INVESTIGATING THE ASSOCIATION OF THE MYOSIN MOTOR MYO2P WITH ITS SECRETORY CARGO

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Cell polarity involves transport of specific membranes and macromolecules at the right time to the right place. In budding yeast, growth is highly asymmetric and requires a constant delivery of secretory cargo to growth sites. This polarized growth depends on an interplay between the cytoskeletal machinery responsible of directed transport and the secretory systems ensuring proper generation and fusion of membrane carriers. Almost all membrane movements are dependent on the myosin-V motor Myo2p, but how Myo2p recognizes and associates with secretory membranes, its only essential cargo, is currently unknown. Here I identify two *myo2* alleles that are sensitive to the levels of Golgi PI4P and of the Rab GTPases Sec4p and Ypt31p. I show that PI4P is enriched in the late secretory compartments and is critical for their association with, and transport by, Myo2p. Similarly, I found a direct interaction between the Myo2p tail and the Rabs Ypt31/32p and Sec4p, disruption of which correlates with uncoupling of the secretory cargo from Myo2p. Although the Myo2p tail failed to bind PI4P directly in our assays, enhancing Myo2p's association with PI4P by fusing it to a PH domain specific for this lipid rescues the PI4P-sensitive *myo2* alleles as well as a Rab-binding

deficient *myo2* mutant. I also present evidence that additional proteins can modulate the interaction between Myo2p and the Rabs, supporting a model where a multicomponent complex makes up the secretory vesicle receptor. Taken together, the data presented here support a model where the Rab GTPase Sec4p, and to a lesser extent Ypt31/32p, and the phosphoinositide PI4P facilitates the recruitment of Myo2p to secretory membranes, resulting in their polarized delivery to sites of growth.

BIOGRAPHICAL SKETCH

Felipe was born in the tiny Caribbean island of Puerto Rico during the waning days of the Generation X. He inherited his first last name Santiago from a microbiologist and his second surname Tirado from a registered nurse. Naturally, coming from a scientific family, at an early age he decided to save the world by becoming a scientist. He received early admission to the Industrial Biotechnology Program of the University of Puerto Rico at Mayagüez, where he was an active member of the Industrial Microbiology and Biotechnology Associations and of the Golden Key Honor Society. He graduated *Magna Cum Laude*, with extensive research and teaching experience that earned him several awards. Of special significance was the Department of Energy Undergraduate Research Fellowship (ERULF) he received that brought him to the Pacific Northwest National Laboratory for a whole spring semester. Under the guidance of Dr. Johnway Gao, he learned about batch fermentation, that people in a government National lab were normal, and that Cornell was the best grad school ever. Making his previous mentor very happy, he was accepted at Cornell in the BMCB Graduate Program, where he joined the laboratory of Dr. Anthony Bretscher, working on the regulation of membrane trafficking using budding yeast as a model system. Although away from his tiny Island, he quickly built another tinier one at Cornell, where he realized that doing a doctorate is not that easy. With the unconditional support of his second Island, he conquered the Ph.D. while preserving his optimism and good humor.

DEDICATORIA

A un pequeño grupo de habitantes en mi Islita… mi familia

Y a todos los habitantes de mi segunda Islita, en especial a su fundadora…

Annie.

DEDICATION

To a small group of inhabitants of my tiny Island… my family

And to all the inhabitants of my tinier Island, especially to its founder… Annie.

ACKNOWLEDGEMENTS

My family and friends know that I can be very wordy when I write, so as a joke I was going to simply say… thanks! However, I laugh and then felt bad as there are so many people without whom this work would have not been completed. For many, I probably never told them how important their friendship was, so I feel that I have to take this opportunity to do just that. Due to space constraints, and for the sanity of the few unlucky that have to read this work, I cannot mention all of them. I will mention, however, the ones that were most responsible for the successes in my graduate career here at Cornell.

Mrs. Colf, as I called Diane during my first months here, was 100% of the times I consulted her reliable, for every question there was an answer. All the past and current member of the Bretscher lab, for being so helpful and welcoming, and becoming a second family. To my committee members, Susan and Eric, for their support and suggestions during our yearly meetings. My gratitude also goes to members of the Henry lab, as they harbored me for a few months to learn and conduct experiments with ^{3}H-labeled phosphoinositides. Lastly but not least, my advisor, otherwise known as Tony, for his continuous guidance and help. Although been immensely busy, he always found time to talk to me. He is an enormous source of information, both technical and historical. I really enjoyed every time I went to his office to ask or show him something and ended sitting down to listen to “science culture” stories, or

showing him how to answer a call in his cellphone. His enthusiasm and sudden bursts of ideas were invaluable for this thesis. I probably have to also mention that I'm indebted to NIH, for awarding me a predoctoral fellowship that has supported me for some time now.

A billion thanks also to my friends Giomar and Abner, Alexis and Jeisa, Juan Camilo and Eli, Vero and Luis, Nelson, Karen, GABA, and all others that have share time with me here in Ithaca. I will miss going to the movies or to BBQs with you guys! Finally, a trillion thanks to God, for allowing me to achieve the most important accomplishment of my career… Annie.

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LIST OF ABBREVIATIONS

AD, *GAL4* activation domain

BD, *GAL4* DNA-binding domain

ccGTD, the region of Myo2p containing the coiled coil and the GTD

CDK, cyclin-dependent kinase

cER, cortical endoplasmic reticulum

CHX, cycloheximide

COG, conserved oligomeric Golgi complex

DRF, Diaphanous-related family

EM, electron microscopy

ERGIC, ER-Golgi Intermediate Compartment

GAP, GTPase-activating protein(s)

GARP, Golgi-associated retrograde proteins

GEF, guanine-nucleotide exchange factor(s)

GTD, the globular tail domain of Myo2p

MOS, hypothetical membrane or secreted protein

PI or PIs, phosphoinositides(s)

P_i, inorganic phosphate

RNPs, ribonucleoprotein particle(s)

SPB, spindle pole body

SR, SRP receptor

SRP, signal recognition particle

SUV, small unilamellar vesicles

TGN, trans-Golgi network

CHAPTER 1

INTRODUCTION TO YEAST'S CELL BIOLOGY AND POLARIZED MEMBRANE TRAFFIC

The dictionary's definition of polarity is '*the condition of having poles*', or in other words, *the possession or manifestation of different sides, attributes, ideas, or any other characteristic*. When we talk about *cell polarity*, we mean the highly organized condition of cells that allows them to carry out their function and be oriented in time and space. Note the different shapes of the four cells in Figure 1.1, each specialized to perform certain functions: the epithelial cells (A) lining the intestine selectively absorb materials from the apical side and move them to the blood at the opposite basolateral side; acinar cells (B) secrete digestive enzymes only from the side facing the pancreatic duct or the stomach, thus ensuring other tissues are not digested; neurons (C) have to transport neurotransmisors and specific receptors to the end of the axon or dendrites; and the useful baker's yeast (D) have to transport lipids and proteins to its daughter bud to grow and later segregate all cellular components between a bigger mother and a smaller daughter cell. These cells are able to perform these and many more essential functions because they can polarize and organize their internal components, and mechanisms to establish such polarity are present in basically all living organisms. One of these mechanisms is the targeting of lipids and proteins to specific places achieved by the selective transport of cargoes by molecular motors moving along the cytoskeleton.

