IDENTIFYING NOVEL REGULATORS OF RHO1 SIGNALING IN *S. CEREVISIAE*

by

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ABSTRACT

Rho GTPases are conserved signaling molecules that regulate a wide range of cellular pathways. Numerous regulators and effectors of Rho contribute to the complexity of Rho signaling in cells. Changes in Rho mediated pathways can often lead to human diseases, including cancer and neurodegenerative disorders. How Rho signaling specificity is regulated is not well understood. Our study uses the model organism *Saccharomyces cerevisiae* to study the regulation of Rho1 signaling. Rho1, the homolog of mammalian RhoA, is a monomeric Rho GTPase that regulates multiple pathways to collectively contribute to cell wall biogenesis. More than fifteen upstream regulators and downstream effectors have been characterized to mediate Rho1 signaling.

A genome wide screen was previously conducted in our lab to identify novel regulators of chitin synthase 3 (Chs3) trafficking by measuring the level of chitin at the cell surface. Rho1 signaling has been implicated in the expression and post translational trafficking of Chs3 via the cell wall integrity (CWI) pathway. Not surprisingly, the top hits from the screen included known regulators of the CWI pathway. The screen also uncovered a new component of the CWI pathway, the putative ORF *ADC2*. Adc2 was physically associated with the RhoGEF Tus1, but not Rom2. It contributed to the localization of Tus1 at the bud neck. Adc2 was also functionally associated with Tus1 in regulating Rho1 signaling. The function of Tus1, but not Rom2, appeared to be dependent on Adc2.

Overall, this study identified Adc2 as a novel regulator of Rho1 signaling. Understanding its specific affinity for Tus1 but not Rom2 may offer insights into the signaling specificity of Rho1. The discovery of Adc2 also raises awareness that additional accessory proteins may be associated with Rho signaling not only in yeast but in humans as well.

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ALS	Amyotrophic lateral sclerosis
ADC2	Arrestin domain containing protein 2
CAR	Cytokinetic actomyosin ring
CDC	Cell division cycle
CHS3	Chitin synthase 3
CNH	Citron homology domain
CR	Congo Red
CW	Calcofluor white
CWI	Cell wall integrity
CWP	Cell wall glycoproteins
DH	Dbl homology
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting required for transport
FH	Formin homology
GAD	Gal4 activating domain
GAP	GTPase activating protein
GBD	Gal4 binding domain
GDI	Guanine dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GlcNAc	β-1, 4-N-acetylglucosamine
GPCR	G Protein-coupled receptor
GS	glucan synthase
GTP	Guanosine triphosphate
HOG	High osmolarity glycerol
HR1	Homology region 1
iMYTH	Integrated-membrane yeast two hybrid
MAPK	Mitogen activated protein kinase
MBS	Myosin-binding subunit
MLC	Myosin light chain
MRX	X-chromosome linked mental retardation
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
ONPG	O-Nitrophenyl-β-galactoside
ORF	Open reading frame
PAK	p21-activated kinases
PBS	Polybasic sequence
PH	Pleckstrin homology domain
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PY motif	Prolin-Tyrosine motif
RBD	Rho binding domain
RHO	Ras homologous
ROK	Rho-associated kinase
TF	transcription factor
TGN	Trans-Golgi network

TOR	Target of rapamycin
TORC2	TOR complex 2
TS	Temperature-sensitive
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family Verprolin-homologous protein
Y2H	Yeast 2 hybrid

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DEDICATION

To my parents. Thank you for your sacrifices so that I can have a better future.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 The Rho GTPase family

1.1.1 Overview of the Rho GTPase family

Rho (Ras homologous) GTPases constitute a distinct family within the Ras superfamily of monomeric GTP binding proteins and are found in all eukaryotic cells (Ridley, 2001). There have been more than 20 mammalian Rho GTPases identified so far, which are categorized into 8 subfamilies based on sequence homology. Rho GTPases are conserved signaling molecules that act as molecular switches to regulate a wide variety of cellular processes, including cytoskeleton reorganization, cell proliferation, transcriptional regulation, vesicle trafficking and more. Due to the importance of Rho, altered Rho signaling can often lead to numerous human diseases, including cancer, neurodegenerative disorders and Down syndrome (Boettner & Van Aelst, 2002). Understanding Rho signaling therefore has tremendous implications for human health.

1.1.2 Rho GTPase regulation

Based on their modes of regulation, all Rho GTPases can be categorized into two groups: classical and atypical. The general regulation of classical Rho GTPases is conserved from humans to yeast (Fig 1.1). Classical GTPases include the majority of Rho GTPases, which cycle between an inactive form and an active form. When bound to GDP, Rho GTPases are localized in the cytosol and remain functionally inactive. When bound to GTP, Rho GTPases are localized at sites of polarized growth and become functionally active. The transition between the two forms is regulated by three groups of proteins: the Rho GDP dissociation inhibitors (GDIs), the guanine nucleotide exchange factors (GEFs) and the GTPase activating proteins (GAPs).

In the inactive state, Rho GTPases are bound to GDIs, a group of negative regulators that maintain and sequester the GDP-bound Rho GTPases in the cytoplasm. The active site of Rho GTPases is masked in this conformation hence rendering them inactive. Rho GTPase activation is regulated by the RhoGEFs through a two-step process that activates and recruits Rho GTPases. RhoGEFs physically recruit the Rho GTPases to sites of polarized growth in cells. Interaction with the RhoGEFs stabilizes the intermediate form of Rho GTPases after GDP dissociation and facilitates the association of GTP with Rho GTPases. The active site of the GTP bound Rho GTPases is unmasked, rendering them active. Active Rho GTPases at sites of polarized growth

can interact with effectors to elicit specific cellular responses via different downstream pathways. The active state of Rho GTPases can be turned off by the GAPs, which negatively regulate Rho signaling by catalyzing the intrinsic ability of Rho GTPases to hydrolyze GTP to GDP. GTP hydrolysis turns off Rho signaling and returns Rho GTPase to an inactive GDP bound state. The GDP-bound Rho GTPase then re-associates with GDI in the cytoplasm.

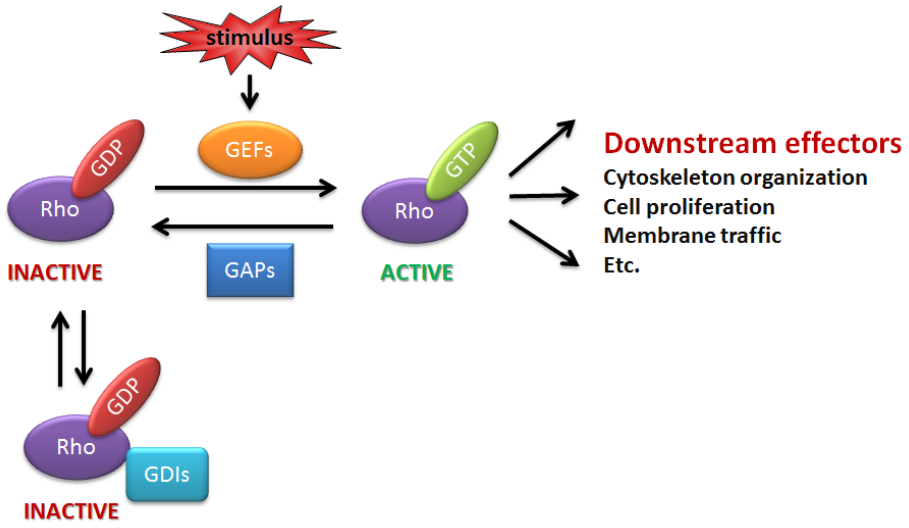

Fig 1.1 The regulation of classical Rho GTPases. Three groups of Rho GTPase regulators have been identified: the GDIs, the GEFs and the GAPs. The GDIs and GAPs are negative regulators that facilitate the formation of inactive GDP bound Rho GTPases. The GEFs are activators that facilitate the signaling of active GTP bound Rho GTPases at sites of polarized growth.

Although most Rho GTPases follow the classical module of regulation, eight members of the family are exceptions and categorized in the atypical group. The regulation of atypical GTPases is independent of GEFs, GAPs and GDIs, because they are not constantly switching between the GDP and GTP bound forms (Heasman & Ridley, 2008). Instead, they display increased affinity for GTP, due to the substitution of residues crucial for GTPase activity. Atypical Rho GTPases are regulated by gene expression, protein turnover and phosphorylation (Chardin, 2006). The regulation and the molecular functions of atypical Rho GTPases are poorly understood.

Most individual Rho GTPases in mammalian cells have multiple GEFs, GAPs and GDIs that share overlapping functions in Rho regulation. This may be a result of evolutionary trait that acts as a failsafe to regulate Rho signaling when some regulators become functionally ineffective. However, Rho regulators are not completely redundant and may play a role in Rho signaling specificity in cells. Each Rho GTPase often regulates multiple pathways by selectively activating its downstream effectors in cells. Evidence suggests that loss of certain Rho regulators can impact specific pathways while other Rho GTPase-regulated pathways are not affected. Studying Rho regulation is an important step towards understanding how a Rho GTPase can selectively activate specific downstream effectors in cells.

1.1.3 Rho signaling in mammalian cells

Initially identified as master regulators of the actin cytoskeleton, additional roles of Rho GTPases have been associated with numerous cellular processes. Rho mediated signaling pathways are generally complex, which is a result of having multiple Rho regulators and effectors. Crystallography studies have shed some light on the interaction between Rho GTPases and their effector proteins. The crystal structures of inactive and active Rho GTPases revealed conformational differences in two surface loops, the switch regions I and II (Ihara et al., 1998). Rho GTPase effectors can discriminate and bind to specific Rho GTPases at these switch regions. However, other sites outside the switch regions have also been shown to mediate effector interactions (Diekmann, Nobes, Burbelo, Abo, & Hall, 1995). Once bound to an active Rho GTPase, the effector is activated and will further propagate Rho signaling in cells. One of the common ways for Rho GTPases to activate effectors is through the disruption of intra-molecular autoinhibition within the effector protein. The binding of Rho GTPases can often lead to the exposure of functional domains of the effector, hence making it active. Individual Rho GTPases normally have multiple effectors in cells. So far, more than thirty effectors for Rho, Rac and Cdc42 have been identified.

Most of our knowledge of the mammalian Rho GTPases has come from studies using dominant negative or constitutively active Rho mutants in other model organisms, such as *Drosophila melanogaster* and *Saccharomyces cerevisiae.* Due to their highly conserved homologs in other organisms, the classical Rho GTPases RhoA Rac1 and Cdc42 are among the best characterized mammalian Rho GTPases (Boureux, Vignal, Faure, & Fort, 2007). The

following sections will review the general function of RhoA, Rac1 and Cdc42 in actin organization in mammalian cells.

RhoA

The Rho subfamily consists of RhoA, RhoB and RhoC, three isoforms with high sequence homology (Heasman & Ridley, 2008). They share overlapping functions in stress-fiber formation, cytokinesis and cell migration in mammalian cells (Pedersen & Brakebusch, 2012; Wheeler & Ridley, 2004). Mice with null mutations in RhoB and RhoC displayed no major developmental defects (Hakem et al., 2005; Liu, Rane, & Liu, 2001). Transgenic mice carrying RhoA- null mutations could not survive during embryogenesis (Pedersen & Brakebusch, 2012). This suggests that RhoA also regulates cellular processes distinct from those regulated by RhoB and RhoC.

Two major effectors of RhoA are ROK and Dia, which are important for RhoA-mediated formation of stress fibres and focal adhesions (Fig 1.2). ROK is a Rho-associated kinase that is activated by RhoA and consequently phosphorylates the myosin light chain (MLC) and the myosin-binding subunit (MBS) (Kawano et al., 1999). The phosphorylation of MLC promotes the assembly of actomyosin filaments. Another target of ROK is the LIM kinase, which negatively regulates cofilin, a family of proteins that dissemble actin filaments in cells. RhoA mediated ROK can stabilize filamentous actin structures through the activation of the LIM kinase (Maekawa, 1999). Dia belongs to the formin-homology (FH) family of proteins and contains two proline-rich motifs in the FH domains . The FH domains of Dia are responsible for binding profilin, a G-actin-binding protein that contributes to actin polymerization and F-actin organization into stress fibres (Wasserman, 1998). Together, the function of RhoA in stress-fibre formation is facilitated by two major Rho effectors ROK and Dia in mammalian cells.

In addition, tissue restricted knockout of RhoA indicated that its function may be cell type specific. RhoA-dependent contractility appears important in epithelial cells. Loss of RhoA resulted in a more open shape of the lens pit due to reduced contraction at the apical side (Plageman et al., 2011). Furthermore, Rock1 and Rock2, effectors of RhoA, are important for eyelid closure, as deletion of these genes caused neonates with omphalocele and open eyes in mouse embryo (Thumkeo, Shimizu, Sakamoto, Yamada, & Narumiya, 2005). Consistent with this observation, keratinocytes derived from these mouse tissues show defective stress fiber

formation, which is important for eyelid closure. RhoA is also implicated in regulating cell junctions in the central nervous system. Deletion of RhoA in the neuroepithelial cells of the spinal cord leads to defect of adherens junctions and apical-basal polarity (Herzog et al., 2011) .

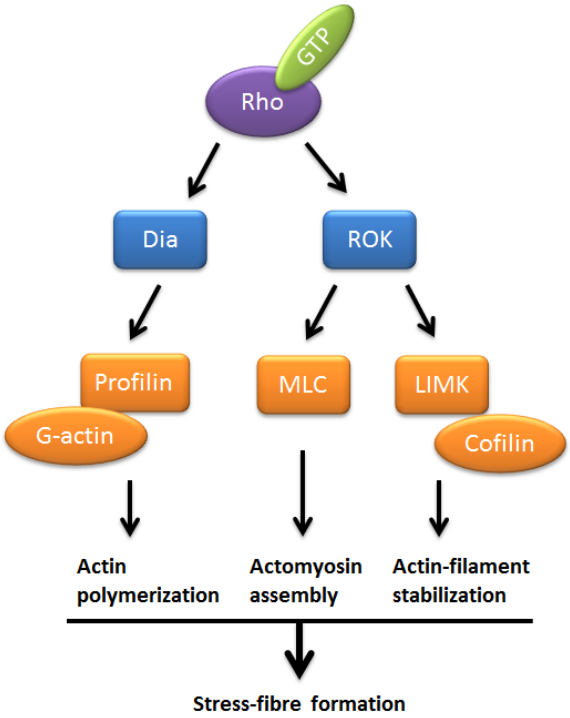

Fig 1.2 RhoA regulation in stress-fibre formation. Two major effectors ROK and Dia are regulated by RhoA to induce stress-fibre formation through different pathways. ROK phosphorylates MLC and LIM kinase to induce actomyosin assembly and actin-filament stabilization, respectively. Dia interacts with profilin to induce actin polymerization. This figure is adapted from the review article by Bishop and Hall, 2000.

Rac1 and Cdc42

Besides RhoA, Rac1 and Cdc42 have been well characterized in mammalian cells. The major functions of Rac1 and Cdc42 are lamellipodia formation, membrane ruffling and filopodia microspike formation (Fig 1.3). One unique effector of Cdc42 is N-WASP, a multi-domain protein capable of binding numerous downstream targets. Once activated by Cdc42, N-WASP acts as a scaffold to recruit profilin and the Arp2/3 complex, which binds to actin monomers and act as a nucleation site for actin polymerization (Miki, Suetsugu, & Takenawa, 1998). One unique effector of Rac1 is PI-4P5K. PI-4P5K mediates the increased level in PIP_2 that leads to actin-filament uncapping in mammalian cells. The process of actin-filament uncapping is

necessary for new actin polymerization. Another Rac1 specific effector is the WAVE protein. Like N-WASP, WAVE also acts as a scaffold to physically interact with profilin and the Arp2/3 complex to contribute to actin reorganization.

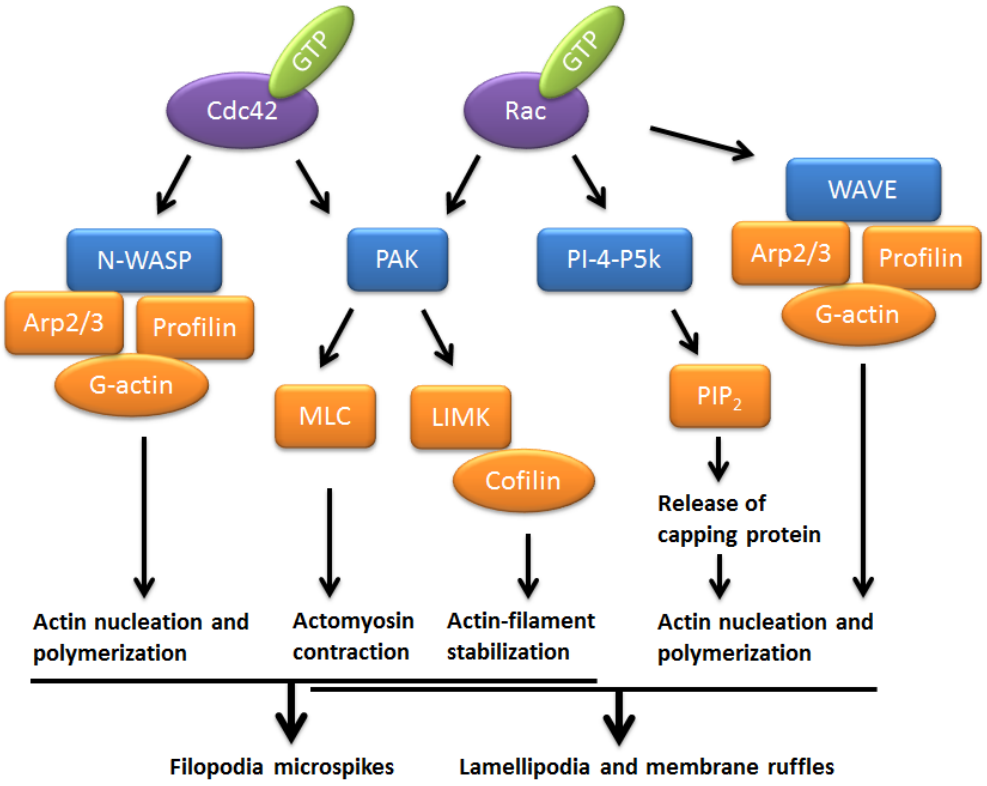

Fig 1.3 The signaling pathways of Rac1 and Cdc42. PAK is a common effector associated with Rac1 and Cdc42 signaling pathways. PAK proteins act through MLC and the LIM kinase to regulate actomyosin contraction and induce actin filament stabilization, respectively. WAVE and PI-4-P5K are unique effectors of Rac1 that together contribute to actin nucleation and polymerization. N-WASP is a unique effector of Cdc42, which also contributes to actin polymerization. This figure is adapted from the review article by Bishop and Hall, 2000.

Besides specific effectors, common targets have also been identified for both Rac1 and Cdc42 in regulating the formation of lamellipodia and filopodia, respectively (Fig 1.3). The PAK cascade consists of three Ser/Thr kinases that regulate a variety of substrates, which collectively contribute to actin reorganization. Two of the PAK substrates are the LIM kinase and MLC, which upon phosphorylation lead to actin filament stabilization and decreased actomyosin contraction, respectively (N. Yang et al., 1998). However, there have been

conflicting reports on the role of PAK in actin reorganization in different cell lines (Lamarche et al., 1996). This suggests that Rac1 and Cdc42 mediated PAK signaling pathways require other tissue specific factors to function properly, hence emphasizing the importance of additional regulatory proteins in Rho GTPase signaling.

Overall, Rho GTPases have a multitude of effectors that can contribute to Rho mediated regulation of actin reorganization and actin independent pathways, including gene transcription, cytokinesis and enzymatic activities. Rho GTPases have been identified as regulators of the JNK and p38MAP kinase pathways (Burbelo, Drechsel, & Hall, 1995; Gallagher, Gutowski, Sternweis, & Cobb, 2004). The activities of numerous enzymes, many associated with lipid metabolism, are affected by Rho GTPases (Hess, Ross, Qiu, Symons, & Exton, 1997; Oude Weernink, Schmidt, & Jakobs, 2004). Thus, the functions of RhoA, Rac1 and Cdc42 cover a vast range of cellular responses that are essential for cell function and survival.

1.1.4 Rho signaling and human diseases

Since Rho GTPases are involved in a wide range of cellular processes, defective Rho signaling can often contribute to human diseases, such as cancer progression, mental disabilities and other diverse and unrelated disorders. Many disease-causing mutations have been identified in genes associated with Rho GTPase signaling, including the GEFs, the GAPs and the effector proteins. However, it is surprising to find that only one gene mutation that directly affects the Rho GTPase has been linked to human disease (RhoH in lymphoma development). Other disease-causing mutations alter the signaling pathways of Rho GTPases in a diverse array of cellular processes.

