}+pRS316 CDC42-NT^{R3}*	this study
BY2487	*MATa rho3Δ::LEU2;ura3-52;his3-Δ200;leu2-3,112;+pRS313 rho3-A17,18+pRS316 CDC42-NT^{R3}*	this study

Table 5: Bacterial Strains used in Chapter Four

Strain	Host	Description
BB442	BL21	*pGEX4T1 SEC9 (aa402-651)*
BB1366	DH5α	*pRS313 RHO3*
BB1367	DH5α	*pRS313 CDC42*
BB1368	DH5α	*pRS313 rho3-NT^{C42}*
BB1516	XL1-blue	*pRS313 RHO3-Q2*
BB1590	DH5α	*pRS313 RHO3-A3,4*
BB1515	XL1-blue	*pRS313 RHO3-A5*
BB1521	DH5α	*pRS313 rho3-A7-11*
BB1592	DH5α	*pRS313 RHO3-A12-15*
BB1591	DH5α	*pRS313 rho3-A16-19*
BB1691	DH5α	*pRS313 rho3-A17-18*
BB1369	DH5α	*pRS313 CDC42-NT^{R3}*
BB1483	DH5α	*pRS315 CDC42-NT^{R3}*
BB1522	DH5α	*pRS313 CDC42-NT^{R3}Q2*
BB1523	DH5α	*pRS313 CDC42-NT^{R3}A5*
BB1524	DH5α	*pRS313 CDC42-NT^{R3}A7-11*
BB1532	DH5α	*pRS315 CDC42-NT^{R3}Q2*
BB1533	DH5α	*pRS315 CDC42-NT^{R3}A5*
BB1534	DH5α	*pRS315 CDC42-NT^{R3}A7-11*
BB1587	BL21	*pGEX6P1PH EXO70*
BB1588	DH5α	*pRS313 CDC42-NT^{R3}A16-19*
BB1589	DH5α	*pRS313 CDC42-NT^{R3}A12-15*
BB1593	DH5α	*pRS315 CDC42-NT^{R3}A12-15*
BB1594	DH5α	*pRS313 CDC42-NT^{R3}A3,4*
BB1596	DH5α	*pRS315 CDC42-NT^{R3}A3,4*
BB1597	DH5α	*pRS315 CDC42-NT^{R3}A16-19*
BB1598	DH5α	*pRS313 CDC42-NT^{R3}A17-18*
BB1731	DH5α	*pRS315 CDC42*
BB1857	DH5α	*pRS315 CDC42-NT^{R3}A6*

REFERENCE

Adamo, J.E., Moskow, J.J., Gladfelter, A.S., Viterbo, D., Lew, D.J., and Brennwald, P.J. (2001). Yeast Cdc42 functions at a late step in exocytosis, specifically during polarized growth of the emerging bud. J Cell Biol *155*, 581-592.

Adamo, J.E., Rossi, G., and Brennwald, P. (1999). The Rho GTPase Rho3 has a direct role in exocytosis that is distinct from its role in actin polarity. Mol Biol Cell *10*, 4121-4133.

Harsay, E., and Schekman, R. (2002). A subset of yeast vacuolar protein sorting mutants is blocked in one branch of the exocytic pathway. J Cell Biol *156*, 271-285.

Irazoqui, J.E., Gladfelter, A.S., and Lew, D.J. (2003). Scaffold-mediated symmetry breaking by Cdc42p. Nat Cell Biol *5*, 1062-1070.

Roth, A.F., Wan, J., Bailey, A.O., Sun, B., Kuchar, J.A., Green, W.N., Phinney, B.S., Yates, J.R., 3rd, and Davis, N.G. (2006). Global analysis of protein palmitoylation in yeast. Cell *125*, 1003-1013.

Roumanie, O., Wu, H., Molk, J.N., Rossi, G., Bloom, K., and Brennwald, P. (2005). Rho GTPase regulation of exocytosis in yeast is independent of GTP hydrolysis and polarization of the exocyst complex. J Cell Biol *170*, 583-594.

Schott, D., Ho, J., Pruyne, D., and Bretscher, A. (1999). The COOH-terminal domain of Myo2p, a yeast myosin V, has a direct role in secretory vesicle targeting. J Cell Biol *147*, 791-808.

TerBush, D.R., Maurice, T., Roth, D., and Novick, P. (1996). The Exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. EMBO J *15*, 6483-6494.

TerBush, D.R., and Novick, P. (1995). Sec6, Sec8, and Sec15 are components of a multisubunit complex which localizes to small bud tips in *Saccharomyces cerevisiae*. J Cell Biol *130*, 299-312.

Wennerberg, K., and Der, C.J. (2004). Rho-family GTPases: it's not only Rac and Rho (and I like it). J Cell Sci *117*, 1301-1312.

Wedlich-Soldner, R., Altschuler, S., Wu, L., and Li, R. (2003). Spontaneous cell polarization through actomyosin-based delivery of the Cdc42 GTPase. Science *299*, 1231-1235.

Wu,H. Turner,C. Gardner,J. Temple,B. and Brennwald, P. (2009). The Exo70 subunit of the exocyst is an effector for both Cdc42 and Rho3 function in polarized exocytosis. Mol.Biol.Cell.

Zhang, X., Bi, E., Novick, P., Du, L., Kozminski, K.G., Lipschutz, J.H., and Guo, W. (2001). Cdc42 interacts with the exocyst and regulates polarized secretion. J Biol Chem *276*, 46745-46750.