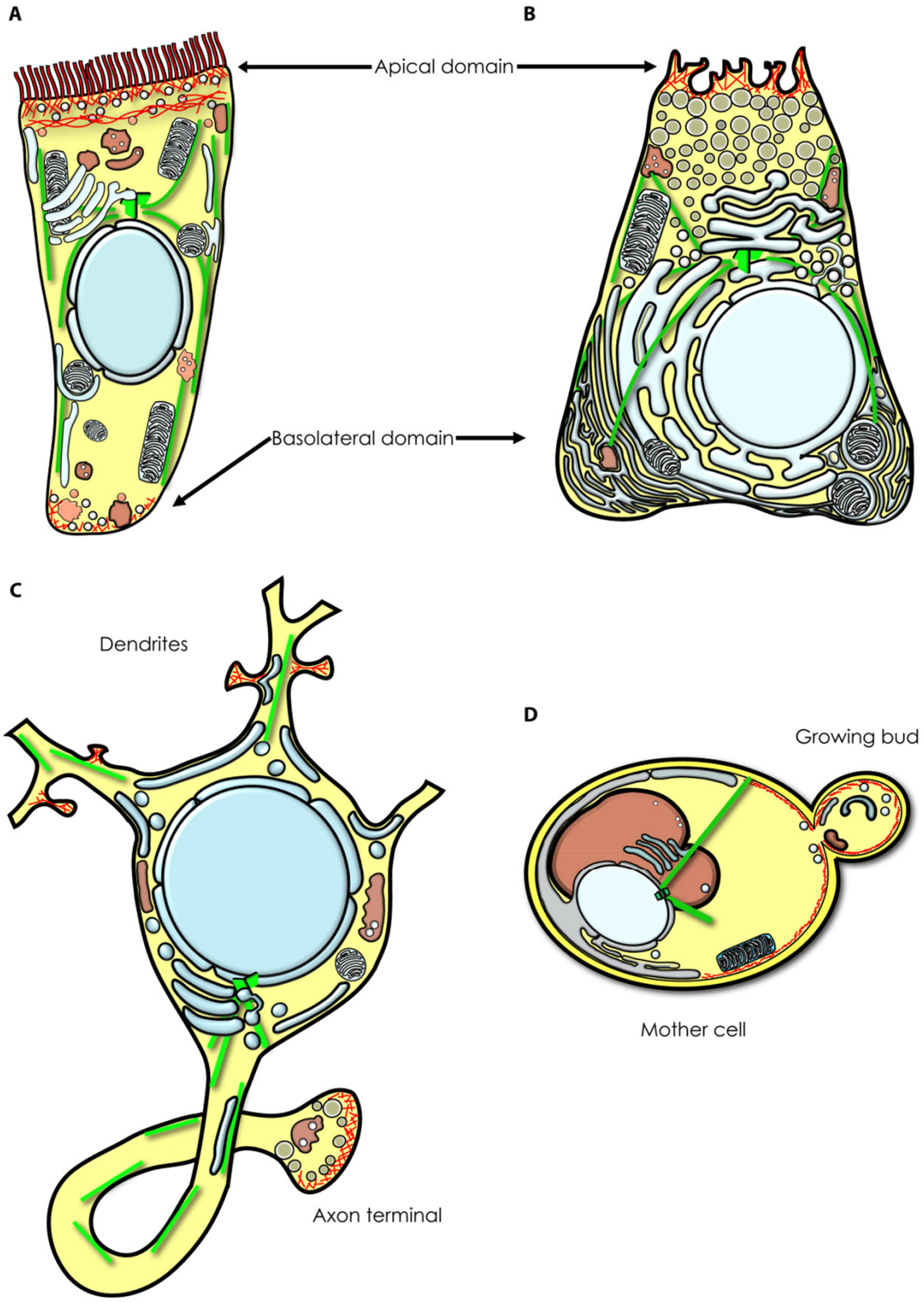


Figure 1.1 Cell polarity is easily seen in the organization of many types of cells such as (A) intestinal epithelial or (B) pancreatic acinar cells, with apical and basolateral domains each with distinct lipid and protein composition and carrying out different functions. Other cells like (C) neurons or (D) budding yeast are highly asymmetric and have sophisticated mechanisms to maintain the asymmetry since it is essential for their function and growth.

Understanding how this selectivity occurs will have repercussions in the study of many processes such as cell growth, division, differentiation, and migration. In this dissertation I explore the role lipids (phosphoinositides, or PIs) and proteins (Rab GTPases) have on the transport of secretory compartments by a molecular motor (myosin-V).

Budding yeast, an excellent model for cell polarity

Due to its simple genome, ease of genetic manipulation, culture, and storage, the budding yeast *Saccharomyces cerevisiae* has been one of the most studied model organism in biology. However, the reason this yeast is a model of choice for polarity studies (in addition to the ones mentioned above) is that in all of its life stages, it displays a highly asymmetric cell shape (Figure 1.2). During vegetative growth, it divides by budding, a process requiring selection of a bud site, establishment of a polarized cytoskeleton, and delivery and segregation of cellular components between a bigger and a smaller cell. Likewise, during mating or under nutrient-deprived conditions, yeast undergo special forms of polarized growth called shmooing (forming of a mating projection) or pseudohypha (a type of filamentous growth), respectively, based on the same principles employed for budding: (1) accumulation of mobile components on a specific region of the cell and (2) re-organization of its cytoskeletal elements in response to external cues. Because these are the same principles observed in all other polarized cells, the molecular mechanisms elucidated here will most likely be applicable to the more complex

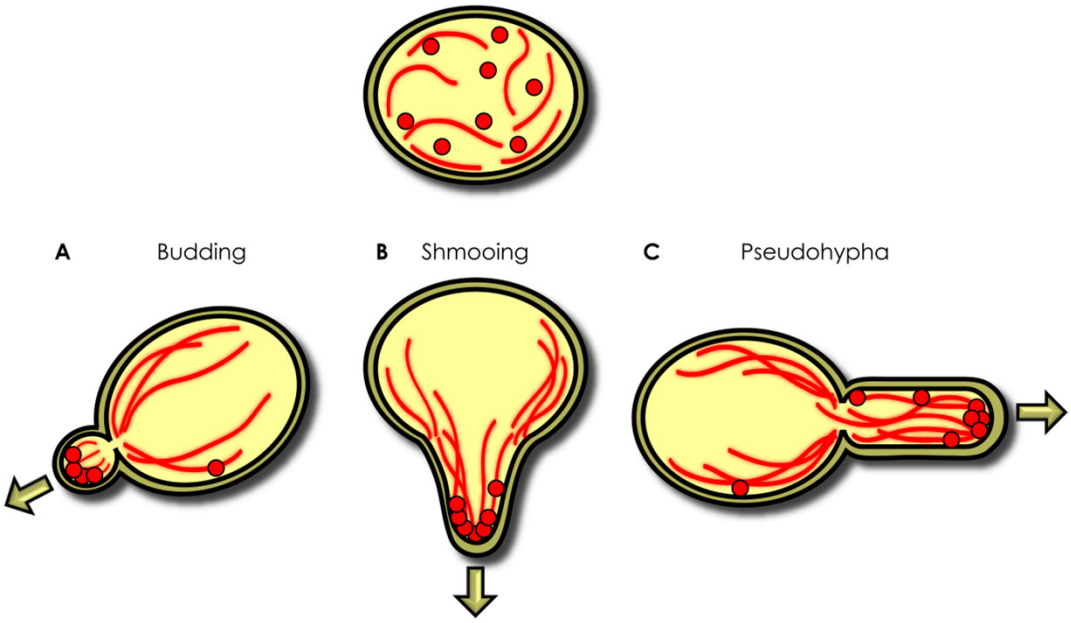


Figure 1.2 Cell polarity is of upmost importance in yeast as in every stage of its life cycle, polarity establishment allows the cell to divide by (A) budding, mate by (B) shmoo formation, or enter a (C) filamentous phase known as pseudohyphae. In red are the two main actin structures, patches (circles) and cables (filaments). Arrows indicate the direction of growth.

processes requiring cell polarization in higher eukaryotic cells. Also, in vertebrate cells, the transport of cargoes between its different domains is shared by many different kinds of motors; dynein and kinesins moving long distances along microtubules, and short-range transport mediated by myosins. However, due to its small size, in yeast all movements required for polarity establishment are mediated by myosin motors, which simplifies the study of the selective transport of cargoes in a single cell.

The actin cytoskeleton: the tracks

A combination of studies during the '80s involving the imaging of actin by immunofluorescence in mutant and wild-type strains, and the use of microtubule-poisoning drugs like nocodazole, clearly demonstrated that actin, and not microtubules, was solely responsible for the polarized delivery of vesicles required for bud growth and cytokinesis (Adams and Pringle, 1984; Kilmartin and Adams, 1984). Not only bud selection, emergence, and growth occurred normally in the absence of microtubules, but actin was always associated with sites of growth. Following these results, many actin-interacting proteins were identified, such as *SAC6* (fimbrin; Adams *et al.*, 1989), *TPM1/2* (tropomyosin; Liu and Bretscher, 1989; Drees *et al.*, 1995), or *CAP1/2* (capping protein; Amatruda *et al.*, 1990), and their functions inferred based on phenotypical characterization of null mutants, all consistent with the idea that actin directs polarized growth (Novick and Botstein, 1985). However, the relationship between the different forms of filamentous actin (patches and

cables) and their role in polarized growth was not clear until the generation in our lab of fast-acting conditional tropomyosin mutants that clearly discriminate between patches and cables (Pruyne *et al.*, 1998). With the discovery of the Arp2/3 complex and formins as the enzymes responsible for actin patches and cables, respectively, a more detailed model for the role of actin in yeast growth was then established (Pruyne *et al.*, 2004). It is now clear that Arp2/3 nucleates branched actin structures required for endocytosis and that formins are responsible for nucleation of the long unbranched actin filaments that give rise to both actin cables and the actomyosin contractile ring. Of these three actin structures, actin cables are the most relevant for the delivery of secretory cargo by myosin-V motors, thus I will discuss only these in more detail below.