Cancer progression

The involvement of Rho GTPase signaling in cancer progression is apparent, as various aspects of oncogenesis are dependent on Rho GTPases. Many RhoGEFs that activate Rho signaling pathways have been classified as oncoproteins. Mutations in regulatory regions of the RhoGEF Vav led to rapid phosphorylation, which up-regulated the activity of Vav (López-Lago, Lee, Cruz, Movilla, & Bustelo, 2000). Consequently, Vav mediated Rho GTPase signaling was hyperactive, leading to actin dependent migratory and invasive behaviors in tumor cells. Downstream effectors of Rho GTPases have also been implicated in cancer development. Cyclin

D1, a key regulator of the G1/S transition during the cell cycle, is transcriptionally regulated by the Rac subfamily of Rho GTPases. Higher levels of cyclin D1 can trigger premature progression through the G1-phase of the cell cycle (Sherr & Roberts, 1999). In fact, the level of cyclin D1 protein was elevated in 50% of mammary carcinomas in breast cancer patients (Boettner & Van Aelst, 2002)

Neurodegenerative disorders

Rho GTPases have been linked to diverse aspects of the nervous system, including neuronal migration, axon guidance and dendrite formation (Luo, 2000). One common disorder is the X-chromosome linked form of mental retardation (MRX), which exists in one of every 500 males and represents 25% of all genetic mental retardation disorders. Patients with MRX experience reduced cognitive function. Detailed analysis revealed that MRX patients have thinner dendritic spines and the spine synapses are weakened. Rho GTPases and their effectors IL1RAPL and PAK3 have been found to directly regulate spine synapses and morphology. Reduced Rho signaling in neurons could lead to the development of MRX in patients.

Amyotrophic lateral sclerosis (ALS) is a paralytic disorder that affects motor neurons in patients. Patients with ALS normally die from respiratory failure, because ALS targets motor neurons in the brain stem and spinal cord, which regulates voluntary actions. Alsin, a RhoGEF in motor neurons, was identified as a potential factor in ALS development (Hadano et al., 2001; Y. Yang et al., 2001). Approximately 10% of ALS patients have a mutation in the *ALSIN* locus. Sequence analysis of Alsin revealed the DH/PH Rho regulatory domains, as well as additional domains important for communication between plasma membrane and intracellular ion channels in neurons (Takeshima, Komazaki, Nishi, Iino, & Kangawa, 2000). However, the role of Alsin-activated Rho signaling in motor neurons is elusive. Rho signaling has been implicated in other human disorders including faciogenital dysplasia, and Wiskott-Aldrich syndrome (Derry, Ochs, & Francke, 1994; Nagata et al., 1998).

Drug development that targets Rho signaling has become is promising in treating many Rho related human diseases. However, the inhibition of Rho GTPases by drug treatment can often lead to cell death and is not a viable option for therapeutic purposes. The ability to selectively down-regulate Rho mediated pathways is a more attractive option. Y-37632, a tumor suppressor drug, is an inhibitor of the Rho GTPase effector ROCK, which specifically regulates

Rho-ROCK mediated pathways. Drug development that specifically targets certain Rho effectors while maintaining Rho signaling in other pathways will be a crucial step in treating Rho related diseases.

Selective activation of different Rho mediated pathways is the hallmark of Rho signaling in all eukaryotes. The complex network of Rho signaling in mammalian cells indicates that Rho GTPases must be tightly and selectively regulated by additional factors. To date, more than forty different regulators and effectors have been identified for RhoA, Rac1and Cdc42 alone (Bishop & Hall, 2000). However, a general paradigm describing how individual Rho GTPases can elicit specific signaling through selective factors is lacking. Identifying novel regulators, as well as examining the links between Rho GTPases and their existing interaction partners, will be crucial in understanding how Rho-GTPase signaling specificity is achieved.

1.2 Rho1 signaling in *Saccharomyces cerevisiae*

1.2.1 The Rho1 GTPase

The signaling of ubiquitously expressed RhoA, Rac1 and Cdc42 GTPases is highly conserved from humans to yeast. In this thesis, I describe my studies of the regulation of Rho signaling in the model organism *Saccharomyces cerevisiae* using genetic and biochemical analyses. Rho1, the *Saccharomyces cerevisiae* homolog of mammalian RhoA, is a monomeric Rho GTPase that regulates a wide range of cellular processes including actin organization, cytokinesis, gene transcription, and protein trafficking. Cells with a Rho1 null mutation are not viable.

1.2.2 The Rho1 GEFs

Rho1 signaling is carried out by numerous regulators and effectors in yeast. Like RhoA, Rho1 acts as a molecular switch that cycles between the GDP bound inactive form and the GTP bound active form. Three RhoGEFs, Rom1, Rom2 and Tus1, physically bind to and activate Rho1 in yeast. All three contain a DH domain, a PH domain and a citron homology (CNH) domain. The DH domains of Rom1/2 and Tus1 have been found to interact with GDP-Rho1 and facilitate the transition to GTP-Rho1(Ozaki et al., 1996a; Schmelzle, Helliwell, & Hall, 2002). The PH domains of Rom1/2 bind to phosphatidylinositol-4, 5-biphosphate (PIP_2), which facilitates the membrane localization of Rom1/2 (Audhya & Emr, 2002). The PH domain of

Tus1 does not bind to phosphoinositides (Yu et al., 2004a). However, Tus1 dependent Rho1 localization at the plasma membrane indicates that the PH domain of Tus1 may bind to other membrane components to ensure proper localization. Membrane bound RhoGEFs recruit Rho1 to sites of polarized growth at the plasma membrane. The membrane association of Rho1 is further stabilized by post-translational prenylation and the presence of a poly basic sequence (PBS) in the C terminus sequence of Rho1 (Yoshida, Bartolini, & Pellman, 2009). Active Rho1 at sites of polarized growth interacts with its effectors to activate downstream pathways. The CNH domain of the RhoGEFs may serve as sites of interaction with other regulatory molecules. However, the role of the CNH domain in Rho1 signaling is not clear.

While Rom1 has been partially implicated in Rho1 and Rho2 functions, Rom2 and Tus1 are identified as the major regulators of Rho1 signaling in yeast. Rho1 signaling is involved in cytokinetic actomyosin ring (CAR) assembly at the bud neck during cytokinesis. Rom2 and Tus1 are regulated by polo-like Cdc5 kinase phosphorylation to ensure proper Rho1 signaling during CAR assembly (Yoshida et al., 2006). Tus1, and likely Rom2, contain Polo kinase binding motifs in their N terminus sequence, which Cdc5 binds to phosphorylate the GEFs. Non-phosphorylated Tus1and Rom2 are not localized at the bud neck in the *cdc5-1* temperature sensitive strain. Consequently Rho1 is no longer recruited by the GEFs and its bud neck localization is abolished as well (Yoshida et al., 2006). The overlapping function of the GEFs likely ensures the proper signaling of Rho1 even when one of the GEFs is functionally inactive. However, it is becoming increasingly clear that Rom2 and Tus1 also possess distinct roles in Rho1 signaling. Recent evidence identified Tus1 specific Rho1 regulation that is independent of Rom2, which suggested the importance of signal differentiation based on individual RhoGEFs. Tus1-mediated Rho1 signaling was found to be associated with the activity of Ycf1, a vacuole transporter responsible for heavy metal detoxification (Paumi et al., 2007). Furthermore, TORC2 signaling was found to be linked to Rho1 activation specifically through Tus1, but not Rom2 (Ho, Lee, Liao, & Chen, 2008a). Cell wall sensors Wsc1 and Mid2, under extra cellular stimuli, can mediate Rho1 signaling via the binding of Rom2, but not Tus1 (Philip & Levin, 2001a). While Tus1 and Rom2 share overlapping functions, they may regulate Rho1 signaling through distinct pathways in yeast.

1.2.3 The Rho1 GAPs

The GAPs are negative regulators that increase the intrinsic rate of GTP hydrolysis of Rho GTPases. Four Rho1 GAPs have been identified in yeast: Bem2, Sac7, Bag7 and Lrg1 (Peterson et al., 1994; Roumanie, Weinachter, Larrieu, Crouzet, & Doignon, 2001; Schmidt, Bickle, Beck, & Hall, 1997). These GAPs facilitate the switch of Rho1 from the active GTP bound form to the inactive GDP bound form. The detailed molecular mechanism of GAP-Rho1 regulation is not well understood. Like the GEFs, the GAPs appear to be important in Rho1 signaling specificity. Lrg7 was found to down-regulate Rho1 signaling in the activation of β-1, 3-glucan synthase, which synthesizes glucan for the cell wall (Watanabe, Abe, & Ohya, 2001). Bem2 and Sac7 were found to down-regulate the Rho1 mediated MAPK pathway, which is associated with the transcription of cell wall biogenesis genes (Schmidt & Hall, 2002).

1.2.4 The Rho1 effectors in yeast

Six direct effectors for Rho1 have so far been identified: Pkc1 protein kinase, β-1, 3-glucan synthase, Bni1 and Bnr1 formin proteins, Sec3 exocyst protein and Skn7 transcription factor. Each effector is activated by Rho1 to further propagate Rho1 signaling in different pathways.

Pkc1

Pkc1 is the yeast homolog of mammalian protein kinase C (D E Levin, Fields, Kunisawa, Bishop, & Thorner, 1990). Pkc1 physically interacts with and is activated by GTP–bound Rho1 (Kamada et al., 1996). Association with Rho1 is facilitated by the presence of two motifs in the Pkc1 N-terminal domain: a cys-rich C1 domain and a homology region 1 (HR1) domain (Schmitz, Lorberg, & Heinisch, 2002). Although Pkc1 is implicated in different pathways, the role of Pkc1 in regulating cell wall biogenesis is best studied. Cells with a deletion of *PKC1* undergo cell lysis, which can be suppressed by osmotic support to maintain cell wall integrity (D E Levin & Bartlett-Heubusch, 1992). Both the inner and outer layers of the cell wall in *pkc1*Δ cells appeared thinner in electron micrographic images under osmotic support (Terry, 1994). Fluorescence microscopy revealed that Pkc1 is co-localized with Rho1 at sites of polarized growth (Andrews & Stark, 2000). Truncation studies further revealed that each regulatory

domain of Pkc1is important for localization to specific subcellular compartments. This suggests that the function of Pkc1 associated with different pathways may be spatially regulated.

β-1, 3-glucan synthase

β-1, 3-glucan synthase (GS) is responsible for synthesizing glucan, an important component of the cell wall in yeast. *FKS1* and *FKS2* encode two catalytic subunits of the GS complex, which share redundant functions in GS activity (Douglas et al., 2000; Mazur & Baginsky, 1996). Cells with deletion of either FKS gene are viable, while the double deletion strain *fks1Δ fks2Δ* undergoes cell lysis. Fks1/2 subunits are transmembrane proteins with a cytosolic central domain. GTP-bound Rho1 was identified as an essential regulatory subunit of the GS complex that physically interacts with Fks1 and Fks2 subunits. Fks1, and perhaps Fks2, is co-localized with Rho1 at sites of cell wall remodeling (Yamochi, 1994). However, the sites of Rho1 interaction with Fks1/2 have not been determined. Interestingly, a recent study of temperature sensitive *rho1* alleles revealed mutant *rho1* alleles defective in GS activity could still regulate Pkc1 activity (Saka et al., 2001). This suggested that multiple modes of Rho1 regulation exist to specifically activate downstream effectors.

Bni1 and Bnr1 formin proteins

Bni1 and Bnr1 are formin proteins that share functions in actin assembly in yeast. Both proteins can interact with profilin, an actin-binding protein that can induce filament formation at the plasma membrane (Sagot, Rodal, Moseley, Goode, & Pellman, 2002). Furthermore, Bni1 also plays a role in the formation of the cytokinetic actin ring (CAR) (Tolliday, VerPlank, & Li, 2002). The activities of Bni1 and Bnr1 are regulated by GTP-bound Rho1. The Rho-binding domain (RBD) in the N-terminal domain of Bni1 and Bnr1 is necessary for Rho1 regulation (Evangelista et al., 2003). In the absence of Rho1 binding, the RBD is involved in an autoinhibitory interaction that down-regulates the activity of Bni1 and Bnr1 (Alberts, 2001).

Sec3

Sec3 is an exocyst protein that is part of a multi protein complex that regulates vesicle transport from the Golgi to the cell surface during polarized exocytosis. The process of exocytosis is important for polarized cell-surface expansion in yeast. Sec3 and other exocyst

components assemble at the site of exocytosis, and facilitate the binding and fusion of the secretory vesicles with the plasma membrane. While the formation of the exocyst complex is induced by vesicle arrival, Sec3 localization at the site of exocytosis is independent of secretory vesicles, the actin cytoskeleton or other exocyst components. Sec3 binds directly to phosphatidylinositol 4, 5-bisphosphate (PIP_2), which is a phospholipid component of the cell membrane. The N-terminal domain of Sec3 is found *in vitro* to directly bind to Rho1 and Cdc42, another essential Rho GTPase in yeast (Guo, Tamanoi, & Novick, 2001). A Sec3 mutant missing the N-terminal domain is no longer localized in a polarized manner. Both interactions with Rho1 and PIP_2 are necessary for Sec3 localization at polarized sites of exocytosis

Skn7

Skn7 is a transcription factor that is regulated by the high osmolarity glycerol (HOG) signaling pathway in yeast (Levin 2011). Skn7 regulates the expression of HOG-responsive genes when cells detect hypo-osmotic or hyper-osmotic conditions via the cell wall surface sensor Sln1. Skn7 is also a potential target of Rho1, which physically binds to an HR1 domain of Skn7 (Alberts, 2001; Ketela, Green, & Bussey, 1999). Several observations further indicate that Skn7 is functionally associated with Rho1 in yeast. The overexpression of Mid2, a known Rho1 activating cell wall receptor, induces a Skn7-LexA-dependent transcriptional reporter (Ketela et al., 1999). *SKN7* was identified as a dosage suppressor of a mutant defective in β-1,6-glucan synthesis, a process closely regulated by Rho1 for cell wall biogenesis (Brown, North, & Bussey, 1993). Overexpression of *SKN7* can also suppress the growth defect caused by loss of *PKC1*, which is a direct downstream target of Rho1 signaling in maintaining cell wall integrity Despite numerous phenotypic observations, the molecular link between Rho1 and Skn7 is still poorly characterized.

1.3 Rho1 and the cell wall integrity (CWI) signaling pathway

1.3.1 Overview of the CWI pathway

Through its numerous regulators and effectors, Rho1 regulates a wide range of cellular responses in yeast. The complex network of Rho1 signaling presents challenges in studying the specific role of Rho1 in an isolated pathway. The function of Rho1 is perhaps best studied in the cell wall integrity (CWI) pathway. The CWI is a major signaling pathway that detects and

responds to cell wall stress in yeast. Although CWI has been implicated in dealing with oxidative stress, pH stress and DNA damage, its major role is to regulate and maintain the integrity of the cell wall (David E Levin, 2011).

Rho1 signaling in the CWI pathway is mediated and amplified by different upstream and downstream components (Fig 1.4). Initial stress is first detected by the cell wall sensors, which mediate Rho1 activation through the RhoGEFs at sites of polarized growth. Next, Rho1 interacts with Pkc1, which activates the MAPK cascade, a group of kinases that are designed to amplify Rho1 signaling. Rlm1 up-regulates the expression of genes associated with cell wall biogenesis. Overall, Rho1 signaling in the CWI pathway allows cells to respond to stress through gene expression. Rho1 signaling has also been shown to act post-transcriptionally to regulate the intracellular trafficking of the membrane protein Chs3 under extra cellular stress (Valdivia & Schekman, 2003). Recent evidence has also implicated Rho1 mediated CWI signaling in cell cycle regulation (Kono et al., 2008; Yoshida et al., 2006). The molecular mechanisms of Rho1 mediated CWI signaling in these pathways are not well understood.

1.3.2 The CWI cell wall sensors

The ability of the CWI pathway to detect extra cellular stimuli is dependent on five cell wall sensors: Mid2, Mtl1 and Wsc1-3 (Ketela et al., 1999; Rajavel, Philip, Buehrer, Errede, & Levin, 1999; Verna, Lodder, Lee, Vagts, & Ballester, 1997). All five are transmembrane proteins that are structurally similar in that they contain a cytoplasmic domain, a single transmembrane domain and a periplasmic ectodomain (Ketela et al 1999; Lodder et al., 1999; Philip and Levin 2001). However, their sequences are not conserved. The cell wall sensors are believed to be capable of detecting mechanical stress, due to the high level of O-mannosylation of the periplasmic ectodomain, which causes stiffening of the proteins (Rajavel et al., 1999, Philip and Levin 2001). Among the five cell wall sensors, Mid2 and Wsc1 appear to be most important in responding to cell wall stress. Deletion of *WSC1* induces cell lysis at elevated temperature, while deletion of *MID2* causes cell death in pheromone-induced morphogenesis (Gray et al., 1997; Rajavel et al., 1999). The *wsc1*Δ *mid2*Δ double deletion strain requires osmotic support to

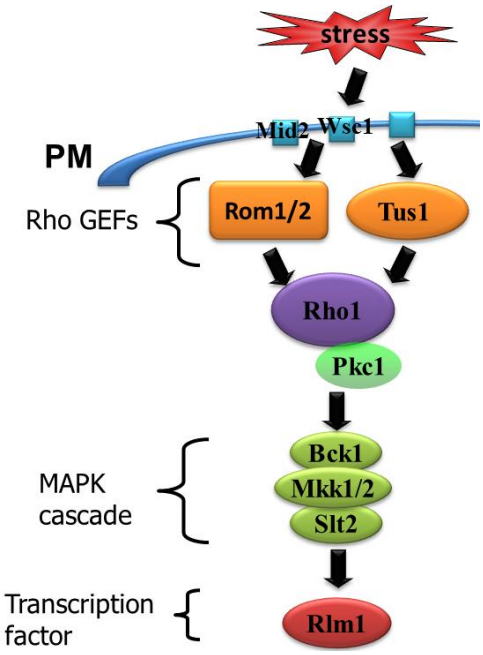

Fig 1.4 Schematic diagram of the CWI pathway. The pathway is mediated by Rho1 and consists of cell wall receptors, the Rho GEFs, Pkc1, the MAPK cascade and the transcription factor Rlm1. The signaling pathway is important for the cell wall biogenesis under extracellular stress.

survive in normal conditions, indicating their complementary roles in regulating cell wall biogenesis (Ketela et al., 1999). In the presence of extracellular stress, cell wall sensors are believed to mediate Rho1 signaling through the RhoGEFs Rom2 and Tus1.

1.3.3 The RhoGEFs Rom2 and Tus1

Rom2 and Tus1 are the activators of Rho1in the CWI pathway. They share complementary functions in Rho1 regulation. The N-terminal domain of Rom2, but not Tus1, can physically bind to the cytoplasmic domains of Wsc1 and Mid2. As a result of this interaction, Wsc1 and Mid2 recruit Rom2 to the plasma membrane at sites of polarized growth. It is unclear if Tus1 is also localized through the cell wall sensors. Wsc1 and Mid2 may also activate the RhoGEFs, as extracts from the *wsc1*Δ and *mid2*Δ cells are deficient in catalyzing GTP-Rho1 formation. Membrane bound Rom1/2 and Tus1 physically recruit and catalyze active GTP-Rho1 formation at sites of polarized growth. Rho1 does not appear to directly interact with the cell wall sensors. Rho1 is switched on by the GEFs and in turn interacts with Pkc1 to

activate the entire CWI pathway. Extracellular stress is detected and integrated by yeast into intracellular Rho1 signaling.

1.3.4 Rho1 and Pkc1 in the CWI pathway

Rho1 is the molecular switch of the CWI pathway. The pathway becomes active when Rho1 is activated by the RhoGEFs Rom2 and Tus1 in cells under extracellular stress. Pkc1, one of the six Rho1 effectors, is recruited and activated by the GTP bound Rho1. The RBD domain of Pkc1 interacts with the active GTP-bound Rho1 at sites of polarized growth. Like *RHO1, PKC1* is an essential gene, which regulates cell wall biogenesis via the CWI pathway. Localization studies have found that they are all localized at the same sites of polarized growth (Andrews and Stark, 2000; Yoshida et al., 2006). It is suggested that Pkc1, Rho1 and the GEFs form a signaling complex at the plasma membrane. The localization of Pkc1 may be solely dependent on Rho1, as direct interactions between Pkc1 and the RhoGEFs have not been identified to date.