Yeast contains two formin isoforms, Bni1p and Bnr1p, both members of the Diaphanous-related family (DRF). It is believed that DRF formins exist in an auto-inhibitory state, where the N- and C-termini are bound to each other, preventing premature or mislocalized polymerization of actin (Goode and Eck, 2007). In order to establish a polarized actin framework, cortical cues need to recruit the formins and once localized, activate them. In yeast, the two formins have distinct, although overlapping functions. Recruitment and activation of the formin Bni1p to the future bud site is carried out by a complex known as the polarisome together with Rho GTPases. The core components of this complex are Spa2p, Pea2p, and Bud6p, all of which localize to sites of growth

and function as a scaffolding platform for the recruitment of Bni1p as well as many other regulators of polarity. Once recruited, Bni1p is activated by the action of Cdc42p or Rho3/4p, and its activity regulated by components of the polarisome itself (i.e. Bud6p; Moseley and Goode, 2005). Activation is achieved by virtue of a Rho-GTP-binding domain in the N-terminal of all DRF formins, that once bound to its corresponding GTPase, disrupts the interaction between the termini, opening the molecule and exposing the catalytic FH1 and FH2 domains. In a similar fashion, another scaffolding platform, dependent on the septins, recruits and activates Bnr1p at the mother-daughter bud junction, forming a second source of polarized cables (Buttery *et al.*, 2007; Gao *et al.*, 2010).

Once the formin is activated at the right site, nucleation and stabilization of actin filaments can occur. This process is dependent on the actin-sequestering protein Pfy1p (profilin; Haarer *et al.*, 1990), which binds actin monomers and recruits them through Bni1p's FH1 domain for rapid actin polymerization by the FH2 domain (Kovar *et al.*, 2003; Romero *et al.*, 2004). While nucleating filaments, Bni1p remains bound to the barbed end (Pruyne *et al.*, 2002), a characteristic that makes formins uniquely suited to organize cables since it protects the fast-growing end from capping protein (which would otherwise terminate polymerization), facilitates the insertion of new subunits, and at the same time anchors the end of the cables to the yeast surface (ensuring that all filaments have the same polarity). As the filaments elongate,

bundling proteins like Sac6p (fimbrin) crosslink many different filaments giving rise to strong bundles of filaments that are stabilized along their sides by Tpm1/2p (tropomyosin; Adams *et al.*, 1991; Pruyne *et al.*, 1998).

Formins are powerful nucleators of actin filaments, and *in vitro*, given the right concentration of reagents, they can nucleate filaments many micrometers long (Kovar and Pollard, 2004; Berro *et al.*, 2007). However, *in vivo*, the size of actin filaments is controlled in a regulated manner, as the length of the filaments in a given structure is fairly constant (e.g. actin cables are a collection of small filaments between 0.3 – 0.5μm; Karpova *et al.*, 1998). Moreover, analysis of Bni1-3XGFP showed speckles moving in a linear, retrograde manner, very similar to the way actin cables grow (Buttery *et al.*, 2007). These observations indicate that a formin inhibitory or displacement factor must exist. One factor capable of doing this is Bud14p, found in a visual screen for genes that when deleted resulted in extremely long cables that kink when they reach the other side of the cell (Chesarone *et al.*, 2009). Bud14p binds strongly to the FH2 domain of Bnr1p *in vitro*, and in doing so it displaces it from the actin filament. Bnr1p, however, is stably associated with the bud neck, and thus another factor would be needed to re-activate the formin by disrupting the Bud14-Bnr1 interaction. Moreover, factors that regulate Bni1p at the bud cortex still remain elusive, although unpublished evidence at meetings have suggested that the mysterious Smy1p could be doing just that (Bruce Goode lab).

The result of this regulated formin recruitment and activation, followed by nucleation, elongation, and stabilization of actin filaments, and finally by inhibition and displacement of the formins, are the long actin cables (composed of mostly uniform length filaments) running along the axis of division in a polarized manner. By controlling when and where cables are generated, the cell can direct the movement of the necessary cargoes mediated by the myosin-V motors translocating along them.

The myosin-Vs: the motors

Most myosins conform to the body plan of an N-terminal head or motor domain, followed by a light-chain binding region, or neck domain that can vary in length, and ending in a C-terminal tail domain that is conserved within a given class (Figure 1.3A; Hodge and Cope, 2000). Yeast has 5 myosin genes, representing the three most ancient conserved myosin classes (Figure 1.3B). Myo1p is the conventional myosin, involved in cytokinesis and orientation of actin cables at the neck region (Bi *et al.*, 1998; Gao and Bretscher, 2009). Myo3p and Myo5p are class I myosins that localize to actin patches and have a role in endocytosis (Geli and Riezman, 1996; Sun *et al.*, 2006). Myo2p and Myo4p are members of the class V myosins, having the same features mentioned above, but characterized for their high-duty cycle motor domain, their long neck (with six light-chain binding sites) and for the presence of a DIL domain in their C-termini (Figure 1.3B: *MYO2* and *MYO4*). These features allow myosin-Vs to perform as processive motors, and their functions seem to

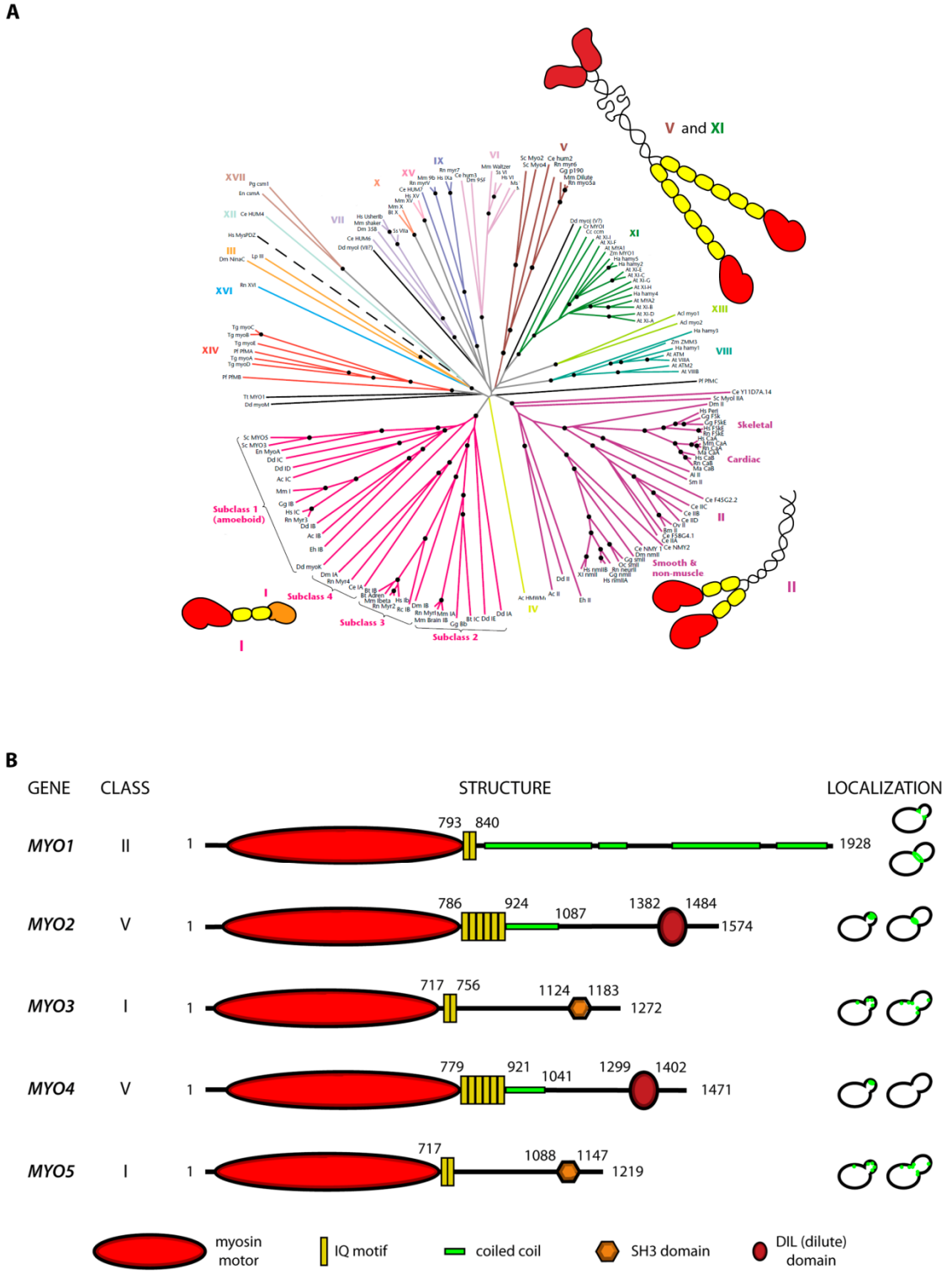


Figure 1.3 The domain structure and function of the myosin superfamily. (A) Phylogenetic tree of 139 myosin genes representing the seventeen classes known by 2000 (Hodge and Cope, 2000). Currently there are 24 classes with several additional orphan sequences. The two-dimensional structure of the three most ancient classes is shown as a cartoon, with class V and XI being grouped together. (B) Representation of the domain structure and localization of the three yeast myosin classes. The different domains present are identified at the bottom.

be conserved virtually in every organism. Although very similar in their structure, Myo2p and Myo4p perform different functions (discussed below) that are related to key sequences and their specific binding partners. I will briefly discuss these structural and functional sequences, and how they determine the myosin's mode of action and possible regulation. As an important note, for metazoan myosin-Vs, the motor and neck region are collectively called the head domain, while the long coiled coil together with the C-terminal globular domain are known as the myosin tail. Maybe for historical reasons, in yeast and plant myosin-XIs (the counterparts of animal myosin-Vs), the four structural domains are kept separate, with the myosin head referring only to the motor domain and the myosin tail denoting only the globular tail domain (GTD).

The myosin-Vs' motor domain is very similar to that of other myosins, and X-ray structures of the chicken Myosin-Va (Myo5A) motor exist in both the pre- and post-stroke conformations (Coureux *et al.*, 2003; Coureux *et al.*, 2004). Limited proteolysis of all myosin motor domains yield three subdomains named 20K, 25K, and 50K according to their molecular weights, with the actin binding interface residing in the 50K region. All myosin-Vs tested so far bind strongly to actin in the absence of ATP and hydrolyze ATP in a state that still has a relatively weak affinity for actin. Although no crystal structure of the acto-myosin complex for myosin-V exists, the current structures of Myo5A showed that the large cleft of the 50K subdomain is significantly more closed and resembles the structure of muscle myosin in the strong actin binding state

(Coureux *et al.*, 2003; Coureux *et al.*, 2004). This structural difference may explain the high affinity for actin during the power stroke cycle (no nucleotide → ATP → ADP+P_i → ADP → no nucleotide). Moreover, binding to actin accelerates the release of P_i (inorganic phosphate; after ATP hydrolysis the byproducts ADP and P_i remain bound), which promotes the transition from the weak to the strong actin-binding states, accounting for the very fast on rate observed for myosin-Vs and actin (De La Cruz *et al.*, 1999). Because for most of the ATPase cycle the motor domain is bound to actin, myosin-Vs are considered a high duty-ratio motor, ensuring that for every encounter with actin, the motor can undertake many steps without dissociating. However, the motor domain is not the only determinant for processivity, as Myo4p (see below) and the fly single Myosin-V are not processive as a single or double motor *in vitro* (Tóth *et al.*, 2005; Dunn *et al.*, 2007), yet their motor domains have all the features other myosin-Vs have. In fact, a chimera consisting of the Myo4-motor with the mouse MyoVa neck and tail domain resulted in a processive motor (Krementsova *et al.*, 2006). An efficient processive motor also requires a lever arm, to amplify the structural changes of the heads, and a way to dimerize, or oligomerize, the motors. Nevertheless, the motor domain structural features are very important since in the original myosin-V mutant, the *myo2-66* allele, a glutamate to lysine mutation that lies in the actin binding interface disrupts all of Myo2p functions (Lillie and Brown, 1994).

A long standing controversy is the finding that Myo2p is not a processive

motor *in vitro* (Reck-Peterson *et al.*, 2001). The Mooseker lab noted that Myo2p does not co-sediment with actin in the presence of ATP, while under the same conditions half of chick Myo5A does. They found that the velocity of actin filaments moving on a bed of immunoadsorbed Myo2p decreased as the concentration of the motor increased (for highly processive motors, the velocity is independent of the concentration). Similarly, in filament landing and movement assays, they found that the minimum number of motors needed to capture and move a filament was 5. They concluded that Myo2p is a nonprocessive motor, a claim inconsistent with the *in vivo* data of imaging secretory vesicle movement or Myo2-GFP itself (Schott *et al.*, 2002 and unpublished data). In a study that reconciles these findings, Kathleen Trybus and colleagues recently proposed that a kinesin-like protein, Smy1p, can serve as a tether between the vesicles and actin (Hodges *et al.*, 2009). They showed that an individual Myo2p cannot move a quantum dot *in vitro*, but if Smy1p is associated with the quantum dot, the whole complex undertakes many steps along actin. Smy1p was found as a multicopy suppressor of *myo2-66* (hence the name; Lillie and Brown, 1992) that has the structural features of a microtubule motor but has lost that activity. They found that Smy1p undergoes an electrostatic-driven diffusional behavior on actin and that it *in vivo* localizes to moving dots, presumably secretory vesicles. They propose that Smy1p is the factor that makes Myo2p processive *in vivo*, consistent with the fact that you need the Smy1p head domain to suppress *myo2-66* (Lillie and Brown, 1998). However, Smy1p is not essential, and Myo2-GFP movement is

unaffected *in vivo* in *smy1*Δ cells (Kirk Donovan, unpublished data), suggesting that either Smy1p is not acting as a tether or there are other redundant factors that can compensate for Smy1p loss. Nevertheless, *smy1*Δ is synthetic lethal with mutations in *SEC2* and *SEC4*, genes involved in vesicular trafficking (see below), and Smy1p function becomes essential in strains with Myo2p defects, strongly suggesting that Smy1p by itself performs an important function in Myo2p-dependent secretory vesicle transport (Lillie and Brown, 1998).

Following the motor domain is the myosin light chain binding region, or neck domain, which consists of a long α-helix of multiple IQ motifs (each with a consensus sequence of IQxxxRGxxxRxx[VLFY]). These IQ motifs are the target for calmodulin and for regulatory or essential myosin light chains and, in fact, chicken Myo5A was first identified as a calmodulin binding protein from brain (Espindola *et al.*, 1992; Espreafico *et al.*, 1992). Shortly thereafter Trisha Davis' lab showed that yeast calmodulin, Cmd1p, also associates with Myo2p through the same region containing the IQ motifs in a Ca^{+2}-independent manner (Brockerhoff *et al.*, 1994). The purification and characterization of chicken Myo5A also demonstrated the existence of additional light chains. Only around 4 to 5 calmodulins purify per heavy chain, with additional bands of 10, 17, and 23 kDa co-purifying with Myo5A (Espindola *et al.*, 2000). Consistent with this, an additional myosin light chain, Mlc1p, was found in yeast by searching the genome for calmodulin homologous sequences, and demonstrated to also bind Myo2p IQ motifs (Stevens and Davis, 1998). In gel

overlay blots, Mlc1p binds more strongly to a construct containing the first two IQs, while calmodulin binds very weakly to this same construct. They also showed that Mlc1p interaction with the IQ motifs stabilizes Myo2p, while Cmd1p has no effect. These results indicate that there is a differential effect on Myo2p depending on which light chain is bound to specific IQ motifs. In fact, a study exploring the effects of the light chain bound to the first IQ motif on the kinetics of chick myosin-V motor domain found that there are significant light-chain-dependent alterations on the kinetics of the ATPase cycle (De La Cruz *et al.*, 2000). Moreover, *in vitro* studies with purified vertebrate myosin-Vs have shown that Ca^{+2} affects the association of calmodulin with the IQ motifs, resulting in a loss of processivity of the motor. Presumably this inhibition of motility is due to a loss of stiffness of the α-helix, which is now unable to transfer the conformational changes of one head to another (Krementsov *et al.*, 2004; Lu *et al.*, 2006). Because only calmodulin is able to respond to Ca^{+2}, the stoichiometry of light chain isoforms bound could have a large regulatory effect in higher myosin-Vs. Likewise, structural studies in yeast showed that the mode of interaction of Mlc1p with IQ1 is distinct from the interaction of Cmd1p with the same motif, suggesting a different mechanism of action for the yeast light chains (Amata *et al.*, 2008). Basically, Cmd1p associates with the IQ1 motif in a very rigid manner using both termini (or lobes) while Mlc1p binds IQ1 using only the C-lobe, and its N-lobe is very dynamic.