1.3.5 The CWI MAPK cascade and Rlm1

The CWI MAPK cascade propagates Rho1 signaling in the CWI pathway. It consists of Bck1, Mkk1/2 and Slt2. Bck1, a functional ortholog of human Erk5, is the direct downstream target of Pkc1 (Truman et al., 2006). The catalytic domain and regulatory domain of Bck1 is phosphorylated by Pkc1 *in vitro* (Levin et al., 1994). Bck1 becomes active and phosphorylates Mkk1 and -2 (Kamada, Jung, Piotrowski, & Levin, 1995). Mkk1/2 then dual-phosphorylates the T-X-Y motif present in the activation loop of Slt2. The linear activation of kinases is believed to amplify Rho1 signaling through Pkc1 in cells. Loss of any MAP kinase does not cause cell death, but results in growth sensitivity to elevated temperature, mating pheromone and the cell wall stress agents calcofluor white (CW) and congo red (CR) (Reinoso-martín, Schüller, Kuchler, Schu, & Schuetzer-muehlbauer, 2003). Cells with *PKC1* deletion are not viable, while cells defective in MARK signaling display temperature-sensitive growth. This suggests that the MAPK cascade must not be the only downstream target of Pkc1.

While Bck1 and Mkk1/2 are cytoplasmic proteins, they have been detected at sites of polarized growth (Van Drogen & Peter, 2002). However, it is unclear whether Bck1 is activated by Pkc1 at the plasma membrane or in the cytoplasm. Slt2 mostly resides in the nucleus, but

under cell wall stress is rapidly localized to the cytoplasm, where it is presumably phosphorylated by Mkk1/2 (Kamada et al., 1995). Slt2 is responsible for the activation of Rlm1, a transcription factor responsible for the expression of many genes associated with cell wall biogenesis.

Rlm1 resides in the nucleus, where Slt2 activates it by phosphorylating the residues in the transcriptional activation domain (Jung, Sobering, Romeo, & Levin, 2002a). Rlm1 contains a MADS DNA-binding domain in its N-terminal sequence. Phosphorylated Rlm1 becomes active and up-regulates gene expression in yeast. A genome wide study of gene expression in response to constitutively active MAPK identified more than 25 genes under the regulation of Rlm1 (Jung and Levin 1999). Most of these genes encode cell wall proteins and are associated with cell wall biogenesis, including *CHS3. CHS3* encodes chitin synthase 3, which synthesizes chitin, a structural component of the cell wall.

1.4 The yeast cell wall and chitin synthase 3

1.4.1 Functions of the yeast cell wall

The cell wall has three major functions in yeast. First, it acts as a protective barrier that prevents cell lysis under environmental stress. The environment is constantly changing for yeast cells growing in the wild. A rainfall can induce a sudden osmotic shock, leading to great influx of water into the cell, which could cause cells to burst (Hohmann, 2002; Smits, Kapteyn, van den Ende, & Klis, 1999). The yeast cell wall establishes a balance by counteracting water intake with turgor pressure against the plasma membrane. The elastic structure of the cell wall is also effective against other types of extracellular stress, including compression and mechanical forces. Second, the cell wall is necessary to establish and maintain cell shape (Klis, Boorsma, & De Groot, 2006). During bud formation, the cell wall must be remodeled to accommodate polarized growth in an actin cytoskeleton dependent manner. Digestive enzymes loosen the cell wall to allow bud emergence and polarized growth, which is tightly regulated to avoid cell lysis. Third, the cell wall can act as a scaffold for many proteins, which have important functions in mating, cell-cell contact, biofilm formation and invasive growth (Cappellaro et al., 1994; Douglas et al., 2007; Reynolds and Fink 2001).

1.4.2 The yeast cell wall structure

The cell wall is the largest organelle in the cell, comprising approximately 10-25% of the cell mass depending on growth conditions (Smits et al., 1999; Aguilar-Uscanga and Francois 2003). The cell wall can be divided into an outer layer and an inner layer. The outer layer is mostly composed of cell wall glycoproteins (CWPs), which function to protect the inner layer from wall-degrading enzymes (Klis et al., 2006). CWPs include glycosylphosphatidylinositol (GPI) proteins and Pir proteins, which interact with components of the inner layer to maintain cell wall stability. The inner layer is mostly composed of β-1, 3-glucan (80-90%) and β-1, 6-glucan (8-18%). Chitin makes up the smallest fraction 1-2% of the cell wall, but is essential for the structure of the cell wall. Together, glucan and chitin contribute to the mechanical strength and elasticity of the cell wall (Smits et al., 1999). Glucan and chitin are synthesized by glucan synthase and chitin synthase respectively, both of which are regulated by Rho1 signaling in yeast. Rho1 interacts with and directly activates Fks1/2, the subunits of glucan synthase. Rho1 signaling regulates the expression of *CHS3* through the CWI pathway, and also the intracellular trafficking of Chs3 through an unknown mechanism(Valdivia & Schekman, 2003).

1.4.3 Chitin and *CHS* family genes

Chitin is a small but essential component of the cell wall. It is a polymer of β-1, 4-N-acetylglucosamine (GlcNAc) that provides elasticity to the inner layer of the cell wall. Chitin makes up 2% of the cell wall mass in vegetative growth, but can increase to as much as 20% under cell wall stress (García-Rodriguez et al., 2000; Valdivieso, Ferrario, Vai, Duran, & Popolo, 2000). Chitin is implicated in septum formation, cell conjugation and spore cell-wall synthesis in yeast.

The chitin synthases that contribute to chitin synthesis have been identified: *CHS1*, *CHS2*, and *CHS3*. While Chs2 level is dependent on the cell cycle, Chs1 and Chs3 are stably expressed. Chs2 is associated with the formation of the primary septum disk. Chs3 is associated with chitin ring formation at early budding. Chs3 is responsible for the majority of chitin synthesis in yeast (Roncero, Valdivieso, Ribas, & Duran, 1988). The expression of *CHS3* is regulated by Rlm1, the transcription factor mediated by Rho1 signaling in the CWI pathway. However the up-regulation of *CHS3* expression does not completely account for the increase of

cell surface Chs3 under cell wall stress conditions. It is suggested that another pathway contributes to the high level of Chs3.

1.4.4 Chs3 trafficking and Rho1 signaling

Under normal growth conditions, most Chs3 is retained in an intracellular pool and is localized to the endosomes and trans-Golgi network. In the presence of stress, a transient redistribution of Chs3 from this intracellular pool to the cell surface occurs to reinforce the cell wall integrity, through higher levels of chitin synthesis. The translocation of Chs3 is a major contributor to increased chitin level in the cell wall under extracellular stress. The stress induced Chs3 trafficking is dependent on Rho1 and Pkc1, but not on Rlm1 (Valdivia & Schekman 2003). This suggests that there are other Rho1-Pkc1 dependent regulators. However, this Rho1 mediated Chs3 trafficking process is not well understood.

1.5 Research question and hypothesis

1.5.1 Understanding Rho1 signaling specificity

Rho GTPase signaling is associated with many cellular pathways. The wide range of roles for Rho begs the question of how signaling specificity is regulated in cells. Since the activation of Rho by the GEFs is conserved from human to yeast, it is not clear how cells can up-regulate specific Rho pathways, while keeping other pathways unaffected. Often the number of Rho mediated pathways is much greater than the number of GEFs present for Rho activation. This suggests that additional regulators may work collaboratively with the GEFs to regulate Rho signaling. To better understand signaling specificity, we decided to study Rho1 signaling in the model organism *Saccharomyces cerevisiae.*

The CWI pathway is perhaps the best studied pathway associated with Rho1 signaling. Yet, there are still many questions unanswered about the CWI pathway. How do the RhoGEFs Rom1/2 and Tus1 activate Rho1 signaling differently under different cell wall stresses? Are there additional accessory proteins that contribute to GEF activation of Rho1? Although some cell wall sensors appear to be specific for different types of cell wall stress, how do they selectively mediate Rho1 signaling through the GEFs?

Our project attempts to answer some of these questions by investigating Rho1 signaling in the model organism *Saccharomyces cerevisiae.* We believe that additional Rho1 regulators

must be present to facilitate the selective activation of Rho1 signaling by Rom2 and Tus1 in yeast. A genome-wide screen was previously conducted in our lab to measure surface chitin level in non-essential knockout collections. We examined the unpublished data from this screen to look for novel regulators of Rho1 that contribute to the cell wall biogenesis through the CWI pathway. Chapter 2 describes the characteristics of an unknown ORF *YPL066W*, including its physical interaction and co-localization with the RhoGEF Tus1. Chapter 3 establishes that *YPL066W* is important for Tus1 localization and functions with Tus1 to regulate Rho1 signaling in parallel with Rom2.

CHAPTER 2: *YPL066W* is part of the Tus1 signaling complex

2.1 Introduction

All cells can respond to environment changes through signaling transduction pathways. In the yeast *Saccharomyces cerevisiae*, a range of extracellular stimuli can be perceived as cell wall stress including heat stress, osmotic shock and physical damage. Rho1, an essential monomeric GTPase, is responsible for maintaining cell wall integrity in response to extracellular stimuli in yeast. Rho1 is the yeast homolog of RhoA, a conserved signaling molecule belonging to the Rho family of GTPases. It is a molecular switch that exists in a GDP bound inactive form and a GTP bound active form. It is recruited and activated at sites of polarized growth by a group of regulators known as the RhoGEFs (David E Levin, 2011).

There are three RhoGEFs that regulate Rho1 activity in *S. cerevisiae:* Tus1 and Rom1/2. All three contain a conserved catalytic DH domain, a lipid binding PH domain and a C-terminal CNH domain. Under cell stress, Rom1/2 interact with cell wall receptors and membrane lipids through the N-terminus and the PH domain, respectively (Philip & Levin, 2001b). The mechanism by which Tus1 is localized at sites of polarized growth is unclear at this time. The GEFs physically recruit Rho1 through the DH domain, which binds to the guanine nucleotide-free form of Rho1 (Rossman, Der, & Sondek, 2005). The binding stabilizes the complex and facilitates the association of GTP, which renders Rho1 active. The function of the CNH domain is not understood.

One of the functions of Rho1 is to regulate the surface level of chitin synthase 3 (Chs3) under extra-cellular stress. Chs3 is a polytopic membrane protein in yeast that synthesizes chitin, which provides integrity and structure for the cell wall. Chs3 is intracellularly stored in the trans-Golgi network and endosomes, but is rapidly transported to the cell surface upon induction. Extra-cellular stress is initially detected by the major cell stress sensors Wsc1 and Mid2, which recruit Rom1/2 and potentially Tus1 to the cell membrane, where Rho1 is recruited and activated (Philip & Levin, 2001b). Active Rho1 can up-regulate Pkc1 activity, which phosphorylates Bck1 of the MAP kinase cascade. Bck1 subsequently phosphorylates Mkk1/2, which in turn phosphorylates Slt2, the final kinase of the MAPK cascade. Phosphorylated Slt2 activates a transcription factor Rlm1 that regulates the expression of numerous cell wall biogenesis genes, including *CHS3*. This stress induced Rho1 signaling pathway is commonly known as the cell

wall integrity (CWI) pathway. The CWI pathway can also regulate the rapid translocation of Chs3 trafficking to the cell surface independent of a change in *CHS3* expression (Valdivia & Schekman, 2003). However, the molecular mechanism is not clear.

Although it is unclear why Rho1 has three RhoGEFs for activation, recent evidence suggested that Rom1/2 and Tus1 are important for regulating specific Rho1 responses under different stimuli (Ho et al., 2008a; Paumi et al., 2007). While Rom1 and Rom2 share overlapping functions, Rom2 is predominantly involved in Rho1 signaling under extracellular stress. Tus1 is recently linked to Rho1 signaling during cell cycle progression (Kono et al., 2008). In this study, we further revealed differentiation in Tus1 and Rom1/2 functions by identifying a putative ORF *ADC2* that is associated specifically with Tus1, but not Rom2. *ADC2* was first identified from a genome wide screen that looked for changes in surface chitin level, as a result of changes in Rho1 signaling. We identified a physical interaction between Tus1 and Adc2, and showed the CNH domain of Tus1 is necessary and sufficient for Adc2 binding. Tus1 and Adc2 co-localize at the bud neck in a cell cycle-dependent manner. In addition, Tus1 is necessary for Adc2 localization and directly recruits Adc2 to the bud neck. Our findings suggest that additional accessory proteins may contribute to the specificity of Rho1 signaling mediated by Tus1 and Rom1/2.

2.3 Results

2.3.1 Validation of the genome wide screen

To look for novel regulators of Rho1 signaling, we examined the unpublished data of a genome wide screen that was previously conducted in our lab to look for changes in chitin level under cell wall stress. We reasoned that changes in chitin level could be a result of changes in the cell surface localization of the chitin synthase Chs3 in response to Rho1 signaling. The detailed protocol of the screen has been published (Burston, Davey, & Conibear, 2008). In this screen, the fluorescence agent Calcofluor White (CW) was used to induce cell wall stress through its interaction with surface chitin, a structural component of the yeast cell wall. Therefore, the fluorescence level of cells grown on CW-containing media could be used to evaluate the level of surface chitin. To validate the results of the genome wide screen, we measured the relative chitin levels of cells lacking known regulators of Rho1 or putative ORFs identified from the genome screen (Table 2.1) using this CW based fluorescence assay. The wild type strain and the non-essential deletion strains, all in the BY4742 strain background, were replicated on YPD +40 µg/ml CW plates using a programmed pinning robot. The plates were scanned daily under white light for growth and UV light for fluorescence (Fig 2.1 A and B). Growth measurement of each strain was necessary to exclude dead or slow-growing strains. The fluorescence intensity, determined by automated image densitometry, was measured from healthy growing cells and normalized to the wild type strain.

The normalized fluorescence value of each strain reflected the surface level of chitin compared to the wild type (Fig 2.1 C). Lower fluorescence suggested that the chitin level was reduced, while higher fluorescence indicated that the chitin level was increased. Consistent with the genome screen, chitin levels were reduced in known regulators *pfa4*Δ*, chs7*Δ*, bck1*Δ*, and slt2*Δ. Pfa4, a member of the DHHC family of putative palmitoyltransferases, is required for the palmitoylation of Chs3. Non-palmitoylated Chs3 is retained in the ER (Lam et al., 2006). Chs7, a chaperone protein for Chs3, is important for the proper folding of Chs3 in the ER. Slt2 and Bck1 are the MAP kinases that regulate the expression of Chs3 via transcription factor Rlm1 (Jung et al., 2002, Jung et al., 1999). The RhoGEFs Tus1 and Rom2 are regulators of Rho1, which is responsible for cell wall biogenesis, including chitin synthesis. The chitin level in *tus1*Δ and *rom2*Δ mutants was slightly lower than the wild type, suggesting that the single deletion of *TUS1* or *ROM2* had little effect on Rho1 mediated cell wall biogenesis (Schmelzle et al., 2002). A

strain containing a deletion of the putative ORF *YPL066W* displayed a similar level to the *tus1*Δ and *rom2*Δ mutants and was 20% lower than the wild type strain.. Deletion strains of the putative ORFS *pef1*Δ and *ykl037w*Δ displayed similar chitin level to the wild type. Overall, the results of the assay were consistent with the genome wide screen: chitin level in the presence of cell wall stress was reduced in known regulators *pfa4*Δ, *bck1*Δ, *slt2*Δ, *rlm1*Δ and *chs7*Δ. We are confident that the genome wide screen was quantatative and sensitive in measuring changes in the surface level of chitin under CW induced cell wall stress

2.3.2 Co-immunoprecipitation confirms *YPL006W* interaction with Tus1

We decided to focus on a putative ORF *YPL066W* as a potential regulator of Rho1 signaling. Past large scale studies predicted physical association of *YPL066W* with a potential Rho1 signaling complex (Babu et al., 2012; Krogan et al., 2006). The complex includes RhoGEFs Rom2 and Tus1, the inorganic pyrophosphatase Ipp1 and Ack1 (Fig 2.1 D). In addition, sequence analysis revealed that *YPL066W* contains arrestin domains and is hence renamed to *ADC2* for arrestin domain containing protein 2. The human homolog Arrdc2 belongs to the Arrdc arrestin family in humans. The arrestin aspect of *ADC2* will be discussed in the next chapter.

To verify the predicted interactions, we epitope-tagged individual components of the complex and carried out co-immunoprecipitation studies. The predicted interaction of Tus1 and Adc2 was confirmed as Tus1-MYC was pulled down by Adc2-HA, but not Ack1-HA (Fig 2.2 A). Pull down efficiency analysis indicated that 70% of total Adc2 and 80% of total Tus1 were pulled down in the IP. The predicted interaction of Rom2 and Ack1 was confirmed as Rom2-MYC was pulled down by Ack1-HA, but not Adc2-HA (Fig 2.2 B). Pull down efficiency analysis indicated that 60% of total Ack1 and 50% of total Rom2 were pulled down in the IP. The interactions of Ipp1 with both RhoGEFs were also confirmed, as Ipp1-HA pulled down Tus1-MYC and Rom2-MYC in separate CoIP experiments (Fig 2.2 C and D). The Ipp1-Tus1 interaction does not depend on Adc2, as the interaction was detected in both the wild type and the *adc2*Δ mutant strain. The interaction data suggested that Adc2 may be a part of a Tus1 signaling complex while Ack1 may be a part of Rom2 signaling complex. Ipp1 may be a common member of both the Tus1 and Rom2 complex.

2.3.3 The CNH domain of TUS1 is sufficient and necessary for Adc2 interaction

Next, we used the Y2H technique to map out the region on Tus1 that is responsible for the Adc2 interaction. Both RhoGEFs Tus1 and Rom2 contain a DH domain for Rho1 interaction and the PH domain for lipid association in the case of Rom2. The function of the CNH domain is not well understood. Using PCR and homologous recombination, we generated different Tus1 truncation mutants fused with a Gal4 Activation Domain (GAD). The interaction of Tus1 full length and truncation mutants were tested with Gal4 Binding Domain (GBD) fused Adc2 (Fig 2.3 B). Positive interactions would reconstitute GAD with GBD, consequently allowing the expression of the reporter gene *HIS3*. Strains were tested for growth on SD-HIS plates. Tus1 mutants missing the N-terminal domain, and both DH and PH domains, grew on SD-HIS plates, suggesting that these domains are not necessary for Adc2 interaction. In addition, the CNH domain by itself was sufficient for Adc2 interaction. The Tus1-Adc2 interaction was abolished in a Tus1 truncation mutant missing the CNH domain. Co-IP confirmed the importance of the CNH domain, as Adc2-HA was pulled down by full length Tus1-MYC, but not Tus1Δ_{CNH}-MYC (Fig 2.3 C). The CNH domain is present in some RhoGEFs and could be the binding site of for other proteins. Based on the physical interaction data, we hypothesized that Adc2 specifically interacts with the Tus1 CNH domain as part of the Tus1-Rho1 mediated signaling pathway (Fig 2.3 D)

2.3.4 Adc2 is co-localized with Tus1 at the bud neck

To further test this hypothesis, we epitope tagged Adc2-GFP at its endogenous locus and looked at its cellular localization using fluorescent microscopy. Previous studies have shown that Rho1, Pkc1, Tus1 are all localized at the bud neck in *S.cerevisae* (Yoshida et al., 2006, Yoshida et al., 2009). The bud neck is the site of polarized growth where the daughter cell emerges and eventually separates from the mother cell during cell division. We observed that Adc2 localization was only visible at the bud neck (Fig 2.4 A). It is important to note that not all wild type cells displayed Adc2 localization. A total of 600 wild type cells carrying Adc2-GFP were counted, and 12.5% of these cells had visible Adc2 localization at the bud neck. This suggests that Adc2 may be temporally and spatially regulated, similar to Tus1 whose localization at the bud neck is cell cycle dependent (Yoshida et al., 2009). The bud neck localization of Adc2

indicated that Adc2 is localized at the same region as other components of the Tus1 signaling complex.

Next, we determined whether Tus1 and Adc2 are co-localized together at the bud neck. Due to the low abundance of both proteins, direct co-localization microscopy of Tus1 and Adc2 was not possible. Instead, we introduced a plasmid carrying the RFP-tagged spindle marker Tub1 to monitor the localization of Tus1-GFP and Adc2-GFP separately during the course of the cell cycle (Fig 2.4 D and E). The length of the spindle as visualized by RFP-Tub1 is indicative of different stages of mitosis. We quantified 200 wild type cells displaying bud neck localization and determined the percentage of localization based on the morphology of Tub1(Fig 2.4 F). Among all cells carrying Adc2-GFP, only 12% of cells displayed Adc2 localization (Fig 2.4 B). Among these 12% of cells, Adc2 localization was often observed in cells displaying minimal tubulin stain and least observed in cells having a short spindle near the bud neck. A similar trend of Tus1 localization at the bud neck was observed in wild type cells. The variation of Tus1 and Adc2 localization compared to the spindle morphology suggested that their localization was cell cycle dependent, and that both proteins were likely present at the bud neck during mitosis when the spindle undergoes changes to facilitate cell division. Furthermore, the identical localization patterns between Adc2 and Tus1 indicated that Adc2 is co-localized with Tus1 temporally, consistent with the hypothesis that Adc2 is part of the Tus1 signaling complex.