In addition of serving as a regulatory region by binding light chains, the

neck domain acts as a "lever arm", which amplifies the small movements of the motor domain into the characteristic 36nm step size of myosin-Vs. This distance is of critical importance because it is the length of the helical repeats of the actin filament, therefore the myosin can walk along without spiraling around. It is well established for various myosin types that the step size increases with the length of the lever arm (Anson *et al.*, 1996; Uyeda *et al.*, 1996; Purcell *et al.*, 2002; Sakamoto *et al.*, 2005), but the definitive demonstration came from *in vivo* studies in our lab where mutants of Myo2p were engineered containing none, two, four, six, or eight IQ motifs and, by measuring the velocity of secretory vesicles labeled with GFP-Sec4, shown to move at proportionately increasing speeds (Schott *et al.*, 2002).

Myo2p's lever arm has also been identified as the binding site for Sro7p, specifically IQ1 *in vitro* and IQ1,5 *in vivo* (Rossi and Brennwald, 2011). *SRO7* was originally identified as a multicopy suppressor of *rho3*Δ (hence the name) in a background conditionally expressing *RHO4* (absence of both Rho proteins is lethal; Matsui and Toh-E, 1992). Sro7p (the yeast's Lgl homolog) associates with Sec9p (the SNAP-25 homolog) and regulates SNARE function (Lehman *et al.*, 1999); however it was first implicated in Myo2p function when it was found that *SRO7* overexpression partially rescues *myo2-66* (Kagami *et al.*, 1998). Later proteomic studies found that the two major Sro7p binding partners are Sec9p and Myo2p (Gangar *et al.*, 2005). Using Myo2p fragments, it was determined that the interaction occurred through the neck region and, in a

continuation of their studies using *in vitro* translated peptides, it was mapped down to IQ motifs 1 and 5 (Rossi and Brennwald, 2011). Previously, Matsui and colleagues explained the relationship between Myo2p and Sro7p on the basis of the known regulation of SNARE function by Sro7p. They speculated that by recruiting more Sro7p to vesicles you prevented mislocalized fusion events. In their recent paper, Rossi and Brennwald extend this hypothesis by proposing that Sro7p acts in a parallel pathway with Sec15p, a component of the exocyst tethering complex, thus regulating both tethering and fusion events. Their basis for this conclusion is the finding that Sec15p and Sro7p behave identically when overexpressed (inducing clusters of vesicles), that both are under the control of active Sec4p, and that both are associated with vesicles and localize to sites of growth in a vesicular traffic-dependent manner. Their data showed that Myo2p is a negative regulator of the tethering abilities of Sro7p, and that there is competition between Myo2p and active Sec4p for Sro7p binding; however they did not explore the effects of Myo2p in the interactions between Sro7p and Sec9p. How Sro7p binding to Myo2p's neck domain affects its function is also unclear, especially since it is known that the neckless *myo2* mutant is viable (Stevens and Davis, 1998).

The heavy chain of myosin-Vs is usually 200 – 210 kDa, but yeast's myosin-Vs are 170 and 180 kDa, mainly due to a shortened coiled coil region. This region is responsible for dimerizing the heavy chains, resulting in a double headed motor complex. In higher myosin-Vs there are three coiled coil regions

interrupted by two unstructured stretches (see Figure 1.3A). Myo2p and Myo4p on the other hand have only one coiled coil region (or rod domain for Myo4p, see below) connecting the neck domain with the GTD. This difference is functionally relevant since in mammalian myosin-Vs, the distal part of the coiled coil region is alternatively spliced, generating isoforms that will bind specific receptors and adaptor proteins. Myo2p, although lacking the variability in its coiled coil, binds the Rab GTPase Sec4p and its guanine exchange factor (GEF) Sec2p through determinants in both the GTD and the coiled coil (this thesis). Myo4p, the other yeast's myosin-V, is not a processive motor because its α-helical region does not form a coiled coil (hereafter termed the rod domain), therefore Myo4p is a single-headed motor (Heuck *et al.*, 2007; Hodges *et al.*, 2008). Furthermore, the adapter protein She3p (discussed below) binds to the rod domain with such high affinity that it has been proposed to be a subunit of the motor (Hodges *et al.*, 2008). Nevertheless, surface plasma resonance (SPR) experiments have shown the additional presence of binding determinants for She3p in the GTD, and an artificially dimerized GTD will bind She3p with slightly less affinity than the rod-tail sequences (Heuck *et al.*, 2007). This finding was, however, put in doubt later when it was shown that *in vivo* a Myo4p construct lacking the GTD could localize cargo while one lacking the rod could not (Bookwalter *et al.*, 2009). Still, closer examination of the data indicated that the GTD contributes to both the association with She3p and with the cargo, as constructs lacking this region do not perform as well as full length Myo4p in various *in vitro* and *in vivo*

assays (Hodges *et al.*, 2008; Bookwalter *et al.*, 2009). Recently, residues in the GTD were identified that are important for the *in vitro* interaction with She3p and when mutated slightly affect the *in vivo* function, demonstrating that the GTD provides a minor but important role in She3p binding. Taken together, these results indicate that regions involved in cargo binding lying outside the GTD can be a feature conserved in myosin-Vs translocation complexes (Heuck *et al.*, 2010).

Myo2p's coiled coil domain is also the binding site for yeast Rho-type GTPase Rho3p (Adamo *et al.*, 1999; Robinson *et al.*, 1999). A fragment of Myo2p was found in a two-hybrid screen for interactors of Rho3p, and the minimal fragment that interacted with Rho3p was mapped down to amino acids 871 to 1130 (the whole of the coiled coil domain and the first 43 aminoacids of the tail domain, which are unstructured in the GTD crystal; see Figure 1.3B). A mutation in Rho3p effector domain specifically abolished the interaction with Myo2p and causes a defect in secretion that is exacerbated at low temperatures. Moreover, after a shift to 14°C, electron micrographs revealed the random accumulation of post-Golgi vesicles throughout the mother cell, indicative of a defect in vesicle transport. However, the mechanism of Rho3p regulation of Myo2p is unknown.

The carboxy-terminal region of all myosin-Vs is known as the cargo binding domain or GTD. The two well-known functions of this domain are (1) cargo binding by associating with receptors on the transported compartment;

and at least in vertebrate myosin-Vs, (2) inactivation of the motor complex by associating with and inhibiting the motor domain (Liu *et al.*, 2006; Thirumurugan *et al.*, 2006). However, the molecular details of these functions are still unclear and, especially for the regulation of cargo binding, the list of players involved in them is far from complete. Moreover, we and others have been unable to demonstrate an interaction between the two termini of Myo2p, suggesting that the regulation of the motor activity by the GTD might be an activity gained later in evolution. Since this activity is not relevant for the focus of this investigation I will not discuss it further (for more information, please see the references above or Taylor, 2007).

The first crystal structure of a myosin-V GTD was that of Myo2p, consisting of two bundles of five α-helices connected through a common long helix (Pashkova *et al.*, 2006). These two bundles correspond to the previously genetically and biochemically characterized subdomains I and II (Catlett *et al.*, 2000; Pashkova *et al.*, 2005; Legesse-Miller *et al.*, 2006). Subdomain I contains the residues involved in vacuole transport while subdomain II contains the ones involved in vesicle transport; however, both domains are nonfunctional by themselves, suggesting that the interaction between the domains is necessary for the correct overall folding of the GTD (Pashkova *et al.*, 2005). This is consistent with the extended interface of interaction between the two bundles and with the last three helices that wrap around both subdomains. At the time of publication, no other protein in the structural databases exhibited a similar

fold; however it later became clear that Myo2p GTD (and that of Myo4p, solved later) had an architecture unique to tethering factors (Richardson *et al.*, 2009; Heuck *et al.*, 2010). This similarity suggests that, by analogy to the interactions within the tethering factors, myosin-V GTDs may play a direct role in tethering the motor to membranes by interacting with these factors.

One of the main tools I utilize in this investigation is the GTD conditional mutants of Myo2p (Schott *et al.*, 1999). These mutants were generated in the lab to shed light on the function of the GTD since the role of this domain was unclear at the time. A controversy arose with the isolation of the new non-conditional *myo2-2* allele, which specifically disrupted vacuole inheritance, a non-essential process in yeast (Catlett and Weisman, 1998). This allele also localized improperly by immunofluorescence and did not have the same genetic interactions as *myo2-66*. Because other vacuole-specific *myo2* alleles were identified that localize correctly, it was suggested that the GTD contains the localization information for the vacuole and for the sites of growth and that they are separate motifs that do not depend on actin. Consistent with this hypothesis was the finding that a small amount of polarized Myo2p remained in cells treated with latrunculin A (LatA; Ayscough *et al.*, 1997) and that a small population of cells overexpressing an HA-GTD exhibited polarized localization of the fusion protein after short induction times (Reck-Peterson *et al.*, 1999). These observations led to a change in the notion that viewed Myo2p as an active motor that marched down actin cables dragging cargoes to one in

which the GTD functioned as a localization determinant for Myo2p and that, once localized, it either captures free vesicles or organizes actin cables at sites of growth. With the generation of our conditional GTD mutants, it was clearly shown that the GTD is responsible for the association of Myo2p with vesicles, that the motor domain is responsible for targeting Myo2p to sites of growth, and that the effect on the actin cytoskeleton seen in *myo2* mutants is a secondary effect of depolarizing secretion. Although it is now clear that the GTD is involved in attaching Myo2p to the different cargoes, when I started this investigation no details existed on how Myo2p is recruited to secretory membranes.

The GTD of myosin-Vs are also subjected to phosphorylation (Karcher *et al.*, 2001; Legesse-Miller *et al.*, 2006), however a generalized function for it has not been demonstrated. In assays using *Xenopus* egg extracts and isolated melanosomes, it was demonstrated that endogenous MyoVA, or its recombinant GTD, dissociates from membranes upon phosphorylation. The phosphorylation site was identified as serine 1650, a residue conserved in higher myosin-Vs and in the filamentous fungi *Ustilago*, but absent in yeast's myosin-Vs. Nevertheless, Myo2p was found to be phosphorylated *in vivo* in a cell cycle-dependent manner and it was shown to occur at the GTD (Legesse-Miller *et al.*, 2006). The residues were identified by mass spectrometry and found to cluster to a small 5 amino acid patch that is not conserved in other GTDs. Although these results suggested a possible mechanism of cargo

binding regulation, mutations of these residues did not have any obvious effect. However, phosphorylation may still represent an important mechanism of regulation since recently a mutation that abolishes the activity of protein phosphatase Ptc1p was found to affect the localization of both myosin-V motors in yeast (Jin *et al.*, 2009). Although it was not clear, the authors speculated that Ptc1p regulates the association of the motors with their cargo and, in the absence of an interaction with the cargoes, the myosin motors became inactivated and delocalized. Further details about Ptc1p role in vacuole inheritance is discussed below. Similarly, the Myo4p subunit She3p is also phosphorylated, and the residues were identified by mass spec (Landers *et al.*, 2009). Phosphodeficient mutants worked fine *in vivo*, but phosphomimetic versions failed to function, indicating that similar to the *Xenopus* case, phosphorylation could negatively regulate association of the myosin with its cargo.

In summary, although very similar in their domain organization, the two yeast myosin-Vs act in different ways, perhaps reflecting their adaptation to their specific *in vivo* functions. Nevertheless, basic principles can be applied for both of them and perhaps for all myosin-Vs, such as binding of adapters and receptors through their coiled coil and globular tail domains to link them to cargo, that proteins can bind other domains to regulate non-cargo events like dimerization or motor activity, and that phosphorylation can regulate cargo association.

Organelles, vesicles and macromolecules: the cargoes

As in all eukaryotic cells, yeast contains many different membrane-bound compartments that carry out specific biochemical reactions necessary for life. Many of these compartments cannot be generated '*de novo*' and therefore need to be inherited. Since budding is a highly asymmetric process, where components need to be segregated between unequal size cells connected by a small neck, active transport of organelles is required. Moreover, in order to grow, the bud must receive a constant supply of lipids and proteins via the delivery of secretory vesicles. Lastly, not only membranous cargo needs to be transported, but macromolecular complexes such as RNA and protein aggregates also are actively segregated. These jobs are shared by the two myosin-V motors in yeast, Myo2p and Myo4p (Figure 1.4). I will briefly first describe the cargoes transported by Myo4p and then go into details on the Myo2p-dependent ones.

messenger RNA (mRNA)

Transport and localized translation of mRNAs is another way cells can establish polarity, and it is a crucial mechanism necessary for the development of metazoans. Although not essential in yeast, we can nevertheless learn about the molecules and features involved as some of them are conserved throughout evolution, like the '*zip code*' sequences present in these mRNAs. According to literature searches, at least 30 transcripts have been shown to be polarized,

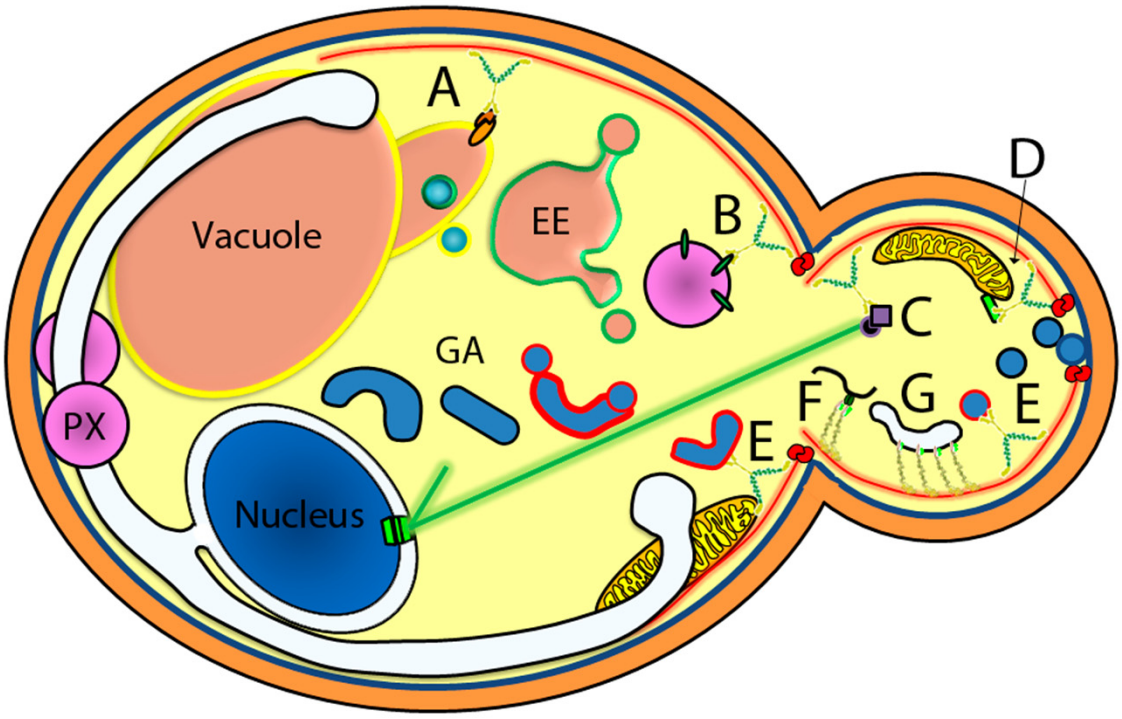


Figure 1.4 The yeast's myosin-Vs cargoes. Myo2p transports (A) vacuole fragments through Vac17p – Vac8p; (B) peroxisomes through Inp2p; (C) microtubule plus ends through Bim1p – Kar9 for orientation of the nucleus; (D) mitochondrial tubes by an unknown mechanism that involves the Myo2-binding protein Mmr1p; (E) and secretory membranes such as the trans-Golgi Network and secretory vesicles, by a mechanism involving Rab GTPases and the lipid PI4P (this thesis). The other myosin-V Myo4p transports (F) mRNA as an ensemble of monomers through the She2/3p complex as well as (G) tubules of cortical ER (independent of She2p). Myo2p associates with another structure, the P bodies, that is not depicted. The red lines along the cortex represent actin cables, and the thick green lines microtubules.