2.3.5 Tus1 is required for Adc2 localization

Next, we asked whether the physical interaction of Tus1 was important for recruiting Adc2. We tagged *ADC2* with GFP at its endogenous locus in the *tus1*Δ and *rom2*Δ mutants in the haploid BY4741 strain background. Adc2 localization was again observed using fluorescence microscopy. A total of 600 cells per strain were counted to calculate the proportion of cells displaying Adc2 localization at the bud neck. The protein level of Adc2-GFP in the wild type and the deletion strains was confirmed by Western blotting, which indicated that the stability of Adc2-GFP was not affected in *tus1*Δ and *rom2*Δ strains (Fig 2.4 C).

We found that Adc2 localization at the bud neck was abolished in the *tus1*Δ strain (Fig 2.4A), which we confirmed by cell counting (Fig 2.4 B). Next, we transformed a complementation plasmid carrying full length *TUS1* into the *tus1*Δ strain to confirm that the abolished Adc2 localization was indeed caused by *tus1*Δ. Mutant *tus1*Δ strain carrying the

p*TUS1* complementing plasmid was able to restore Adc2 localization at the bud neck in approximately 13.5% of all *tus1*Δ cells, which was similar to the percentage of the wild type cells displaying Adc2 bud neck localization (Fig 2.4 A and B). This strongly indicated that Tus1 is necessary for Adc2 localization, and may even physically recruit Adc2 via its CNH domain.

Next, we wanted to examine Adc2-GFP localization in the *Tus1*Δ_{CNH}*-MYC* strain to study the importance of the CNH domain on Adc2 localization. We first checked the localization of Tus1Δ_{CNH}-GFP in the wild type strain to ensure that Tus1Δ_{CNH} is still being properly localized. Surprisingly, Tus1 localization at the bud neck was completely abolished in the absence of its CNH domain (data not shown). Western blotting confirmed that Tus1Δ_{CNH}-MYC was stable in cells (data not shown). This suggests that the CNH domain plays a role in Tus1 localization, which has not been reported before. The PH domain is thought to be sufficient for membrane localization, at least in the case of Rom1/2 (Philip & Levin, 2001b). Future work will be needed to determine the role of the CNH domain in Tus1 localization.

Our physical interaction data so far have not indicated any interaction between Adc2 and Rom2. As expected, we did not find Adc2 localization to be affected in the *rom2*Δ strain (Fig 2.4 A). Like the wild type cells, approximately 12% of *rom2*Δ cells displayed Adc2 localization at the bud neck (Fig 2.4B). Unlike Tus1, Rom2 does not appear to play a role in Adc2 localization. This is consistent with the hypothesis that Adc2 is a specific component of the Tus1 signaling complex in yeast.

2.4 Discussion

A novel genome wide screen was initially conducted in our lab to identify regulators of Chs3 trafficking in non-essential knockout collections (Burston et al., 2008). The screen evaluated the changes in chitin level in different strains as a result of reduced Chs3 trafficking to the cell surface. The screen identified known Chs3 trafficking regulators, as well as factors required for Chs3 activity. Numerous putative ORFs whose deletion led to reduced Chs3 trafficking were also identified. To look for novel regulators of Rho1 signaling, we re-examined the unpublished data of the screen. We reasoned that reduced level of chitin could be a direct result of lowered biogenesis of chitin, a process that is regulated by Rho1 signaling through *CHS3* transcription and Chs3 intracellular trafficking.

2.4.1 Small scale fluorescence assay validated the genome wide screen

As proof of concept, we conducted the same fluorescence assay on small scale to evaluate surface chitin level in mutants of known Rho1 signaling regulators, as well as putative ORFs identified from the screen. Mutant strains of known Rho1 regulators *tus1*Δ and *rom2*Δ, as well as Rho1 downstream effectors *bck1*Δ, *slt2*Δ, and *rlm1*Δ displayed lower surface chitin levels compared to the wild type strain. Both *tus1*Δ and *rom2*Δ strains had slightly lowered chitin level than the wild type indicating that Rho1 signaling was not greatly affected. This suggested that loss of one GEF does not severely compromise Rho1 signaling as long as the other GEF is still present. This is consistent with the idea that both Tus1 and Rom2 can activate Rho1 signaling independent of each other. However, the slight reduction in chitin level also suggested that Tus1 and Rom2 may have unique functions in Rho1 signaling, which cannot be completely complemented by each other.

Bck1, Slt2 and Rlm1 are part of the downstream components of Rho1 signaling in the cell wall integrity pathway, which regulates *CHS3* expression. The chitin level in the *bck1*Δ, *slt2*Δ, and *rlm1*Δ mutant strains were lower than the *tus1*Δ and *rom2*Δ strains. This suggests that while Rho1 signaling can be independently activated by Rom2 and Tus1 in parallel, the downstream targets of Rho1 are shared by both GEFs. Rho1 signaling regulates *CHS3* expression through the MAP kinases Bck1, Slt2 and the transcription factor Rlm1. The fact that the chitin level was lower in the *bck1*Δ and *slt2*Δ strains than in the *rlm1*Δ mutant suggests that

Swi4, another downstream transcription factor of Slt2 and Bck1, may play a role in chitin biogenesis through an unknown mechanism.

The putative ORF *ADC2* identified from the previous screen was validated by the small scale fluorescence assay. The slight reduction in chitin level suggested that Adc2 may potentially contribute to chitin biogenesis in yeast. A recent genome scale study on membrane protein interactions identified Adc2 as a potential signaling molecule involved with Tus1 and Rom2 in Rho1 signaling (Babu et al. 2012). The chitin level in the *adc2*Δ was similar to the chitin level in *tus1*Δ *and rom2*Δ strains and higher than the *bck1*Δ*, slt2*Δ*,* and *rlm1*Δ strains. This suggested that Adc2 may be associated with Tus1 and/or Rom2 and is upstream of Bck1, Slt2 and Rlm1. If Adc2 is functionally associated with both Tus1 and Rom2, it must not be essential for both RhoGEFs or the loss of *ADC2* should have a more severe defect in chitin biogenesis compared to single deletion of *TUS1* and *ROM2*. Adc2 could also be functionally associated with either Tus1 or Rom2 such that the loss of *ADC2* would therefore be similar to the loss of *TUS1* or *ROM2*. We hypothesized that Adc2 may be a potential Rho1 regulator through the RhoGEFs Tus1 and/or Rom2.

2.4.2 Adc2 physically interacts with the RhoGEF Tus1, but not Rom2.

A recent study indicated that Adc2 was part of a signaling complex with Tus1, Rom2, Ipp1 and Ack1 (Babu et al., 2012). Adc2 was found to interact specifically with Tus1, but not Rom2. We further identified the CNH domain of Tus1 to be sufficient and necessary for the Adc2 interaction. While the function of the CNH domain is not clear, it may be important for Tus1 to interact with other proteins. A recent study indicated that the CNH domain of Tus1 interacts with Ycf1, a heavy metal transporter regulated by Tus1 mediated Rho1 signaling. It was hypothesized that Ycf1 recruits Tus1 through physical interaction, and Tus1 recruits and activates Rho1 at the Ycf1 transporter (Paumi et al., 2007). The study further revealed that Rom2 was not functionally associated with Ycf1, and no interaction was observed between the CNH domain of Rom2 and Ycf1.

While both RhoGEFs are sufficient for activating Rho1, the CNH domain may hold the key to understanding how Tus1 and Rom2 can selectively regulate Rho1 signaling in different pathways, such as the regulation of Ycf1. Since both Rom2 and Tus1 contain a CNH domain near the C-terminus, there must be different binding motifs to allow binding specificity. Perhaps

it contains unique sites for specific protein interactions that can spawn signaling specificity in Tus1 that is different from Rom2. While Tus1 interacts with Rho1 to form the core components of the complex, additional proteins may bind to the CNH domain of Tus1 to ensure Rho1 signaling is specific to certain pathways. Adc2 could be a Tus1 specific protein that is part of the Tus1 signaling complex.

While Adc2 is found to be Tus1 specific, Ack1 specifically interacts with Rom2. It is plausible that Ack1 is a Rom2 specific protein that plays a similar role as Adc2 does for Tus1. However, there is not any direct evidence suggesting that both Adc2 and Ack1 share genetic interactions. Sequence comparison of *ADC2* and *ACK1* did not reveal any significant homology (data not shown). Although Ipp1 had direct interactions with Rom2 and Tus1, the potential role of Ipp1 in Rho1 signaling is not clear at the time. Ipp1 could be a common component of Tus1 and Rom2 signaling complexes, which suggests that it likely is not a contributing factor for Rho1 signaling specificity. Therefore, the rest of the project will focus on Adc2 as a potential Tus1 specific protein that may contribute to Tus1-Rho1 signaling in yeast.

2.4.3 Tus1 recruits Adc2 to the bud neck

The localization studies revealed that Adc2 is localized at the bud neck in wild type cells. In previous studies, Tus1, Rho1 and Pkc1 have been found to localize at the bud neck (Yoshida et al., 2006; Yoshida, Bartolini, & Pellman, 2009; Denis et al. 2005). Our results suggested that Adc2 is localized at sites of polarized growth, where Tus1 activates Rho1 to regulate Pck1 activity in cells. It is likely that the physical interaction of Adc2 with the Tus1 CNH domain may have a functional role in the formation of a complex that includes Tus1, Rho1, Pkc1 and Adc2. The binding of Adc2 may activate Tus1 to recruit Rho1. Alternatively, Adc2 binds to Tus1 after Tus1-Rho1 association, and the Adc2 binding enhances the stability of the Tus1 complex. Future work will be needed to investigate the molecular mechanism of Adc2 interaction with Tus1.

Tus1 and Rho1 have been implicated in CAR assembly during cell division (Yoshida et al., 2006). Cell cycle dependent kinase Cdc5 binds and phosphorylates Tus1, which is localized at the bud neck during anaphase. Tus1 then recruits Rho1 to the bud neck, where Rho1 signaling is carried out for the assembly of the septum ring that is important for cell division. Rho1 localization at the bud neck is cell cycle dependent, and can be affected by Cdc5 through Tus1

phosphorylation. To further examine whether Adc2 is part of the Tus1 complex, we studied temporal regulation of Tus1 localization and Adc2 localization separately. Our results suggested that Adc2 localization coincides with Tus1. The cell cycle dependent localization of Adc2 suggests that it is dependent on Tus1 localization.

To test this hypothesis, we looked at the Adc2 localization in the *rom2*Δ and *tus1*Δ strains. Adc2 localization at the bud neck was completely abolished in the *tus1*Δ strain but remained unaffected in the *rom2*Δ strain. Tus1 therefore is important for the bud neck localization of Adc2. This result further supported our hypothesis that Adc2 is a Tus1 specific protein that may be functionally associated with Tus1 to regulate Rho1 signaling. It is possible that the CNH domain of Tus1 physically recruits Adc2 to the bud neck. We are attempting to replace the CNH domain of Rom2 with that of Tus1 to examine whether the CNH domain is sufficient for Adc2 localization. Our results also showed that Tus1 Δ_{CNH} –GFP is mis-localized when its CNH domain is truncated. Although the CNH domain has never been implicated in Tus1 localization, our results suggest that the CNH domain is necessary for Tus1 localization through Adc2 or other proteins via an unknown molecular mechanism.

2.4 Figures

2.1 Known regulators of chitin biogenesis and putative ORFs identified from the screen

Name	Rank	Function
SLT2	1	MAP kinase in the CWI pathway, phosphorylates Rlm1
BCK1	2	MAP kinase in the CWI pathway, phosphorylates Mkk1/2
PFA4	11	Palmitoyltransferase; palmitoylates Chs3 in the ER
TUS1	31	RhoGEF in the CWI pathway, regulates Rho1 activity
ROM2	36	RhoGEF in the CWI pathway, regulates Rho1 activity
WSC1(SLG1)	40	Cell wall sensor in the CWI pathway
PEF1	42	Penta-EF-hand protein required for cell wall abscission
RLM1	48	MADS-box transcription factor, activated by Slt2
YPL066W/ADC2	56	Putative protein of unknown function
CHS7	64	Chaperone protein, facilitates Chs3 folding in the ER
YKL037W	74	Putative protein of unknown function

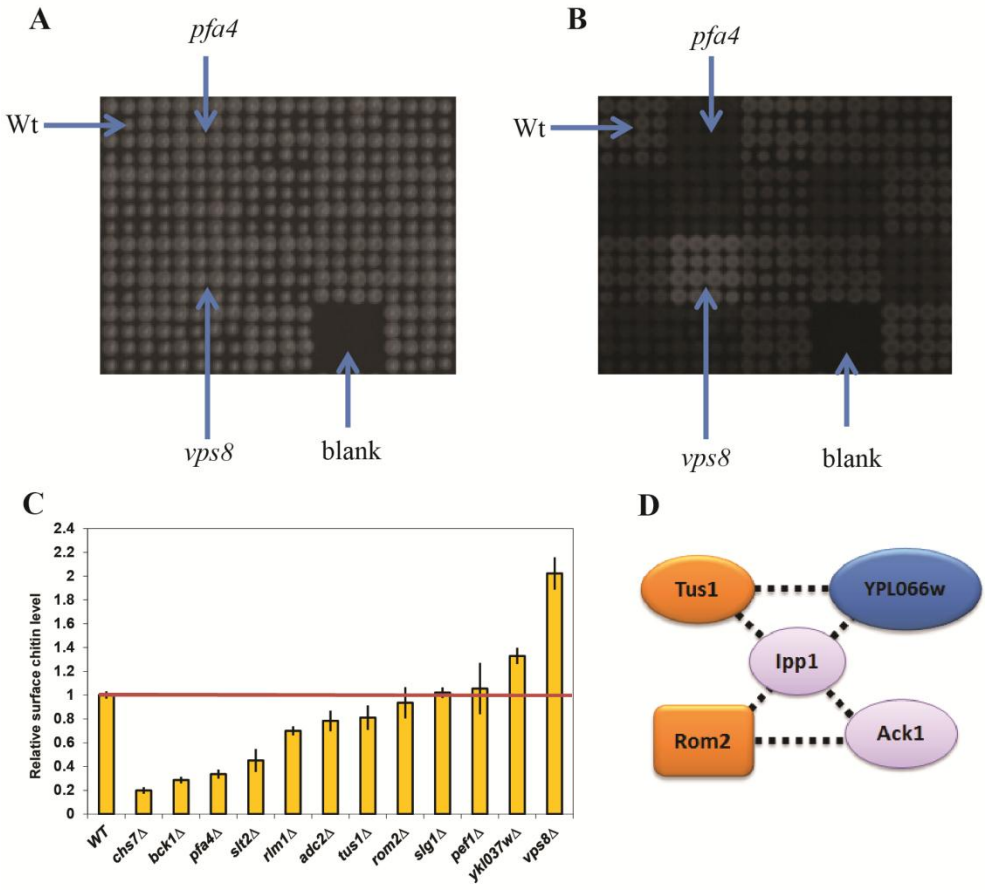

Figure 2.1. Small scale fluorescence assay validated the genome-wide screen.

(A-B) Each dot is a single colony of a strain. Sixteen replicates of each strain are clustered together on the plate. Seven clusters of each strain were randomly distributed on YPD+CW plates. The plates were scanned under white light for growth (A) and UV light for fluorescence (B). Mutants defective in Chs3 trafficking exhibited reduced fluorescence intensity relative to the wild type, but appeared normal in growth. Wild type and mutants with high *(vps8)* and low *(pfa4)* levels of Chs3 trafficking are indicated. **(C)** The median chitin surface level, calculated from all replicates, of each strain was normalized to the wild type. Red line indicates the level of the wild type strain. **(D)** Predicted interaction cluster of *YPL066W* (Babu et al., 2012)

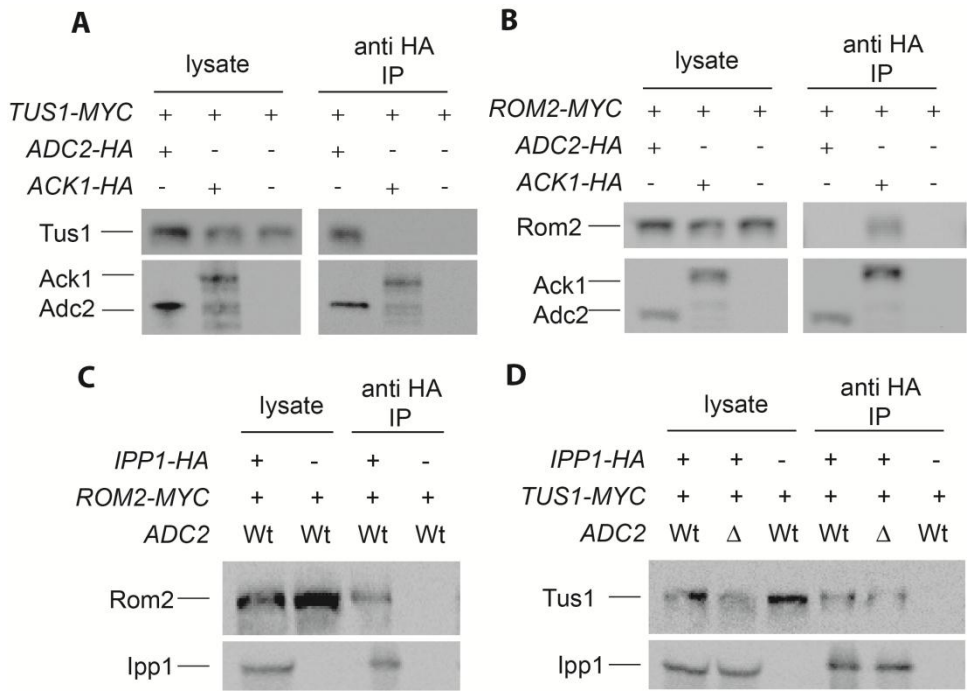

Figure 2.2. Adc2 is physically associated with Tus1, but not Rom2.

(A-D) Wild type cells of BY4742 strain background were grown to midlog phase and were processed for immunoprecipitation (IP). IP was performed with anti HA antibodies, whereas Western blotting was performed with both anti-HA and anti-MYC antibodies. **(A)** Tus1-13MYC is pulled down with Adc2-3HA, but not Ack1-3HA. **(B)** Rom2-13MYC is pulled down with Ack1-3HA, but not Adc2-3HA. **(C)** Rom2-13MYC is pulled down with Ipp1-3HA. **(D)** Tus1-13MYC is pulled down with Ipp1-3HA, independent of Adc2.