including several mRNAs encoding cell polarity and secretion proteins (Takizawa *et al.*, 2000; Aronov *et al.*, 2007). All are dependent on the SHE system, a complex composed of Myo4p and the She2/3p proteins (*MYO4* was originally known as *SHE1*; Takizawa and Vale, 2000). Myo4p and She3p form a heterodimer through Myo4p's rod domain (consequently Myo4p is nonprocessive, see above) that bind She2p, which in turn binds as a homodimer to the zip codes in the mRNA molecule. These zip codes are cis-acting elements contained within the RNA molecule itself. Most of the studied yeast's mRNAs that are polarized contain one or more zip codes that, unlike metazoans, can be in the coding sequence. *ASH1* mRNA, for example, has four zip codes, with the first three present within the coding sequence of the transcript, and a fourth overlapping the coding sequence and the 3'UTR (Böhl *et al.*, 2000). Because most mRNAs have multiple zip codes, and each zip code may bind two Myo4p/She3p complexes bridged by the She2p dimer, and *in vivo* many mRNAs are co-transported on the same particle, the moving ribonucleoprotein complex can be moved processively by an ensemble of Myo4p monomers. It was suggested then that the regulation of the mRNA transport depends on achieving a critical mass of mRNAs to recruit enough motors to move the particle (Chung and Takizawa, 2010). However, it may not be that simple since She3p was found to be a phosphoprotein and, although not affecting its interaction with Myo2p or She2p, phosphomutants are defective in polarizing mRNA, suggesting a novel, phosphorylation-dependent way to regulate the Myo4p-mRNA complex formation (Landers *et al.*, 2009). The only

known physiologically relevant functions of mRNA transport in yeast are mating type switching and in diploid pseudohyphal growth. When haploid cells in the wild divide, the mother cell will undergoes mitotic recombination at the *MAT* locus, resulting in a switch of the mating type. In order for recombination to occurs, the HO endonuclease must cleave the DNA at specific sequences of the *MAT* locus, so by controlling the expression of the nuclease yeast cells can control recombination. Ash1p is a transcription factor that represses the HO endonuclease and, because its mRNA is transported into and translated in the daughter cell only, inhibits recombination in the daughter cell (Sil and Herskowitz, 1996). Ash1p was also found to have a role in the pseudohyphal growth of diploid cells, where it acts as an activator. Homozygous *ash1*Δ diploids do not form pseudohypha while its overexpression induces exaggerated filamentous growth (Chandarlapaty and Errede, 1998). Moreover, during filamentous growth Ash1p is polarized to the nuclei of the growing tip cell, which is necessary to induce the expression of a set of proteins required for invasive growth (Pan and Heitman, 2000).

cortical endoplasmic reticulum (cER)

In yeast, the ER has two morphologies and two localizations: (1) flat sheets surrounding the nucleus to form the perinuclear ER, and (2) sheets and tubules associated with the cortex to form the cER. Although these two domains are interconnected by cytoplasmic tubules, they are inherited by distinct mechanisms. The nucleus-associated ER is inherited in a

microtubule-dependent manner coupled to nuclear segregation, thereby rendering inheritance of cER non-essential, since enough cER may be dragged with the perinuclear ER into the bud when nuclear division occurs. On the other hand, the cER inheritance is actin-dependent and occurs as tubules transported into the bud by Myo4p/She3p. In fact, the ER tubules that are dragged into the bud by Myo4p emanate from the perinuclear ER, consistent with the idea that in the absence of an inherited cER membrane, the perinuclear ER can give rise to a cER. Although this process requires She3p, it is independent of She2p, and the specific cER receptor has not been identified. There is also a subsequent phase to anchor the delivered tubules to the bud tip, a process requiring the exocyst, VAP proteins, reticulons, translocon components, and few other proteins of unknown function (Wiederkehr *et al.*, 2003; De Craene *et al.*, 2006; Loewen *et al.*, 2007). Once attached, the cER spreads around the growing bud to form a tubular network, a process requiring the activity of the serine/threonine phosphatase Ptc1p (Du *et al.*, 2006). Since is not an essential process, the physiological significance of Myo4p-dependent inheritance of the cER remains obscure.

Vacuole membranes

The yeast vacuole is the functional counterpart of the mammalian lysosome. It plays a wide variety of roles essential for yeast homeostasis: protein degradation, ion and metabolite storage, pH regulation, detoxification, and first-aid center in response to cellular stresses such as osmotic shock or

nutrient deprivation. After reading this assortment of functions, it must be a surprise for the reader to know that vacuole inheritance is not essential. The reason for this disparity is that yeast can generate a vacuole *'de novo'* possibly by directing vacuolar proteins from the Golgi into endosomal compartments, so although its inheritance is not important, in practice vacuoles are essential organelles. This fact, that vacuole inheritance is not essential, presented a huge advantage for the genetic analysis of the process and allowed for the easy isolation of mutants (Weisman *et al.*, 1990; Shaw and Wickner, 1991; Wang *et al.*, 1996). These mutants, collectively known as *vac* mutants, were grouped into three classes based on the morphology of the vacuole, but were all defective in segregation of vacuolar membranes into the bud. The most interesting ones were the class I mutants, with normal looking and functional vacuoles, but yet they were not transported into the bud. The first indication of the mechanism of vacuole inheritance came from the study of actin and myosin mutants (Hill *et al.*, 1996). Visualization of the vacuole in a subset of actin mutants (the ones affecting myosin-binding) and in the *myo2-66* strain showed that inheritance was affected. Moreover, they noted that actin cables were in many instances lying along vacuolar membranes and that the vacuolar segregation structure was always associated with Myo2p staining. This was the first conclusive report that an organelle in yeast was being transported along the cytoskeleton by the myosin Myo2p, which was later confirmed with the identification of the *myo2-2* allele (discussed above; Catlett and Weisman, 1998).

The first clue into the identity of the vacuolar receptor for Myo2p came from the discovery of the gene corresponding to the class I mutant *vac8* (Wang *et al.*, 1998). Wild-type Vac8p is myristoylated and palmitoylated at its N-terminus, and those modifications are necessary for its association with vacuolar membranes. Moreover, mutation of these lipid modification sites also gives rise to defects in vacuole inheritance, suggesting that Vac8p could act as the vacuole-specific receptor. However, no physical interaction was found between Vac8p and Myo2p, indicating that if Vac8p was indeed needed for vacuole transport, at least another protein must be present to act as a bridge between Vac8p and Myo2p. This adapter protein was found by three independent approaches: (1) an overexpression suppression screen of vacuole-specific *myo2* mutant, guessing that if the interaction between the adapter protein and the *myo2* allele was weakened, increasing the levels of the adapter might strengthen it to near normal levels (Ishikawa *et al.*, 2003); (2) an extragenic suppressor screen of another *myo2* point mutant, reasoning that if a point mutation in Myo2p disrupted its interaction with the adapter protein, a compensatory mutation in the adapter protein could restore the interaction with the *myo2* mutant (Ishikawa *et al.*, 2003); (3) a yeast two-hybrid screen using Vac8p as bait (Tang *et al.*, 2003). All of these screens yielded *VAC17*, another of the class I vac mutants isolated previously, and uncovered the core components of the vacuole-specific Myo2p receptor: Myo2p GTD binds the N-terminal domain of Vac17p, which in turn through its C-terminal region binds Vac8p, which is anchored at vacuolar membranes via its lipid modifications

(Tang *et al.*, 2003). Genetic evidence, however, indicates that the receptor may not be as simple as this linear series of interactions (Myo2p-Vac17p-Vac8p), since extragenic suppressors of vacuole specific *myo2* mutants or of *vac17-1* mapped to areas outside their respective binding regions. Moreover, *vac17*(S57F) supports vacuole inheritance in all *myo2* vacuole-mutants without restoring the interaction between Myo2p and Vac17p. Nevertheless, this work has provided a very detailed molecular picture of how motors are recruited to their cargo and how that interaction is regulated. Interestingly, a similar situation exists in the melanosome system of mammalian cells, where despite all the data demonstrating a linkage between melanosomes (a specialized lysosome) and Myo5A through melanophilin and Rab27a (Wu *et al.*, 2002), an extragenic mutation exists (dsu, for '*dilute* suppressor') that restores melanosome transport in all of the coat color mouse mutants (*dilute*, *leaden*, and *ashen*) in a myosin-Va-independent way (O'Sullivan *et al.*, 2004).