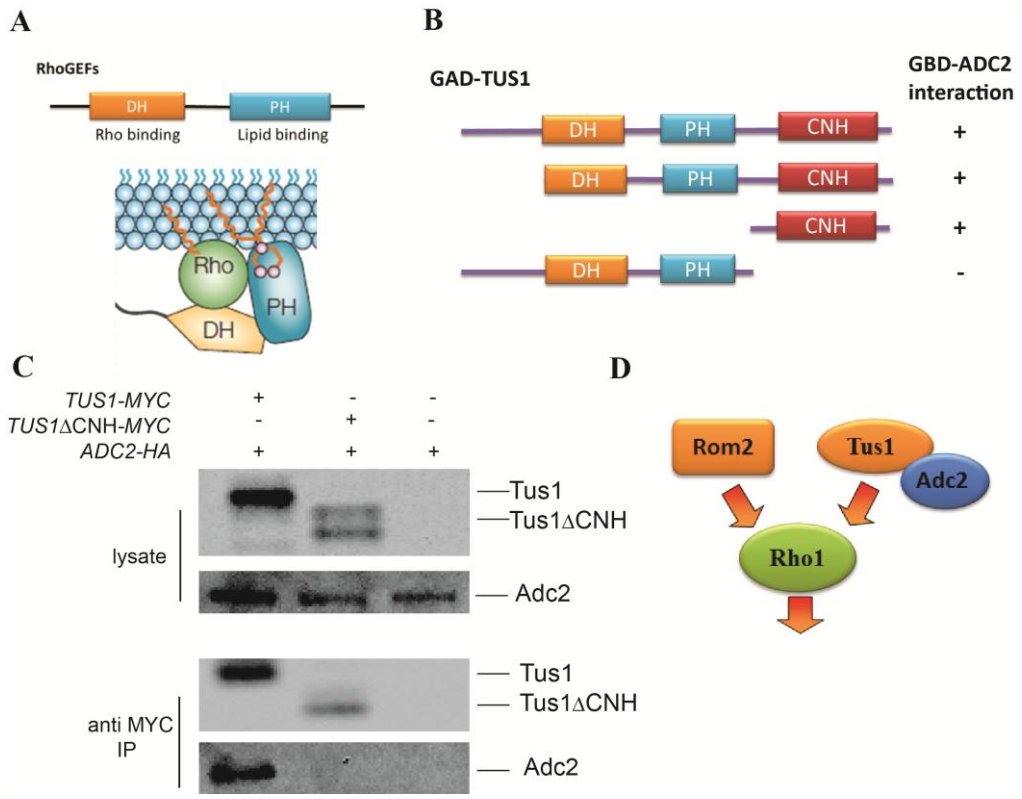

Figure 2.3. The CNH domain of Tus1 is sufficient and necessary for Adc2 interaction.
(A) Schematic of RhoGEF-Rho1 interaction. **(B)** Yeast two-hybrid assay between full length GAD-Tus1 and Adc2-GBD, and between GAD-Tus1 truncation mutants and Adc2-GBD **(C)** IP was performed with anti MYC antibodies, whereas Western blotting was performed with both anti-HA and anti-MYC antibodies. Adc2 was pulled down by Tus1 full length, but not Tus1ΔCNH. **(D)** Schematic of Adc2 being a part of Tus1-Rho1 mediated pathway

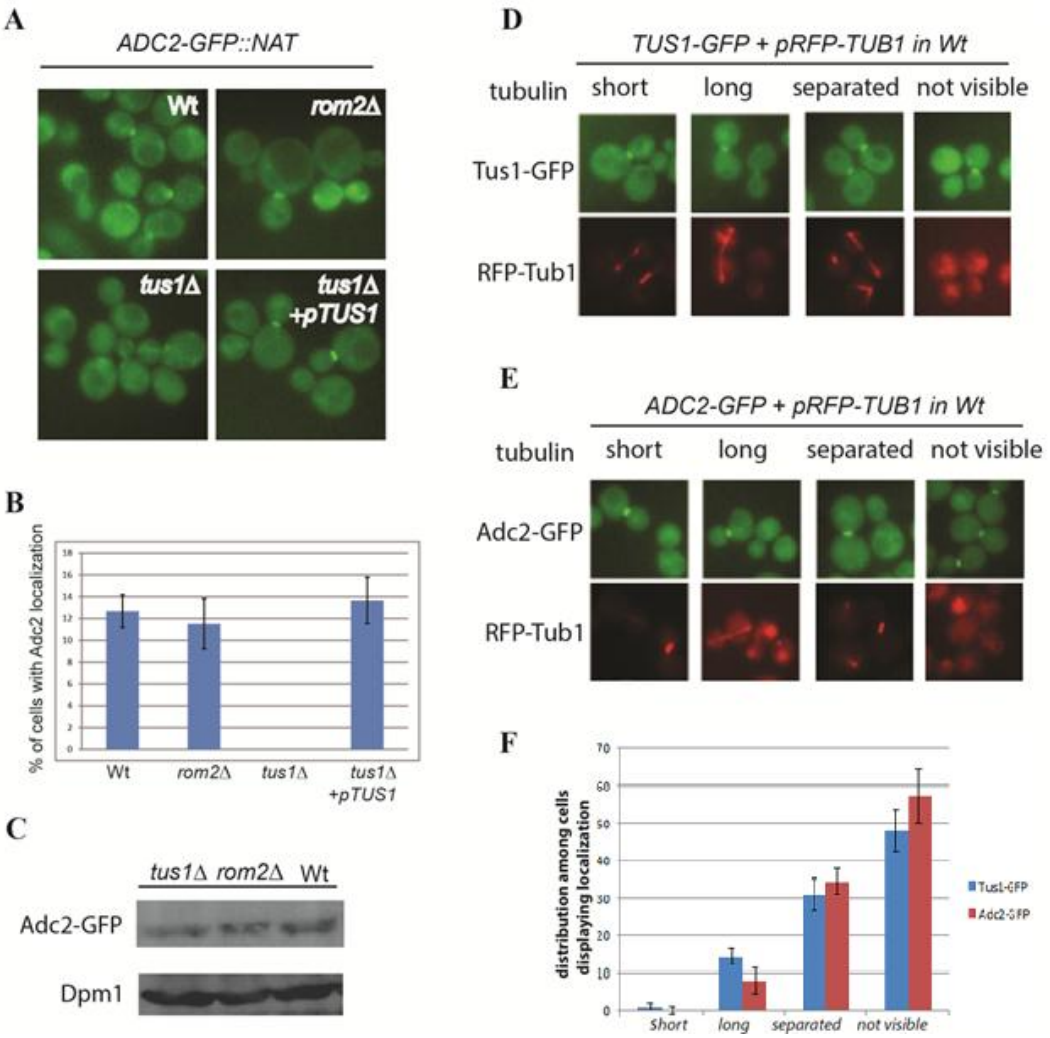

Figure 2.4. Tus1 is necessary for Adc2 localization at the bud neck

(A) Adc2-GFPlocalization in the wild type and mutants **(B)** Cell counting analysis quantified the percentage of cells displaying Adc2-GFP localization at the bud neck. **(C)** Western blotting using anti-GFP antibodies to monito levels of Adc2-GFP in indicated background. Dpm1 was used as a loading control. **(D-E)** Tus1-GFP and Adc2-GFP localization at different stages of cell cycle, as indicated by the morphology of the spindle labeled with RFP-tubulin. **(F)** Quantification of the percentage of total cells displaying Tus1 and Adc2 localization based on spindle morphology.

2.5 Materials and methods

Construction of plasmids and yeast strains

The *MATα* yeast strain BY4742 and its gene deletion derivatives were purchased from Open Biosystems (Huntsville, AL). Gene knockout and epitope tagging of genomic ORFs were generated using high-efficiency transformation of a PCR product flanked with 50-55bp of homology to the region of interest. Unless otherwise stated, plasmids construction was based on homologous recombination after co-transforming yeast with linearized plasmid and DNA fragments. Recombinant plasmids were recovered in *E. Coli.* Yeast strains and plasmids used for this chapter are listed in the appendix A (Table A1).

Fluorescence based genome wide screen

Yeast deletion collections of homozygous diploid, *MATα* haploid and *MATa* haploid in BY strain background were obtained through Open Biosystems. Manipulation and screening of the deletion collections was performed as described (Burston et al., 2008). Colony arrays on 96 stock plates were pinned sixteen times to YPD+CW plates creating 1536 arrays using a Virtek automated colony arrayer (BioRad, Hercules, CA). After incubation at 30 °C for 3 days, growth images of colony arrays were acquired using an Epson 2400 flat-bed scanner. Fluoresence images of colony arrays were acquired using a Biorad Fluor-S Max^{TM} multi imager using UV epifluorescence and the 530 DF60 filter. Densitometry on growth and fluorescence images used spot-finding program GridGrinder (gridgrinder.sourceforge.net) and values were modified to background subtraction to eliminate absent or slow-growing strains. The average values of each deletion strain was normalized to that of the wild type strain.

To validate the screen, selective deletion strains of *MATα* haploid BY strain background were used in a small scale fluorescence assay. The technique used for the assay is identical to the genome wide screen.

Co-immunoprecipitation

The equivalent of 30 OD_{600} cell pellet made from log phase spheroplasts were re-suspended in 1ml cold lysis buffer (30mM HEPES, 2mM EDTA, 100mM NaCl, 1mM DTT, 1% Tween-20) with Roche complete protease inhibitor. The lysis buffer containing cell pellets was incubated with rabbit αHA (1:200 ABM) and 30µl Protein A-Sepharose (50% slurry in PBS). Proteins

were identified by western blotting using mouse αMYC (1:5000 Upstate) and mouse αHA (1:1000 Covance) and goat α mouse HRP-labelled secondary antibodies (BioRad). Blots were developed with ECL (Pierce, Rockford, IL) and luminescent images were obtained with a Fluor S Max Multi-imager.

Yeast two-hybrid screening

Linearized pGAD vector and PCR product flanked with 55bp homology to the region of interest were cotransformed into PJ694a strain. Linearized pGBD vector and PCR product flanked with 55bp homology to the region of interest were cotransformed into PJ694α strain. PJ694a and PJ694α strains carrying the recombinant plasmid were mated on SC-URA-LEU plates (Sigma-Aldrich). Positive two-hybrid interactions were detected on SC-HIS+5mM 3AT plates.

Fluorescence microscopy

GFP-tagged proteins were verified by western blotting using mouse αGFP antibodies (1:1000 Roche). Yeast cells expressing GFP-tagged proteins were grown to log phase in minimal selective media at 30°C. 1ml of the cells was harvested through gentle spinning for 1 min and 900µl of the supernatant was discarded. Cell pellet was re-suspended in 100µl remaining media and 2µl was spotted onto glass cover slides. Cells were viewed using a 100x oil-immersion objective on a Zeiss Axioplan2 fluorescence microscope. Exposure time varied from 300ms-2000ms. Images were captured with a CoolSnap camera using MetaMorph software and adjusted using Adobe Photoshop. Cell counting was carried out using MetaMorph counting tools.

CHAPTER 3: *YPL066W* IS FUNCTIONALY ASSOCIATED WITH TUS1

3.1 Introduction

Rho1 signaling is an important process that is spatially and temporally regulated in yeast. Active Rho1 is localized at sites of polarized growth. The localization of Rho1 is regulated by the RhoGEFs Tus1 and Rom1/2. In the absence of the RhoGEFs, cells become inviable and cannot be rescued by a constitutively active Rho1 mutant (Yoshida et al., 2006). This indicates that the active Rho1 mutant cannot function when it is not properly localized in the absence of RhoGEFs. In essence, the activation of Rho1 by the RhoGEFs is a two-step process. First, the physical interaction of Rho1 with the DH domain facilitates the formation of GTP bound Rho1. Second, the RhoGEFs maintain the proper localization of Rho1 at sites of polarized growth, where GTP bound Rho1 can elicit downstream signaling.

Although Tus1 and Rom1/2 can physically interact with Rho1 at the DH domain, the mechanism by which they are localized to sites of polarized growth may differ. In the case of Rom1/2, the PH domains bind to phosphatidylinositol-4.5-biphosphate (PIP_2) and are responsible for their proper localization to the plasma membrane (Audhya et al., 2002). However, the PH domain of Tus1 does not appear to bind PIP_2 (Yu et al., 2004). Furthermore, the localization of Rom1/2 is facilitated by the interaction at their N-terminal regions with the cytosolic domains of Wsc1 and Mid2, two major cell wall receptors of the CWI pathway. Unlike Rom1/2, Tus1 interaction with Wsc1 and Mid2 has not been detected through the Y2H assay. The mechanism by which Tus1 is localized to sites of polarized growth is unclear.

One of the pathways regulated by Rho1 is the cell wall integrity (CWI) pathway. The CWI pathway regulates cell wall biogenesis when cells undergo extracellular stress including heat and biochemical stress. Rlm1 is the transcription factor associated with the CWI pathway. Rlm1 is related to members of the MEF2 transcriptional regulators in mammalian cells. It shares sequence similarity with MEF2 isoforms, and displays same DNA-binding specificity *in vitro* (Dodou and Treisman, 1997). Rlm1 regulates the expression of at least 25 genes associated with cell wall biogenesis, including *CHS3* (Jung, Sobering, Romeo, & Levin, 2002b). *CHS3* encodes chitin synthase 3 (Chs3), which synthesizes chitin at the cell surface to maintain the integrity of the cell wall. Rho1 therefore can regulate cell wall biogenesis through Rlm1 transcription factor of the CWI pathway.

In Chapter 2, we showed that YPL066w, which we named *ADC2* (arrestin domain containing 2), interacts specifically with Tus1, and not with Rom2. Arrestins are conserved molecules that function in signaling transduction pathways. They are categorized into three groups based on sequence homology: visual arrestins, beta arrestins and alpha arrestins. *ADC2* appears to be a new member of the yeast alpha arrestin family and is most closely related to mammalian Arrdc2. The functions of alpha arrestins in mammalian cells include scaffolding signaling complexes, binding to cell wall receptors and inducing E3 ligase dependent ubiquitination. Alpha arrestins contain arrestin N and C domains that are important for substrate binding. The PY motif found in the C terminus of alpha arrestins is responsible for interaction with the WW domains commonly found in E3 ligases. A recent study found Arrdc alpha arrestins can act as ubiquitin adaptors between HECT ubiquitin ligases and the core ESCRT machinery through the PY motif in mammalian cells (Rauch and Martin-Serrano, 2011).

Ten alpha-arrestins have been identified in *Saccharomyces cerevisiae*, although their functions are only beginning to emerge. Two studies have found that alpha-arrestins regulate endocytosis and protein turnover of several yeast membrane receptors (Nikko et al, 2008; Lin et al, 2008). Another study revealed that alpha-arrestins Aly1 and Aly2 regulate the translocation of Gap1from the TGN to the plasma membrane in response to nutrient signaling (O'Donnell et al., 2010). The intracellular trafficking pattern of Gap1 is similar to Chs3, in that the cell surface levels of both proteins are enhanced in response to intracellular signals. As more evidence emerges, the function of alpha-arrestins in signaling transduction is becoming more lucid. However, no direct evidence has been found to link alpha arrestins with Rho1 signaling in yeast.

In this study, we identified Adc2 as an arrestin like protein based on its sequence analysis with its homologs across species. The PY-like motif identified in Adc2 is functionally important for the localization of Tus1 at the bud neck. However, we failed to identify a connection between Adc2 and two major cell wall receptors of the CWI pathway. Genetic interaction assays further indicated that Adc2 is functionally associated with Tus1 in regulating Rho1 signaling complementary to Rom2.

3.2 Results

3.2.1 Sequence analysis suggests that Adc2 is an arrestin like protein

Sequence analysis using BLAST and PFAM identified an arrestin C domain in *ADC2* sequence. Further sequence comparison with Adc2 homologs across species identified an arrestin N domain and a potential PY motif near the C terminus (Fig3.1 A). The arrestin C and N domains are important for arrestin structure in mammalian cells and therefore provide specificity for arrestin-substrate interactions (Gurevich et al., 2006). Adc2 belongs to a family of alpha-arrestins in yeast. Alpha-arrestins commonly contain a conserved PY motif (PY or PPxY) that is mainly responsible for the binding of WW domain of E3 ligase (Nikko et al., 2008). The PPxxY is a non-canonical form of PY motif, which is also capable of binding to E3 ligases (Djiane et al., 2011). Sequence analysis identified numerous Adc2 homologues across species, but their functions are poorly understood. The *pombe* homolog of Adc2 has been implicated in the formation of actomyosin ring in fission yeast. The human homolog of Adc2 is Arrdc2, whose functions remain elusive, although some Arrdc proteins have recently been linked to the ESCRT machinery (Rauch & Martin-Serrano, 2011).

3.2.2 Adc2 is important, but not necessary, for Tus1 localization

One of the functions of arrestins is to act as a scaffold protein to facilitate the formation of a complex (Gurevich & Gurevich, 2006). Arrestins can often interact with cytosolic proteins for signaling complex formation, including the MAP kinase cascade. We asked whether Adc2 plays a role in the formation of the Tus1 signaling complex, and specifically the localization of Tus1.

We epitope-tagged Tus1 with GFP+ at its endogenous locus in the wild type and *adc2*Δ mutant in the haploid BY4741 strain background. Tus1 has previously been found at the bud neck (Yoshida et al., 2009). Using fluorescence microscopy, we found that Tus1-GFP is localized at the bud neck in the wild type cells, consistent with the previous study (Fig 3.1 B). Roughly 11% of 600 wild type cells displayed Tus1 localization (Fig 3.1 C). Next, we looked at Tus1-GFP in the *adc2*Δ deletion strain. The number of cells displaying Tus1 localization at the bud neck was reduced, with less than 4% of 600 *adc2*Δ cells displaying Tus1 localization at the bud neck. A mutant *adc2*Δ strain carrying a pRS416-*ADC2* complementation plasmid was able to restore Tus1 localization back to wild type levels (Fig 3.1 C). Fewer numbers of *adc2*Δ cells

displaying Tus1 localization indicated that Adc2 is important, but not necessary, for Tus1 localization.

3.2.3 The PPxxY sequence is functionally relevant to Adc2

Next we asked if the PPxxY sequence found in Adc2 is functionally important. Canonical PY motifs consist of Pro-Tyr or Pro-x-Tyr sequences that interact with the WW domain of E3 ubiquitin ligases. The PY motif is functionally important for alpha-arrestins to act as adaptors to scaffold ligases and ESCRT machinery. Using site directed mutagenesis, we mutated both prolines and tyrosine to alanines (PPxxY→AAxxA) and placed mutant adc2$_{AAxxA}$ in a pRS416 plasmid. To study the functional importance of PPxxY in Adc2, we compared Tus1 localization in *adc2*Δ strains transformed with either the pRS416-*ADC2* complementation plasmid or the pRS416-*adc2$_{AAxxA}$* mutant plasmid (Fig 3.1 B and C). Although Tus1 localization was visible in both strains, the plasmid carrying wild type *ADC2* restored Tus1 localization to 10% of *adc2*Δ cells, which was similar to the wild type. However, pRS416-*adc2$_{AAxxA}$* mutant plasmid failed to restore Tus1 localization and behaved just like an *adc2*Δ deletion strain (Fig 3.1 C). The P-value was less than 0.05, suggesting that the difference was statically significant. This indicated that the PPxxY motif in Adc2 is functionally relevant.

Based on microscopy data, we hypothesized that Tus1 recruits Adc2 to the bud neck, and Adc2 in turn contributes to Tus1 localization. As an alpha-arrestin like protein, Adc2 may interact with additional factors to stabilize the Tus1 complex. A previous study using the Y2H assay revealed that Mid2 and Wsc1 cell wall receptors interact directly with Rom2 (Philip & Levin, 2001b). However, it is not clear whether Tus1 localization is also dependent on these receptors. We examined Adc2 and Tus1 localization in the *mid2*Δ and *wsc1*Δ strains (3.2 A and B). Mid2 and Wsc1 are transmembrane sensors that act as major stress sensors for the CWI pathway (Ketela et al., 1999, Gray et al., 1997). The percentage of cells displaying Tus1 and Adc2 localization in the *mid2*Δ and *wsc1*Δ mutants was similar to the wild type (Fig 3.2 C). However, this does not prove that Tus1 and Adc2 localization is independent of these cell wall receptors, as they may act redundantly in localizing Tus1. Alternatively, other cell wall receptors that have yet to be identified may interact with Adc2 to influence the stability of Tus1 localization.

3.2.4 Adc2 is in the same functional pathway as Tus1

Our physical interaction and microscopy data so far have suggested that the uncharacterized protein Adc2, initially identified from our genome wide screen, is physically and perhaps functionally associated with Tus1, but not Rom2 (Fig 2.3 D). To further test this hypothesis, we studied the genetic interaction of Adc2 with Tus1 and Rom2 by generating *tus1*Δ *rom2*Δ*, adc2*Δ *tus1*Δ*,* and *adc2*Δ *rom2*Δ mutants in a haploid BY4742 strain background. When deletion of a gene disrupts a pathway, loss of another gene in the same pathway should not have any further effect. However, deletion of a second gene in a parallel pathway will have an additive effect. Based on phenotypic readouts, genes that co-function within a same pathway can often be distinguished from genes that function in parallel.

One phenotypic assay to evaluate Rho1 signaling propagation is cell growth at elevated temperature. High temperature can induce cell lysis in CWI mutants that have weakened cell wall due to compromised Rho1 signaling (Levin 2011). We performed a growth assay to examine the integrity of the cell wall in the wild type and mutant strains at elevated temperature (Fig 3.3 A). Serial dilutions of each strain were spotted onto SC-URA plates and grown overnight at 30 °C and 37 °C. The wild type strain grew well at both 30 °C and 37 °C. Like the wild type, single *tus1*Δ and *rom2*Δ mutants did not display cell lysis, suggesting that one RhoGEF can compensate for the absence of another RhoGEF to preserve Rho1 signaling. However, the *tus1*Δ *rom2*Δ strain grew well at 30 °C, but displayed severe growth defect at 37 °C. Consistent with previous studies, Rho1 signaling is severely compromised when both RhoGEFs are absent, and cells are therefore more likely to lyse due to weakened cell wall. The strong defect in the *tus1*Δ *rom2*Δ double mutant reaffirmed that Rom2 and Tus1 regulate Rho1 activity in parallel. The *adc2*Δ single mutant grew like the wild type, while the *adc2*Δ *rom2*Δ strain displayed a severe growth defect at 37 °C, which could be suppressed with a pRS416-*ADC2* complementation plasmid. The growth defects of *adc2*Δ *rom2*Δ and all other mutants were suppressed by the addition of cell wall stabilizer sorbitol, indicating that the growth defect was indeed caused by cell lysis (Fig 3.3 B). This suggests that the cell wall in the *adc2*Δ *rom2*Δ strain was weakened, likely due to compromised Rho1 signaling, and that Adc2 may contribute to Rho1 activity in parallel to Rom2. Next, we studied the *adc2*Δ *tus1*Δ strain to determine if Adc2 also worked in parallel to Tus1. The fact that the *adc2*Δ *tus1*Δ mutant grew much like *adc2*Δ and *tus1*Δ single mutants suggest that Adc2 and Tus1 co-function within the same

pathway, since loss of a second gene in the same pathway does not show additive effect. Parallel to Rom2, Adc2 appeared to be functionally associated with Tus1 in maintaining cell wall integrity.

In the CWI pathway, Rho1 signaling propagates through Pkc1, which can activate the MAPK cascade composed of Bck1, Mkk1/2 and Slt2. The MAPK cascade is necessary for the activation of Rlm1, a known transcription factor downstream of Rho1 (Fig 3.4 A). Activated Rlm1 is responsible for the expression of many cell wall biogenesis genes, including *CHS3*. To determine if Rho1 signaling was compromised in *adc2*Δ *rom2*Δ double mutant, we examined the activity of Rlm1. A 2x Rlm1-binding site was inserted at the promoter region of *lacZ*, placing *lacZ* expression under Rlm1 regulation. This Rlm1 reporter plasmid was transformed into the wild type and the mutant strains (Fig 3.4 B). The gene *lacZ* encodes the enzyme β-Galactosidase, which cleaves the synthetic compound o-nitrophenyl-β-D-galactoside (ONPG) to yield galactose and o-nitrophenol. O-nitrophenol produces a yellow color in liquid, which can be quantified at 420nm λ. Since the expression of *lacZ* is under Rlm1 regulation, the intensity of the yellow color reflects the activity of Rlm1. All strains were grown at 30 °C with 40μg/ml CW induction for 3 hr. The average Rlm1 activity of each strain was measured and normalized to the wild type (Fig 3.4 C). The *bck1*Δ mutant displayed minimal Rlm1 activity, consistent with previous findings that Bck1 is necessary for Rlm1 activation. The *tus1*Δ, *rom2*Δ and *adc2*Δ single mutants displayed slightly lowered Rlm1 activity compared to wild type. This is consistent with the overlapping functions of these RhoGEFs in Rho1 regulation. As expected, the activity of Rlm1 was greatly reduced in double mutant *tus1*Δ *rom2*Δ, consistent with compromised Rho1 regulation in the absence of both RhoGEFs. This additive defect confirmed that Rom2 and Tus1 regulate Rho1 in parallel. A similar effect was also observed in the *adc2*Δ *rom2*Δ double mutant, suggesting that Adc2 contributes to Rho1 signaling in parallel to Rom2. Next, we studied the *adc2*Δ *tus1*Δ mutant to determine if Adc2 functions in parallel to Tus1. The fact that Rlm1 activity in the *adc2*Δ *tus1*Δ strain was similar to *adc2*Δ and *tus1*Δ single mutants suggests that Adc2 and Tus1 function in the same pathway. Overall, the genetic interactions of *tus1*Δ, *rom2*Δ and *adc2*Δ strongly suggest that Adc2 is functionally associated with Tus1, and contributes to Rho1 signaling in parallel to Rom2.

3.3 Discussion

3.3.1 Adc2 is an alpha-arrestin like molecule in yeast

Alpha arrestins are signaling molecules that are highly conserved from mammalian cells to yeast. Sequence analysis identified ADC2 as an alpha-arrestin like protein containing arrestin N and C domains and a non-canonical PY motif with PPxxY sequence near the C terminus. Arrdc2, the human homologue of Adc2, is an alpha arrestin domain containing protein that has a PY motif with PPxY sequence. The PY motif in alpha arrestins is generally composed of PY or PPxY protein sequence, which have been shown to interact with the WW domain of ubiquitin E3 ligase. However, non-canonical PPxxY sequence has also recently been found to interact with the WW domain as well (Djiane et al., 2011). Alpha arrestins can act as a scaffold for E3 ligases, which can often lead to subsequent ubiquitination by the ligase. In fact, 9 out the 10 alpha arrestins in *Saccharomyces cerevisiae* are known to interact with the Rsp5 E3 ligase (Kee et al., 2006; Gupta et al., 2007; Lin et al., 2008). It is worth exploring the association of Adc2 with Rsp5 in future work. Besides the PY motif, alpha arrestins often contain arrestin N and arrestin C domains for substrate specificity. It is possible that these two domains are responsible for the specific interaction with Tus1, but not Rom2. Currently, we are attempting to modify the Adc2 domains and examine mutant Adc2-Tus1 interaction using the Y2H assay.

3.3.2 Adc2 regulates Tus1 localization

In the previous chapter**,** we have shown that Tus1 recruits Adc2 and is necessary for the Adc2 localization at the bud neck. Here, we present the interesting case that Adc2 in turn is important, but not necessary, for the Tus1 localization. Studies have so far only identified two important factors for Tus1 localization at sites of polarized growth during different states of the cell cycle: Cdc5 in the M phase and Cdc28 in the G1/S phase (Kono et al., 2008; Yoshida et al., 2006). Both of which are cell cycle dependent kinases that phosphorylate the N terminal domain of Tus1. While sequence analysis did not indicate Adc2 to be a kinase, it revealed that Adc2 is an alpha arrestin like protein. From the arrestin perspective, Adc2 may contribute to Tus1 localization in a number of ways. Different functions of alpha arrestins have been identified in signaling transduction pathways, including scaffolding additional proteins, interacting with cell wall proteins and recruiting ubiquitin ligases (Donnell, Apffel, Gardner, & Cyert, 2010; Gurevich & Gurevich, 2006; Lin, MacGurn, Chu, Stefan, & Emr, 2008).

Alpha-arrestins have been implicated in scaffolding MAR kinase Erk1/2 in mammalian cells (Gurevich & Gurevich, 2006). Tus1 mediated Rho1 signaling activates Pkc1 which regulates the MARK cascade in yeast. It is unclear whether Pkc1 and the MAP kinases come in direct contact with Tus1. However, the localization of Pkc1 and MAP kinases Bck1 and Slt2 has been shown at sites of polarized growth(Drogen, Peter, & Boveresses, 2002). While the crystal structure of the CNH domain is lacking, sequence based structure prediction indicated that the CNH domain is an exposed elongated structure that could be used for protein-protein interactions. It is plausible that Adc2 could act as a scaffold to first bind to the CNH domain and then recruit Pkc1 and/or MAP kinases to the Tus1-Rho1 complex. Additional lipid binding proteins may also interact with Tus1 to stabilize the Tus1–Rho1 signaling complex at sites of polarized growth. Slm1 is a phosphoinositide $PIP_{(2)}$ binding protein that is regulated by the TORC2 in yeast. Its interaction with Tus1 has been identified in a study that was looking at cross talk between Tor2 signaling and Rho1 signaling (Ho, Lee, Liao, & Chen, 2008b). It is unclear whether Adc2 recruits Slm1 to Tus1 and facilitates interaction with $PIP_{(2)}$.

Binding to cell wall sensors could also increase the stability of Tus1 signaling complex at the membrane. Adc2 may act as a bridge that connects Tus1 with membrane proteins. In the absence of Adc2, Tus1 localization could become transient and is dislodged from the membrane. Previous evidence suggested that Mid2 and Slg1, two major CWI cell wall sensors, physically recruit Rom2 to the cell membrane (Philip & Levin, 2001a). We found that the localization of Tus1 and Adc2 was not affected in *mid2*Δ or *wsc1*Δ mutant. However, the data is inconclusive and cannot suggest that Mid2 and Wsc1 do not contribute to Tus1 localization. RhoGEFs are known to interact with multiple cell wall receptors in cells. Mid2 and Slg1 may share overlapping functions with other cell wall sensors in recruiting Tus1 to the membrane. Loss of one sensor can be compensated by others and therefore would not show notable defect in Tus1 localization. A recent study has identified Sho1 as a potential physical binding partner of Tus1 (Tonikian et al., 2009). Sho1 is a transmembrane osmosensor involved in the HOG pathway in yeast. It is the first cell wall sensor that is implicated in Tus1 localization. Future work is needed to determine whether theTus1 physically interacts with Sho1 and if such interaction requires Adc2.

The fact that a complementation plasmid carrying $ADC2_{AAxxA}$ mutation in the PPxY motif could not rescue Tus1 localization defect indicated that the PPxxY sequence is important for

Adc2 function. While most alpha arrestins in yeast interact with Rsp5, Adc2 does not seem to be ubiquitinated (data not shown). This indicated that the PY motif in Adc2 may have functions independent of E3 ligases in cells. The PPxxY sequence may simply be a part of the region in Adc2 that interacts with Tus1. Mutations in PPxxY could reduce Adc2 binding affinity for Tus1, and therefore cause Tus1 mislocalization. The PY motif, in some cases, has been found to interact with the ESCRTs machinery(Rauch & Martin-Serrano, 2011b). The potential role of Adc2 in ESCRTs mediated transport is not clear.

Although the molecular mechanism by which Adc2 facilitates Tus1 localization is not well understood at this time, it is apparent that Adc2 is important to Tus1 mediated Rho1 signaling in yeast. The proper localization of RhoGEFs is essential for recruiting Rho1 to sites of polarized growth. Mislocalized Tus1in the *adc2*Δ strain therefore could not properly recruit Rho1. While Rho1 may still be activated by Tus1 in the *adc2*Δ strain, active Rho1 mislocalized from sites of polarized growth cannot function properly (Yoshida et al., 2006). This is consistent with the idea that Rho1 signaling is tightly regulated both temporally and spatially (David E Levin, 2011). Adc2 may be a novel regulator of Rho1 signaling through its role in Tus1 localization.

3.3.3 Adc2 is functionally associated with Tus1

To further verify that Adc2 is part of the Tus1 signaling pathway, we studied the genetic interaction of Adc2, Tus1 and Rom2. The single deletion strains of *TUS1*, *ROM2* and *ADC2* did not affect cell growth, suggesting that their role in Rho1 signaling can be compensated by others. However, we were able to observe strong defect at elevated temperature in the *tus1*Δ *rom2*Δ and the *adc2*Δ *rom2*Δ strains, but not in the *adc2*Δ *tus1*Δ strain. Since Rho1 is an essential protein in yeast, the viability of the *tus1*Δ *rom2*Δ strain suggested that Rho1 signaling is not completely lost and may be preserved through another RhoGEF Rom1. Previous studies found that Rom1 is partially implicated in the regulation of Rho1 and Rho2 in yeast (Ozaki et al., 1996b; Schmelzle et al., 2002). This suggested that all three RhoGEFs share overlapping but complementary functions in Rho1 regulation.

The growth defects of the *tus1*Δ *rom2*Δ and the *adc2*Δ *rom2*Δ strains were suppressed by the addition of cell wall stabilizing agent sorbitol, suggesting that Tus1, Rom2 and Adc2 had direct effect on cell wall biogenesis. Cells with inefficient Rho1 signaling cannot maintain the

integrity of the cell wall under heat stress and therefore lysed at elevated temperature. Our results suggested that Rho1 signaling is compromised in the absence of both RhoGEFs Tus1 and Rom2. Furthermore, the strong defect in the *adc2*Δ *rom2*Δ strain indicated that Adc2 and Rom2 contribute to Rho1 regulation in distinct pathways. Lack of growth defect in the *adc2*Δ *tus1*Δ strain suggested that both Adc2 and Tus1 are functionally associated in regulating Rho1 signaling. Combined with the localization studies, it is likely that Adc2 contributes to Rho1 signaling by regulating the localization of Tus1. In the absence of *ADC2*, Tus1 cannot be properly localized to sites of polarized growth, where it can recruit and activate Rho1.

The result of the Rlm1 activity assay further supported the idea that Adc2 and Tus1 are functionally associated and together contribute to Rho1 signaling in yeast. Low Rlm1 activity in the double deletion mutants suggested that Adc2 and Tus1 regulate Rho1 signaling through Rlm1 activation in parallel to Rom2. While they may activate Rho1 differently, Tus1 and Rom2 mediated Rho1 signaling must act on a common effector Bck1, as Rlm1 activity is completely gone in the absence of *BCK1*. This is consistent with previous studies that Rlm1 is regulated by Rho1 through a linear cascade of MAP kinases Bck1, Mkk1/2 and Slt2 (Jung et al., 2002b).

The goal of our study is to understand Rho1 signaling specificity in yeast. Evidence have emerged that Tus1 is specifically associated with pathways that are independent of Rom2. Connections between Tus1 specific Rho1 signaling have been established with TOR2 signaling pathway, CAR assembly during cell division and Ycf1 activity in heavy metal detoxification pathway (Ho et al.; 2008, Yoshida et al.; 2006, Paumi et al., 2007). It is likely that additional GEF specific proteins are needed to achieve specific Rho1 signaling in cells. Our results suggested that Adc2 is an upstream regulator of Rho1. It is physically and functionally associated with Tus1, but not Rom2. It contributes to the proper localization of Tus1 at sites of polarized growth through an unknown mechanism. The arrestin like domains have given new insights to future studies on the functional roles of Adc2. Understanding the significance of Adc2 association with Tus1 is important to identify more RhoGEF specific accessory proteins not just in yeast but in mammalian cells as well. In humans, family of Rho GTPases account for a large number of human diseases, including cancer and neurodegenerative illness (Boettner & Van Aelst, 2002). Understanding Rho signaling specificity will have implications in treating these diseases by selectively regulating and/or inhibiting RhoGEF specific proteins. This is likely

to cause less side effects compared to directly targeting the Rho GTPases or the RhoGEFs, which are associated with a multitude of pathways.

3.4 Figures

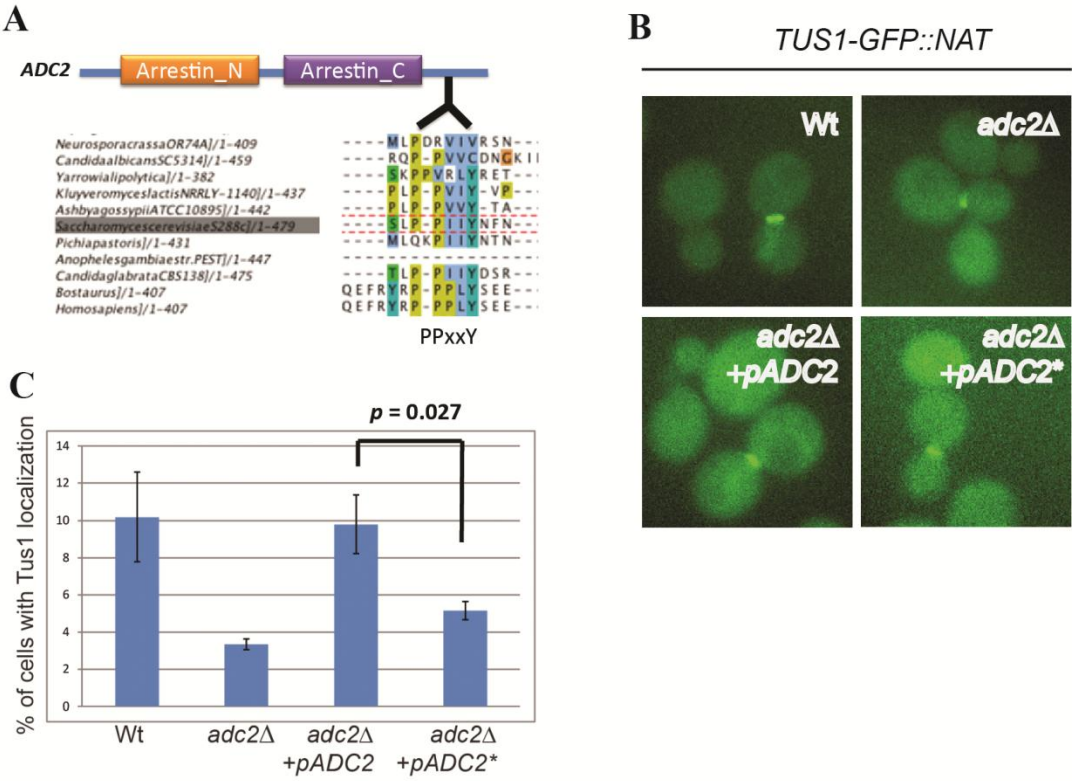

Figure 3.1. Adc2 is an arrestin like protein that contributes to Tus1 localization

(A) Sequence comparison with *ADC2* homologs identified the arrestin N and C domains. A PY like motif sequence PPxxY was also identified **(B)** Cells carrying *TUS1*-GFP were grown to midlog phase in SD-URA medium prior to visualization under a fluorescence microscope. Tus1 localization was observed in the wild type strain and the *adc2*Δ strain. pADC2 is a pRS416 based plasmid carrying the full length *ADC2*. pADC2* is a pRS416 based plasmid carrying ADC2 with PPxxY mutations to AAxxA. **(C)** Cell counting analysis quantified the percentage of cells displaying Tus1-GFP localization

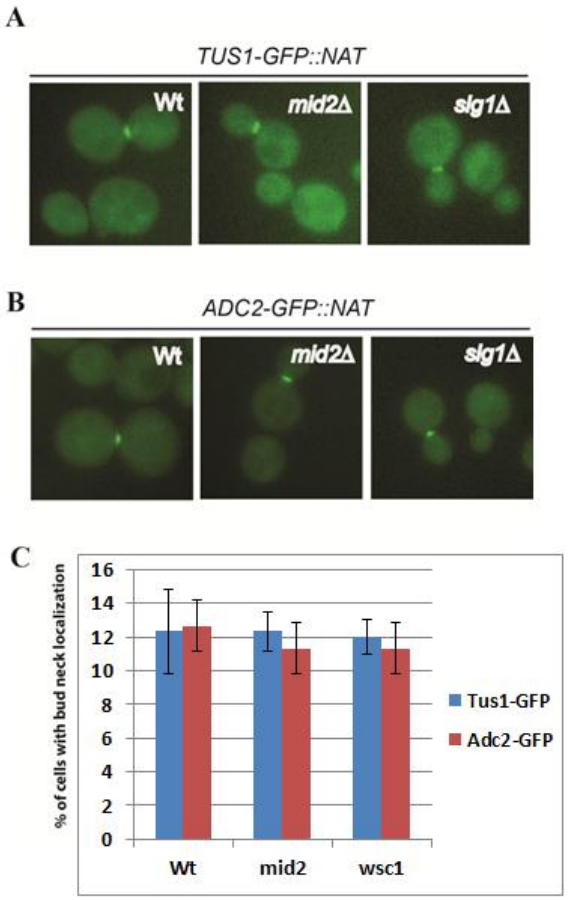

Figure 3.2. Adc2 and Tus1 localization are not affected in the *mid2*Δ and *slg1*Δ strains

(A-B) Tus1 and Adc2 were separately tagged with GFP under endogenous locus in in the wild type and the *mid2*Δ and *slg1*Δ strains in the BY4741 background. Slg1 is the alias name of Wsc1. Cells were grown until midlog phase prior to visualization under a fluorescence microscope **(C)** A total of 200 cells of each strain were counted. The fraction of cells displaying bud neck localization was calculated.

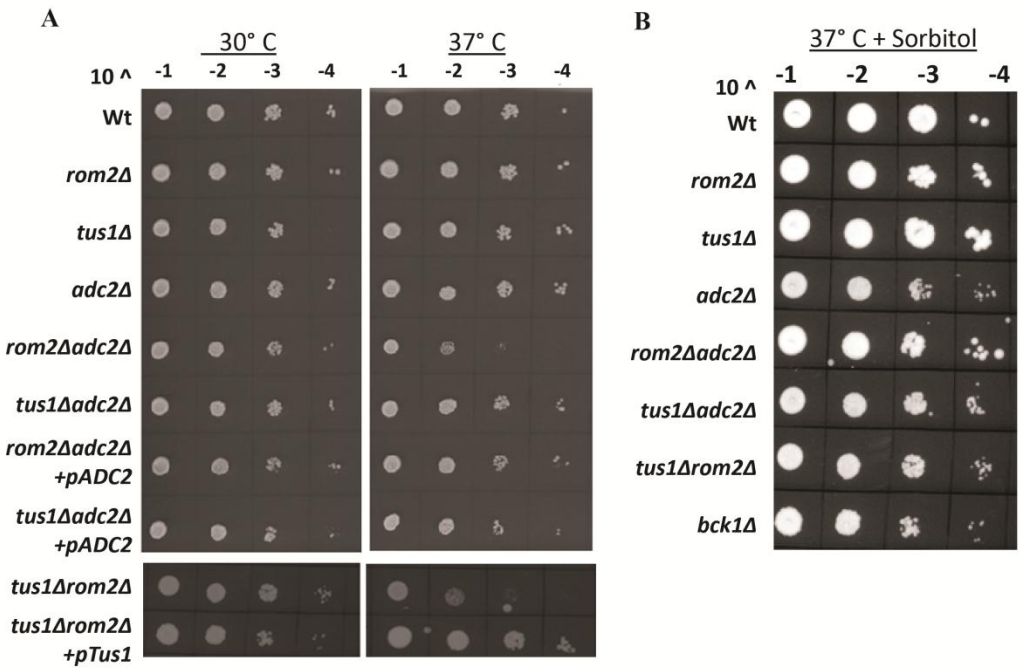

Figure 3.3. Adc2 is functionally associated with Tus1 in maintaining cell wall integrity

(A) A growth assay was used to evaluate mutant strains at elevated temperature. The wild type and mutant strains were grown overnight in SD-URA medium. Series of dilutions from 0.3 O.D to 0.00003 OD of each strain culture were generated. 1µl of each dilution was dotted on SD-URA plates, which were incubated at 30°C and 37°C. **(B)** Mutant strains were also dotted on SD-URA + sorbitol plates. Sorbitol is a cell wall stabilizing agent that prevents cell lysis due to weakened cell wall. All mutants were able to grow on SD-URA +sorbitol at elevated temperature.

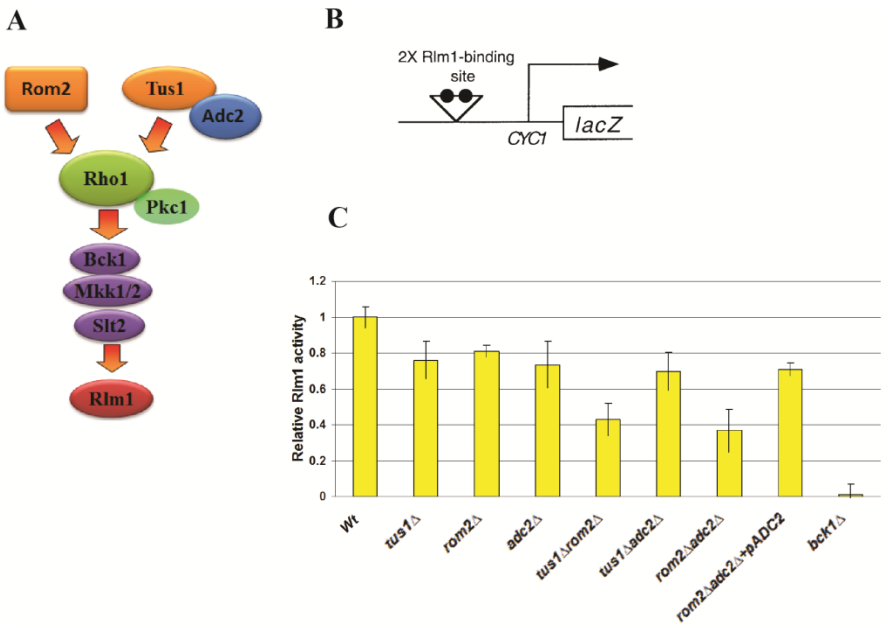

Figure 3.3. In parallel to Rom2, Adc2 contributes to Rlm1 activity via Tus1.

(A) Schematic of Rho1 signalling propagation. Rho1 activates Rlm1 via the Pkc1-MAPK mediated pathway. (B) Schematic of the reporter plasmid. A 2x Rlm1-binding site was inserted at the promoter region, placing *lacZ* expression under Rlm1 regulation. (C) Overnight culture of each strain was re-inoculated into fresh SD-URA-LEU+40CW µg/ml medium. Cells were grown to 1 O.D under CW induction at 30 °C. Cells were harvested and treated with the substrate ONPG. The intensity of the yellow color was measured at wavelength 420 by a standard spectrometer. The average Rlm1 activity of each strain was normalized to the wild type.

3.5 Materials and methods

Construction of plasmids and yeast strains

The *MATα* yeast strain BY4742 and its gene deletion derivatives were purchased from Open Biosystems (Huntsville, AL). Gene knockout and epitope tagging of genomic ORFs were generated using high-efficiency transformation of a PCR product flanked with 50-55bp of homology to the region of interest. Plasmid construction was based on homologous recombination after co-transforming yeast with linearized plasmid and DNA fragments. Recombinant plasmids were recovered in *E. Coli.* Yeast strains and plasmids used for this study are listed in the appendix (Table B1).

Fluorescence microscopy

GFP-tagged proteins were verified by western blotting using mouse αGFP antibodies (1:1000 Roche). Yeast cells expressing GFP-tagged proteins and plasmids carrying RFP-Tub1were grown to log phase in minimal selective media lacking uracil at 30°C. 1ml of the cells was harvested through gentle spinning for 1min and 900µl of the supernatant was discarded. Cell pellet was re-suspended in 100µl remaining media and 2µl was spotted onto glass cover slides. Cells were viewed using a 100x oil-immersion objective on a Zeiss Axioplan2 fluorescence microscope. Exposure time varied from 300ms-2000ms. Images were captured with a CoolSnap camera using MetaMorph software and adjusted using Adobe Photoshop. Cell counting was carried out using MetaMorph counting tools. Error analysis was carried out using average values obtained from 3 separate trials.

Spot assay for yeast growth

Equal OD_{600} of different cell strains collected from 30°C overnight cultures were used to make 10-fold serial dilutions over a 1000-fold range. 2µl of each dilution was spotted onto appropriate plates and incubated at 30°C and 37°C overnight. Colony growth was scanned using an Epson 2400 flat-bed scanner.

Rlm1 activity assay

Equal OD_{600} of different cell strains collected from 30°C overnight cultures were re-inoculated in appropriate fresh minimal media containing 40µg/ml CW at 30°C. Cells grown to mid-logged

phase were harvested and re-suspended in 1 ml of Z buffer (60mM Na_2HPO_4 $7H_2O$, 40mM NaH_2PO_4 H_2O, 10mM KCl, 1mM $MgSO_4$ $7H_2O$, pH7). 15 µl of chloroform and 40 µl of 0.1% SDS were added to the Z buffer and vortexed at top speed for 15 seconds. Samples were pre-incubated at 30°C for 5 min. Reaction was started by adding 0.2ml of o-nitrophenyl-β-D-galactoside (4mg/ml) and carried out for 30 seconds. 0.5ml of Na_2CO_3 was added to stop the reaction. The reaction mixture was centrifuged and the supernatant was measured at λ420 using an ultrospec 3000 UV/visible spectrophotometer (Pharmacia Biotech). Three replicates of each strain were tested during each trial. Three trials were performed on different days.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

4.1 Overview

The overall goal of this work was to understand the requirement for signaling specificity of Rho1, a yeast homolog of mammalian RhoA. Since Rho1 is involved in a wide range of pathways, it was not possible to systematically study the entire spectrum of Rho1 signaling. Instead, we focused on one aspect of Rho1 signaling in the CWI pathway, a well characterized Rho1 signaling pathway that is responsible for cell wall biogenesis under extracellular stress in yeast. A sensitive and quantitative fluorescence screen was previously conducted to measure surface chitin level in non-essential knockout collections, hoping to identify novel regulators of Rho1 signaling in regulating cell wall biogenesis. Bioinformatic approaches were used to functionally categorize top candidates, which included known Rho1 regulators and effectors, as well as putative ORFs. Through this work, *ADC2*, one of the putative ORFs identified from the screen, was predicted to be a Tus1 specific accessory protein that contributes to Rho1 signaling specificity in yeast. A model that describes the functional role of Adc2 in contributing Rho1 signaling through the CWI pathway is shown in Figure 4.1

Chapter 2 described the characterization of the putative ORF *ADC2* in yeast. A small scale fluorescence assay was first carried out to validate the genome wide screen. Consistent with the genome data, known regulators of Rho1 in the CWI pathway and Adc2 contributed to chitin synthesis on the cell surface. Next, we examined the physical interaction of Adc2 and its predicted binding partners. The results showed that Adc2 specifically binds to Tus1, but not Rom2. Both Rom2 and Tus1 are the GEFs that activate Rho1 signaling in the CWI pathway. Furthermore, the CNH domain of Tus1 was found to be necessary and sufficient for Tus1-Adc2 interaction. Like Tus1, Rho1 and Pkc1, Adc2-GFP was localized at the bud neck in a cell cycle dependent manner. This study also revealed that Tus1 is necessary for the localization of Adc2 at the bud neck.

Chapter 3 focused on the functional roles of Adc2 in Rho1 signaling. Although Tus1 is necessary for Adc2 localization, Adc2 contributes to Tus1 localization as well. Since Tus1 physically recruits and activates Rho1, disruption in Tus1 localization caused by loss of *ADC2* could affect Rho1 signaling. Sequence analysis of *ADC2* and its homologs across species revealed that Adc2 is an alpha-arrestin like protein that contains the arrestin N and C domains and a PY like motif (PPxxY) at the C terminus. Site directed mutagenesis of the PPxxY sequence

indicated that it is functionally relevant to Adc2's role in Tus1 localization at the bud neck. Genetic interaction studies further confirmed that Adc2 contributes to Rho1 signaling through Tus1, but not Rom2. The *rom2*Δ *adc2*Δ strain had compromised Rho1 signaling similar to the *rom2*Δ *tus1*Δ strain, while the *tus1*Δ *adc2*Δ strain behaved similarly to the single *tus1*Δ and *adc2*Δ mutants. While Tus1 and Rom2 are known to have complementary functions, our results indicated that Adc2 contribute to Tus1 mediated Rho1 signaling complementary to Rom2 in yeast.

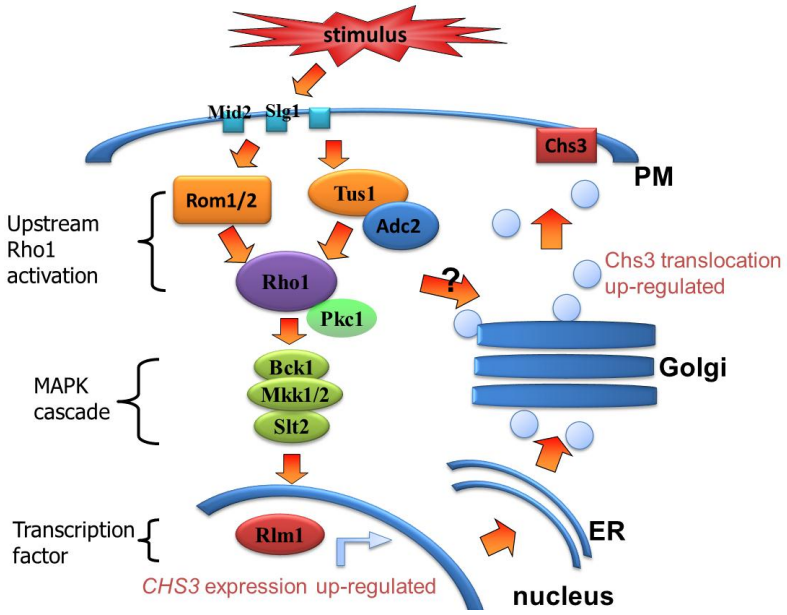

Fig 4.1 Rho1 mediated CWI pathway in yeast. Different cell wall stresses can be detected by a set of sensor proteins at the cell surface, and followed by the membrane localization of the RhoGEF Tus1 and Rom1/2. Adc2 is believed to facilitate the localization of Tus1, enabling it to recruit and activate Rho1. Active Rho1 next activates the Pkc1-MAPK-Rlm1 cascade which up-regulates the expression of cell wall biogenesis genes, including *CHS3*. The CWI pathway also plays a direct role in the rapid trafficking of Chs3 to cell surface through an unknown mechanism.

Collectively, our results show Rho1 signaling is dependent on additional proteins besides the RhoGEFs Tus1 and Rom2. Adc2 is identified as a novel regulator that contributes to Rho1 signaling through Tus1, but not Rom2. Although the exact molecular mechanism of Adc2 regulation in Rho1 signaling is not clear, our results are consistent with the view that Rho1

signaling specificity is not merely achieved by the GEFs but could be a product of coordinated regulation between the GEFs and additional accessory proteins in yeast. We have shown that Adc2 is a Tus1-specific protein that contributes to Rho1 signaling, yet there are many new questions waiting to be answered. How does Adc2 contribute to Rho1 signaling through Tus1? Is it merely through the regulation of Tus1 localization or is there something more? Do other alpha-arrestins function in Rho1 signaling? Lastly, is Adc2 functionally associated with Tus1 in all Rho1 signaling pathways? These questions will be discussed in this chapter.

4.2. Adc2 contributes to Rho1 signaling through Tus1

4.2.1 The importance of Tus1 localization in Rho1 signaling

There are two general functions of the Rho GEFs that collectively contribute to proper Rho signaling in cells. First is that the Rho GEFs are localized to sites of polarized growth where they directly bind and recruit Rho GTPases. Second is that they facilitate the association of GTP with Rho GTPases at sites of polarized growth. Mutations in the catalytic site of the GEFs render them incapable of activating Rho. However, cells with active but mis-localized Rho1 are not viable (Yoshida et al., 2006). Therefore, the proper localization of Tus1 and Rom2 is crucial for regulating Rho1 signaling in cells.

Although Rom2 and Tus1 both contain DH/PH domains, they are localized to sites of polarized growth through distinct mechanisms (Levin, 2011). Rom2 localization is dependent on its association with the PIP_2 and the cell wall receptors (Philip & Levin, 2001; Yu et al., 2004). However the exact mechanism for Tus1 localization is not well understood. Recent studies have identified two binding partners of Tus1: Slm1 and Sho1 (Ho et al., 2008a; Tonikian et al., 2009b). Slm1 is a lipid binding protein that specifically targets phosphoinositide PIP_2. Sho1 is a transmembrane osmosensor involved in the high osmolarity glycerol (HOG) pathway in yeast. The lipid association properties of Slm1 and Sho1 could contribute to Tus1 localization at sites of polarized growth.

The CNH domain of Tus1 is a likely binding site for protein interactions. Our Y2H data suggested that the CNH domain is necessary and sufficient for Adc2 interaction. To test whether the Y2H interactions were affected by variations in the "prey" and "bait" level in cells, we examined protein levels of GBD-Adc2 full length and GAD-Tus1 full length and truncation mutants. Western blotting using GBD specific antibodies showed that the level of GBD-Adc2

was similar to GBD only in cells. However, we could not evaluate the protein level of GAD-Tus1 full length and truncation mutants by western blotting due to lack of GAD specific antibodies. Therefore, we could not determine whether the GAD-Tus1 mutant with the CNH truncation was properly expressed in cells, which could affect Adc2 interaction. Currently, we are testing other GAD specific antibodies to look at the protein level of Tus1 truncation mutants.

4.2.2 How does Adc2 contribute to Tus1 localization?

While the exact mechanism of Tus1 localization is not understood, it is known that Tus1 localization at the bud neck during CAR assembly requires Cdc5, a polo-like cell cycle dependent kinase. Cdc5 binds to the two Polo binding motifs (Ser/Ser, Thr/Pro) in the N terminus of Tus1. Upon binding, Cdc5 phosphorylates the N terminal consensus sites of Tus1. Tus1 in a *cdc5 ts* conditional mutant strain and a Tus1-ST mutant incapable of binding Cdc5 are both not localized to the bud neck (Yoshida et al., 2006). As expected, Rho1 localization at the bud neck is also completely gone in the *cdc5* mutant strain. While it is not clear if or how Adc2 is involved in Tus1 localization during CAR assembly, we present two hypotheses based on our physical interaction and microscopy findings.

One hypothesis is that Adc2 contributes to the phosphorylation of Tus1 through CNH binding to make the phosphorylation sites more accessible for Cdc5 (Fig 4.2). Autoinhibition is one of the common regulatory mechanisms exhibited by Rho GEFs in mammalian cells. The inactive GEFs can mask active sites through intramolecular binding with their own domains. It has been reported that the N terminal domain binds to the DH domain in some GEFs to prevent interaction with Rho GTPases. While the function of the CNH domain in Tus1 and Rom2 is not known, it is plausible that it may be involved in autoinhibitory mechanisms. Assuming that the N terminal domain binds to the DH domain to exert an autoinhibitory regulation of Tus1, the binding of Adc2 at the CNH domain may cause conformational changes that relieve Tus1 from the autoinhibitory state. The phosphorylation sites in Tus1 associated with Adc2 would therefore become more accessible to Cdc5. In essence, Adc2 binds to Tus1 to facilitate the phosphorylation and subsequent localization of Tus1. This would explain why Tus1 is necessary for Adc2 localization, since Adc2 is bound to the CNH domain of Tus1, getting a “free ride” while Tus1 is phosphorylated and localized to the bud neck. This would also explain why the deletion of *ADC2* affected Tus1 localization, because the CNH domain cannot relieve Tus1 from

its inactive state. To test the importance of Adc2 to Tus1 phosphorylation, we could compare the motility shift of Tus1 caused by Cdc5 phosphorylation in the wild type and the *adc2*Δ strain. A full length Tus1 and Tus1Δ_{CNH} should also be included to detect the difference in the phosphorylation level.

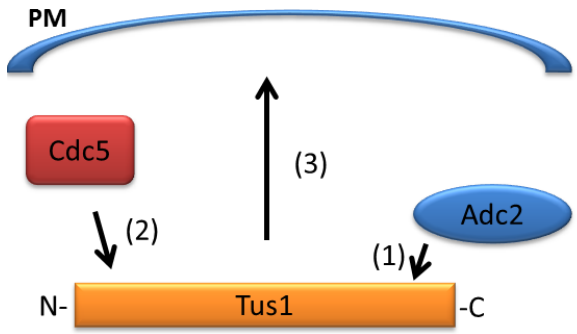

Fig 4.2 Hypothesis I: Adc2 contributes to Tus1 phosphorylation, which leads to Tus1 localization. (1) Adc2 is initially bound to the CNH domain, which could relieve Tus1 from an autoinhibitory interaction. The binding renders Tus1 to be more susceptible to Cdc5 phosphorylation **(2)** Cdc5 phosphorylates the N terminus of Tus1, which is prerequisite for Tus1 localization at the bud neck during CAR assembly. **(3)** Phosphorylated Tus1carrying Adc2 is recruited to the sites of polarized growth. The mechanism of this pathway is not well understood.

A second hypothesis is that Adc2 is not involved in the phosphorylation of Tus1, and only contributes to Tus1 localization stability once it is localized to the bud neck (Fig 4.3). Tus1 recruits Adc2 to the bud neck through its CNH domain. It is unclear at this time if Adc2 recruitment occurs prior to, during, or after Rho1 recruitment to the bud neck. However, it is clear that Adc2 is important for the stability of Tus1 localization, as Tus1 is partially mis-localized in an *adc2*Δ deletion strain. Since no interaction has been identified between Tus1 and membrane proteins, Adc2 may act as a scaffold that bridges Tus1 with cell wall receptors. Lipid association through Adc2 could strengthen Tus1 localization and facilitate Rho1 signaling. However, we have yet to identify potential membrane proteins, as the localization of Tus1 and Adc2 were not affected by deletion of *WSC1* or *MID2*, both of which contribute to Rom2 membrane association. If Adc2 is indeed a scaffold protein, one of the proteins it may interact with is Rsp5, an E3 ligase in yeast. Nine out of ten alpha arrestins in yeast have been found to

interact with Rsp5, a major E3 ligase involved in signaling. PY motifs are commonly found in alpha-arrestins and bind to the WW domain in E3 ligase. We identified a non-canonical PY motif (PPxxY) in the C terminus of Adc2.

Our microscopy data found that the mis-localization of Tus1 in the *adc2*Δ strain was rescued by a complementation plasmid carrying full length *ADC2*, but not $ADC2_{AAxxA}$ with the PY motif mutations. This indicated that the PPxxY sequence in Adc2 is functionally important for the localization of Tus1. Rsp5 could contribute to Tus1 localization through Adc2. To test this hypothesis, we could compare the localization of Tus1 in the wild type strain, the *rsp5* ts conditional mutant and the *adc2*Δ deletion strain. If Rsp5 is involved in Tus1 localization, we should see mis-localization of Tus1 in an *rsp5* mutant. However, the PPxxY motif of Adc2 may simply be part of region that is responsible for Tus1 interaction. To test this, we could use the Y2H assay to compare the interactions between Tus1-Adc2 and Tus1-$Adc2_{AAxxA}$. If a Tus1-$Adc2_{AAxxA}$ interaction is not observed, it would be important to epitope tag the $Adc2_{AAxxA}$ mutant and evaluate its protein level in cells. There is a possibility that $ADC2_{AAxxA}$ is not expressed or expressed at a lower level. As a result, there is not enough $Adc2_{AAxxA.}$ to fully restore Tus1 localization in the *adc2*Δ strain.

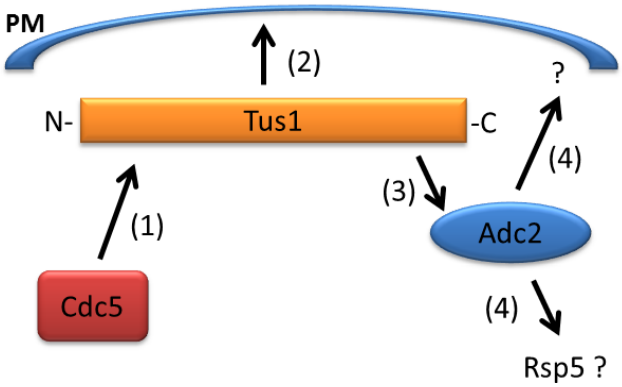

Fig 4.3 Hypothesis II: Adc2 contributes to Tus1 localization after Cdc5 phosphorylation. **(1)** Cdc5 initially binds to and phosphorylates the N terminus of Tus1. **(2)** Tus1 is phosphorylated and recruited to the plasma membrane through an unknown molecular mechanism. **(3)** Tus1 recruits Adc2 to the plasma membrane as well. **(4)** Adc2 may act as a scaffold protein that recruits additional proteins for the stability of Tus1.

Overall, both hypotheses emphasize the significance of the Adc2 interaction with the Tus1 CNH domain. Either way, Adc2 is important for the localization of Tus1. Loss of *ADC2* leads to the mis-localization of Tus1, which can no longer recruit Rho1 to sites of polarized growth. While Rom2 can regulate Rho1 localization in overlapping pathways with Tus1, specific Tus1 mediated Rho1 signaling pathways will not be complemented by Rom2. Therefore, Adc2 is functionally associated with Tus1 and contributes to Tus1 mediated Rho1 signaling pathways. Our studies found that Tus1Δ_{CNH}-GFP with a CNH domain truncation is expressed but no longer localizes to the bud neck. This is the first time to our knowledge that the CNH domain has been shown to be important for Tus1 localization. Future studies are needed to address the regulation of Tus1 localization in yeast. While Tus1 localization may be one important function of Adc2, we will further discuss the other possibilities by which Adc2 could contribute to Rho1 signaling in later sections.

4.2.3 Identifying Adc2 domains important for Tus1 interaction

Tus1 and Rom2 share overlapping functions and high sequence homology in yeast. Both have a DH domain, a PH domain and a CNH domain. It is therefore interesting that RhoGEF specific accessory proteins were identified. We wonder what makes Adc2 specific for Tus1 but not Rom2. Adc2 domains important for Tus1 interaction could be identified using the yeast 2 hybrid assay. Sequence comparison with Adc2 homologs in other species has revealed that Adc2 contains arrestin N and C domains. The N and C domain of alpha arrestins in mammalian cells are implicated in substrate specificity. Using sub-cloning methods, we could systematically truncate the arrestin N domain and other regions in Adc2 to see if the Tus1 interaction is affected in the Y2H assay. By identifying specific interacting regions in the proteins, we can better understand the interaction between Adc2 and Tus1.

4.2.4 How does Adc2 contribute to Rho1 signaling in the CWI pathway?

Besides contributing to Tus1 localization, Adc2 may also regulate the function of Tus1 by facilitating the formation of a Tus1-Rho1-Pkc1 complex. The Tus1 DH domain binds Rho1 at the plasma membrane, where Rho1 physically interacts with and activates Pkc1. The CNH domain of Tus1 may enhance the Rho1-Pkc1 interaction through Adc2 interaction. To test this hypothesis, we could use a GST-RBD pull-down assay to investigate the role of Adc2 in Pkc1-

Rho1 and Pkc1-Tus1 interactions. RBD is the Rho1 binding domain that is present in Pkc1. We are interested to see how much active Rho1 and Tus1 are pulled down by GST-RBD in the wild-type and the *adc2*Δ strain. Similar pull-down assays have been previously performed to look at the temporal regulation of Rho1 (Kono, 2008). If Adc2 plays a role in Tus1-Rho1-Pkc1 complex formation and stability, we would expect to see a lower amount of active-Rho1 and Tus1 pulled down by Pkc1 in the *adc2*Δ mutant compared to the wild-type.

Adc2 specifically contributes to Tus1 mediated regulation of Rho1 signaling in yeast. If Tus1 and Rom2 have complementary roles in Rho1 regulation, and if Adc2 is required for Tus1 function, the double mutant *adc2*Δ *rom2*Δ should behave like the *rom2*Δ *tus1*Δ double mutant. While the results of the growth assay and Rlm1 activity assay are consistent with this hypothesis, we wanted to have one more assay to reaffirm our findings. We therefore looked at Slt2 to evaluate Rho1 signaling mediated by Adc2, Tus1 and Rom2. Slt2 is the upstream activator of Rlm1. It is activated through dual phosphorylation by Mkk1/2, which is phosphorylated by Bck1. Bck1 is the downstream target of Rho1- Pkc1. We hypothesized that the level of phosphorylated Slt2 would be lowered in the double mutant *rom2*Δ *adc2*Δ, which would explain the reduced activity of Rlm1 in the *rom2*Δ *adc2*Δ strain. The wild type cells and the double mutant cells were grown and induced in YPD + 40µg/ml CW prior to western blotting using anti-Slt2p antibodies that specifically recognize the dual phosphorylated Slt2. Surprisingly, preliminary data has shown an increase in dual phosphorylation of Slt2 in the *rom2*Δ *adc2*Δ strain. This suggested that the activity of Rlm1 is not solely related to the level of dual phosphorylation of Slt2.

The unexpected finding prompted us to further look into the role of phosphorylated Slt2. Rho1 signaling in the CWI pathway regulates two transcriptional programs through Slt2: Rlm1 and Swi4/6. Rlm1 regulates the expression of many cell wall biogenesis genes, including *CHS3.* Swi4/6 regulates the expression of *FKS2* under chronic cell wall stress (Jung and Levin, 1999). Both Rlm1 and Swi4/6 can be activated by dual phosphorylated Slt2. However Rlm1 activation also requires dual-phosphorylated Slt2 to be catalytically active. Since high level of dual phosphorylated Slt2 was observed in the *rom2*Δ *adc2*Δ strain, we ask whether Slt2 is catalytically inactive, which should still be able to activate Swi4/6 transcription factors. To do this, a β-gal assay similar to the one we conducted for Rlm1 activity could be carried out. A *lacZ* reporter plasmid can be modified to include a Swi4/6 binding region in the promoter, which

places the *lacZ* gene under the regulation of Swi4/6. We expect that the activity of Swi4/6 to be up-regulated and is independent of Tus1 Rom2 mediated Rho1 signaling. With these experiments, it could provide further insight into the role of Adc2 in the CWI pathway.

4.2.5 Is Ipp1 important for Tus1 signaling?

Ipp1 was predicted to be part of Tus1 signaling complex with Adc2 (Babu et al. 2012). Our Co-IP results confirmed the interaction between Ipp1 and Tus1, as well as Ipp1 and Adc2. Furthermore, Ipp1 interacted with Rom2 as well. Ipp1 is a cytoplasmic inorganic pyrophosphatase that catalyzes the conversion of pyrophosphate into two phosphate ions. This reaction is exergonic and is often used to drive an unfavorable biochemical reaction to completion (Terkeltaub, 2012). Ipp1 is an essential protein that has been implicated in lipid metabolism, including lipid synthesis and degradation. The lipid aspect of Ipp1 may suggest that it plays a role in the lipid association of Tus1 and Rom2. However, whatever role it may have is likely not specific to Tus1 or Rom2, since Ipp1 physically interacts with both GEFs. For this reason, we did not consider Ipp1 important for Rho1 signaling specificity mediated by Tus1 and Rom2.

However, it is still important to examine the role Ipp1 in Rho1 signaling. *IPP1* is an essential gene and was not included in the original genome-wide screen that looked for regulators of Rho1 signaling in the non-essential knockout collections. Now that we have the temperature sensitive *ipp1* mutant strain, we can look at Tus1/Rom2 localization and Rlm1 activity in future work.

4.2.6 Is Adc2 functionally associated with Tus1 in all Rho1 pathways?

Besides the CWI pathway, Tus1-mediated Rho1 activation can regulate other pathways in yeast (Paumi, 2007, Yoshida, 2009, Ho, 2008). We wonder if Adc2 is associated with Tus1 in other Tus1-Rho1 pathways. One of the binding partners of Tus1 outside the CWI pathway is Ycf1. Ycf1 is a vacuolar membrane transporter that detoxifies heavy metals by transporting them into the vacuole for further metabolism. The integrated split-ubiquitin membrane yeast two-hybrid (iMYTH) technique found that Ycf1 physically interacts with the CNH domain of Tus1. To determine if Adc2 is required for all Tus1 functions, we could use the iMYTH technique to determine if Adc2 is necessary for the Ycf1-Tus1 interaction and if it also interacts with Ycf1.

The wild-type and the *adc2*Δ strain could be tested for the Tus1-Ycf1 interaction. If Adc2 is important for the interaction, the *adc2*Δ mutant would display growth defects on the selection plate. We could also use the iMYTH to detect a potential Ycf1-Adc2 interaction as well.

The Tus-Rho1 signaling cascade is a potential downstream effector of TORC2 in yeast (Ho et al., 2008b). TORC2, one of the TOR complexes, regulates actin organization and cell integrity. Co-immunoprecipitation revealed a physical interaction between Tus1 and Slm1, one of the effectors of TORC2. The *avo3-2* mutant displayed growth defects at high temperature, which can be suppressed by *SLM1* over-expression in a Tus1 dependent fashion. We could use over-expression studies and double KO mutants to determine whether Adc2 is involved with the Slm1-Tus1 association. We could ask if over-expressing *SLM1* could suppress the growth defects of the *avo3-2* in the *adc2*Δ strain at high temperature. These experiments would elucidate whether Adc2 is functionally associated with Tus1 beyond the CWI pathway.

4.2.7 Future perspectives

The main theme of our study is that Rho signaling is a complex process that is regulated by the RhoGEFs and additional factors. Our findings highlight the significance of different regulators and effectors in achieving Rho signaling specificity. Instead of directly regulating Rho, cells can regulate the RhoGEFs to induce or down-regulate Rho signaling. Studying the importance of RhoGEF regulation and the functions of their specific factors in yeast can be further applied to understanding Rho signaling in mammalian cells. Rho signaling in humans has been implicated in numerous diseases, including cancer and neurodegenerative disorders. Identifying Rho1 regulators and effectors is important for pharmaceutical drug development that specifically targets individual Rho mediated pathways. However, future work is needed to understand the general theme of achieving specificity of Rho signaling.

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APPENDIX A: Supplemental material for chapter 2

Table A1. Plasmids and Yeast Strains used in this study

Type	Name	Description/Genotype	Source	Notes
Plasmid	pHW202	TUS1 930-1307 in pGAD vector[a]	This study	Y2H assay
Plasmid	pHW203	TUS1 425-721 in pGAD vector	This study	Y2H assay
Plasmid	pHW204	TUS1 1-930 in pGAD vector	This study	Y2H assay
Plasmid	pHW205	TUS1 420-1307 in pGAD vector	This study	Y2H assay
Plasmid	pHW199	ROM2 1040-1356 in pGAD vector	This study	Y2H assay
Plasmid	pHW65	TUS1 full length in pGAD vector	This study	Y2H assay
Plasmid	pLC907	pGAD-c2 vector	This study	Y2H assay
Plasmid	pHW67	ADC2 full length in pGBD vector[b]	This study	Y2H assay
Plasmid	pHW196	ADC2 1-172 in pGBD vector	This study	Y2H assay
Plasmid	pHW197	ADC2 1-193 in pGBD vector	This study	Y2H assay
Plasmid	pLC906	pGBDU-C2 vector	This study	Y2H assay
Plasmid	pHW219	pTUS1 (CEN,URA) MOBY ORF	C.Boone	microscopy
Yeast strain	LC861	*PJ694a MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	This study	Y2H assay
Yeast strain	LC862	*PJ694a MATα trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	This study	Y2H assay

[a.b.] James, P., Halladay, J. & Craig, E. A. (1996) Genomic libraries and a host strain designed for highly efficient two- hybrid selection in yeast. Genetics 144: 1425-1436

Type	Name	Description/Genotype	Source	Notes
Yeast strain	LC3107	BY4742 *Mat α his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study	
Yeast strain	HWY81	BY4742 ADC2-GFP::NatR	This study	microscopy
Yeast strain	HWY82	BY4742 ADC2-GFP:NatR *tus1*Δ*::*KanR	This study	microscopy
Yeast strain	HWY83	BY4742 ADC2-GFP:NatR *rom2*Δ*::*KanR	This study	microscopy
Yeast strain	HWY119	BY4742 ADC2-GFP:NatR *tus1*Δ*::*KanR *pTUS1*	This study	microscopy
Yeast strain	HWY182	BY4742 ADC2-GFP:NatR pRFP-TUB1	This study	microscopy
Yeast strain	HWY184	BY4742 TUS1-GFP:NatR pRFP-TUB1	This study	microscopy
Yeast strain	HWY48	BY4742 ADC2-HA::KanR	This study	Co-IP
Yeast strain	HWY56	BY4742 ADC2-HA::KanR TUS1-MYC::HIS	This study	Co-IP
Yeast strain	HWY60	BY4742 ROM2-MYC::HIS	This study	Co-IP
Yeast strain	HWY62	BY4742 ADC2-HA::KanR ROM2-MYC::HIS	This study	Co-IP
Yeast strain	HWY166	BY4742 ADC2-HA::KanR ROM2-MYC::HIS *tus1*Δ::NatR	This study	Co-IP
Yeast strain	HWY168	BY4742 ADC2-HA::KanR IPP1-MYC::HIS	This study	Co-IP

Type	Name	Description/Genotype	Source	Notes
Yeast strain	HWY172	BY4742 ROM2-MYC::HIS IPP1-HA::KanR	This study	Co-IP
Yeast strain	HWY87	BY4742 ACK1-HA::KanR ROM2-MYC::HIS	This study	Co-IP
Yeast strain	HWY88	BY4742 ACK1-HA::KanR TUS1-MYC::HIS	This study	Co-IP
Yeast strain	HWY170	BY4742 IPP1-MYC::HIS	This study	Co-IP
Yeast strain	HWY200	BY4742 IPP1-HA::KanR TUS1-MYC::HIS *adc2*Δ::NatR	This study	Co-IP
Yeast strain	HWY173	BY4742 TUS1-MYC::HIS IPP1-HA::KanR	This study	Co-IP
Yeast strain	HWY 72	BY4742 ACK1-MYC::HIS	This study	Co-IP
Yeast strain	HWY189	BY4742 TUS1Δ_{CNH}-MYC::HIS ADC2-GFP::NatR	This study	Co-IP
Yeast strain	HWY4	BY4742 *chs7*Δ::KanR	This study	CW assay
Yeast strain	HWY9	BY4742 *rom2*Δ::KanR	This study	CW assay
Yeast strain	HWY11	BY4742 *bck1*Δ::KanR	This study	CW assay
Yeast strain	HWY14	BY4742 *tus1*Δ::KanR	This study	CW assay
Yeast strain	HWY17	BY4742 *pfa4*Δ::KanR	This study	CW assay

Type	Name	Description/Genotype	Source	Notes
Yeast strain	HWY10	BY4742 *slt2*Δ::KanR	This study	CW assay
Yeast strain	HWY85	BY4742 *adc2*Δ::KanR	This study	CW assay
Yeast strain	HWY5	BY4742 *ykl037w*Δ::KanR	This study	CW assay
Yeast strain	HWY2	BY4742 *vps8*Δ::KanR	This study	CW assay
Yeast strain	HWY7	BY4742 *pef1*Δ::KanR	This study	CW assay

APPENDIX B: Supplemental material for chapter 3

Table B1. Plasmids and Yeast Strains used in this study

Type	Name	Description/Genotype	Source	Notes
Plasmid	pHW219	pTUS1 (CEN,URA) MOBY ORF	C.Boone	Spot assay
Plasmid	pHW105	p5586 (empty vector, CEN,URA) MOBY ORF	C.Boone	Spot assay
Plasmid	pLC698	pRS416 (CEN,URA)	This study	
Plasmid	pHW130	pRS416-ADC2$_{AAXXA}$ (CEN,URA)	This study	microscopy
Plasmid	pHW93	pRS415-ADC2 (CEN,LEU)	This study	microscopy
Plasmid	pHW94	pRS416-ADC2 (CEN,URA)	This study	microscopy
Plasmid	pHW201	pRFP-TUB1 (CEN,URA)	V.Measday	microscopy
Plasmid	pHW79	p*lacZ* (2u, URA, containing 2x Rlm1 binding site)[a]	D.Levin	Rlm1 assay
Plasmid	pHW80	p*lacZ* (2u, URA,)[b]	D.Levin	Rlm1 assay
Plasmid	pHW131	p*lacZ* (2u, LEU, containing 2x Rlm1 binding site) –marker swapped with pHW79	This study	Rlm1 assay
Yeast strain	LC3107	BY4742 *Mat α his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study	
Yeast strain	HWY178	BY4742 TUS1-GFP::NatR	This study	microscopy

[a.b] Jung, U. S., Sobering, A. K., Romeo, M. J., & Levin, D. E. (2002). Regulation of the yeast Rlm1 transcription factor by the Mpk1 cell wall integrity MAP kinase. *Molecular microbiology*, *46*(3), 781–9

Type	Name	Description/Genotype	Source	Notes
Yeast strain	HWY89	BY4742 TUS1-GFP::NatR *adc2*Δ*::*KanR	This study	microscopy
Yeast strain	HWY95	BY4742 TUS1-GFP::NatR *adc2*Δ*::*KanR	This study	microscopy
Yeast strain	HWY180	BY4742 TUS1-GFP::NatR *adc2*Δ*::*KanR pADC2 (pHW93)pTUB1(pHW201)	This study	microscopy
Yeast strain	HWY183	BY4742 TUS1-GFP::NatR *adc2*Δ*::*KanR pTUB1(pHW201)	This study	microscopy
Yeast strain	HWY184	BY4742 TUS1-GFP::NatR pTUB1(pHW201)	This study	microscopy
Yeast strain	HWY190	BY4742 TUS1-GFP::NatR *mid2*Δ*::*KanR	This study	microscopy
Yeast strain	HWY192	BY4742 TUS1-GFP::NatR *wsc12*Δ*::*KanR	This study	microscopy
Yeast strain	HWY179	BY4742 pTUB1 (pHW201)	This study	microscopy
Yeast strain	HWY181	BY4742 ADC2-GFP::NatR *tus1*Δ*::*KanR pTUB1(pHW201)	This study	microscopy
Yeast strain	HWY182	BY4742 ADC2-GFP::NatR pTUB1(pHW201)	This study	microscopy
Yeast strain	HWY107	BY4742 p5586 (pHW105)	This study	Spot assay
Yeast strain	HWY108	BY4742 *adc2*Δ::KanR p5586 (pHW105)	This study	Spot assay
Yeast strain	HWY110	BY4742 *tus1*Δ::KanR p5586 (pHW105)	This study	Spot assay

Type	**Name**	**Description/Genotype**	**Source**	**Notes**
Yeast strain	HWY220	BY4742 *rom2*Δ::KanR p5586 (pHW105)	This study	Spot assay
Yeast strain	HWY221	BY4742 *bck1*Δ::KanR p5586 (pHW105)	This study	Spot assay
Yeast strain	HWY114	BY4742 *rom2*Δ::NatR *adc2*Δ*::*KanR p5586(pHW105)	This study	microscopy
Yeast strain	HWY162	BY4742 *rom2*Δ::NatR *tus1*Δ*::*KanR pTUS1(pHW219)	This study	microscopy
Yeast strain	HWY223	BY4742 *rom2*Δ::NatR *tus1*Δ*::*KanR p5586(pHW105)	This study	microscopy
Yeast strain	HWY117	BY4742 *tus1*Δ::NatR *adc2*Δ*::*KanR p5586(pHW105)	This study	microscopy
Yeast strain	HWY149	BY4742 pHW131+pRS416	This study	Rlm1 assay
Yeast strain	HWY150	BY4742 *rom2*Δ::NatR *adc2*Δ*::*KanR pHW131+pRS416	This study	Rlm1 assay
Yeast strain	HWY151	BY4742 *bck1*Δ*::*KanR pHW131+pRS416	This study	Rlm1 assay
Yeast strain	HWY155	BY4742 *rom2*Δ::NatR *adc2*Δ*::*KanR pHW131+pADC2(pHW94)	This study	Rlm1 assay
Yeast strain	HWY156	BY4742 *tus1*Δ*::*KanR pHW131+pRS416	This study	Rlm1 assay
Yeast strain	HWY157	BY4742 *adc2*Δ*::*KanR pHW131+pRS416	This study	Rlm1 assay
Yeast strain	Hwy158	BY4742 *tus1*Δ::NatR *adc2*Δ*::*KanR pHW131+pRS416	This study	Rlm1 assay

Yeast strain	HWY159	BY4742 *rom2*Δ*::*KanR pHW131+pRS416	This study	Rlm1 assay
Yeast strain	HWY164	BY4742 *rom2*Δ::NatR *tus1*Δ*::*KanR pHW131+pRS416	This study	Rlm1 assay