As noted above, the inheritance of the vacuole is a cell-cycle regulated process. As soon as a bud site is selected, the large vacuole fragments into several smaller "vesicles" that will then form a segregation structure. Right after bud emergence occurs, the segregation structure moves into the growing bud and then disappears before nuclear elongation. The delivered vacuolar "vesicles" remain associated with the bud tip away from the mother-daughter neck until, after cytokinesis, when they fuse with each other to form one or two large vacuoles in both the mother and daughter cells (although this number

varies in the different background strains, see Gomes de Mesquita *et al.*, 1991). This cycle clearly indicates that the transport of the vacuole is a highly regulated process, and the obvious target for this regulation is the receptor complex. In fact, Vac17p levels are coordinated with the cell cycle, peaking at G1 (unbudded cells) and the beginning of S phase (nascent bud) and dropping to the lowest levels during cytokinesis (Tang *et al.*, 2003). Although *VAC17* mRNA levels also are cell-cycle regulated, block of vacuole inheritance increased Vac17p levels, indicating that the protein levels were coupled to delivery of the vacuole independent of the mRNA levels. Analysis of the Vac17p sequence revealed a PEST domain (stretches of proline, glutamate, serine, and threonine, hence the name) that could potentially regulate the protein levels by rapid degradation. Indeed, when the PEST sequence was deleted, the levels of Vac17p increased and, most notably, no longer oscillated with the cell cycle. Consistent with the role of this motif in regulating vacuole inheritance, in a *vac17*ΔPEST strain, the vacuole is delivered to the bud, but then is brought back to the mother-daughter neck junction. These observations, together with other imaging evidence of Vac17p localization, indicated that Vac17p was being specifically degraded in the bud after the vacuole is delivered to its correct destination (Tang *et al.*, 2003). It was then hypothesized that this regulated degradation released Myo2p from the vacuole, but what is the signal for the degradation?

Vac17p in immunoblots runs as a doublet, and they demonstrated that it

is as result of its phosphorylation (Peng and Weisman, 2008). Moreover, phosphorylation was also cell-cycle dependent, with minimal phosphorylation in G1 and peaking in M phase, exactly matching the previous results with protein levels. By using an ATP analog-sensitive *cdc28* mutant (the only cell-cycle cyclin-dependent kinase in yeast, CDK), overexpressing different cyclins, and mutating predicted CDK sites on Vac17p, they showed that yeast's CDK directly phosphorylates Vac17p on at least 4 residues. However, phosphorylation is not only the signal for degradation, since in the phosphomutant *vac17*-4A (or in *vac17*-4D/E, personal communication), where the four CDK sites are mutated to alanine (or to aspartate/glutamate), vacuole inheritance is only partially affected, and the defect is augmented if combined with the *myo2* phosphomutants. Because two of the four Vac17p's CDK sites reside in the Myo2p-binding domain (S119 and T149), they tested if phosphorylation affected the interaction with Myo2p. Although they only reported the results obtained in a pull-down with the *vac17*-4A protein, they stated that CDK phosphorylation of Vac17p promotes the association with Myo2p, consistent with the fact that the phosphorylation pattern mimics vacuole inheritance. The other two CDK sites are on or close to the PEST domain (S178 and T248), so they could possibly affect the turnover of Vac17p, however this possibility was not explored in the study. Although is clear that Vac17p is phosphorylated in a regulated manner by yeast's CDK, the actual purpose or the mechanism of action of these modifications still are undefined.

Another of the class I vac mutants, *vac10-1*, was subsequently cloned and identified as a point mutation in the gene *PTC1*, encoding the serine/threonine phosphatase mentioned above (required for late stages of cER inheritance; Jin *et al.*, 2009). Phosphatase-dead versions of Ptc1p, including the *vac10-1* mutation, are defective in vacuole movement, as well as movement of practically every other myosin-V cargo (except for late Golgi and microtubules). Surprisingly, *ptc1*Δ in their background strain cause both myosin-Vs to mislocalize, which they attributed to the fact that the levels of their cargo receptors are decreased (Vac17p, Mmr1p, and Inp2p protein levels are decreased, but not Vac8p levels – however, they did not test receptors of unaffected cargoes, like Ypt11p or Kar9p) and unable to associate with the motors. They found that in the *ptc1*Δ, Vac17p-3XGFP is now all around the vacuole rather than at the leading edge and that a Myo2p-Vac17p fusion protein could restore vacuole transport in the *ptc1*Δ. They concluded that Ptc1p controls the association of Myo2p with the vacuole receptor complex, and hypothesize that it may control the association with other cargo receptors as well.

Peroxisome membranes

Peroxisomes are organelles characterized by their biochemical functions: production and decomposition of H_2O_2 (hydrogen peroxide), β-oxidation of fatty acids, and synthesis of carbohydrates from fats. Like the vacuole, these functions are essential, especially in nutrient-challenging environments, so it

must also be a surprise to know that inheritance of peroxisomes is not essential under laboratory conditions. Similarly to the vacuole, peroxisomes can be generated '*de novo*', although in this case, the source of the membranes and the mechanism of protein targeting still is ill-defined and it is under great deal of debate. Due to this controversy, I will only review the major findings that lead to the discovery of its main Myo2p-receptor.

The first report that peroxisomes are inherited in a Myo2p-dependent manner came from the analysis of peroxisome inheritance *in vivo* by time-lapse microscopy (Hoepfner *et al.*, 2001). By looking at a GFP-peroxisomal marker (GFP-PTS1), they noticed that peroxisomes (on average 9 per cell) behaved as a migratory organelle, with half of them retained in the mother at fixed positions, while the other half becoming fixed at the bud tip after movement. They reasoned that fission of peroxisomes could have an effect on its inheritance, so they looked at GFP-PTS1 in mutants of the dynamin-related genes (*VPS1*, *DNM1*, and *MGM1*) and found that the normal morphology and number of peroxisomes was only affected in *vps1*Δ. Even here, where each cell would only have 1 – 3 big peroxisomes, tubular extensions would form and move into the growing bud. Use of microtubule inhibitors or mutants had no effect on the inheritance pattern; however the authors notice a strong resemblance to actin dynamics in the movement of peroxisomes. Peroxisomes would always associate with sites of growth in the bud and a subset of peroxisomes in the mother would lie along phalloidin stained actin cables, which was especially

evident in *vps1*Δ cells. Moreover, in Lat-A treated cells, or in the GTD *myo2* mutants, peroxisome movement and inheritance was abolished, clearly indicating that they are a cargo of Myo2p.

This first study also showed that only half of the peroxisomes moved into the bud, while the other half was retained in the mother at fixed positions associated with the cortex. A protein responsible for that anchoring was later identified as Inp1p (Fagarasanu *et al.*, 2005). In *inp1*Δ cells, peroxisomes are not associated with the cortex and are highly mobile, and very frequently buds will acquire the whole population of peroxisomes, although they also saw incidences where in buds with the right amount of peroxisomes, just before cytokinesis, the peroxisomes would return to the mother cell. Consistent with its role in anchoring, overexpressing Inp1p makes peroxisomes become static on the cortex and a high frequency of buds not receiving any, while Inp1p also decorates the cortex (but it was unclear if it is associating with the plasma membrane or the cortical ER), suggesting the presence of additional factors that, consistent with the fact that peroxisomes do not have any cellular location preference, must be widely localized throughout the whole cell cortex.

Right after Inp1p was discovered, the same group reported the identification of the peroxisomal-myosin receptor, Inp2p (Fagarasanu *et al.*, 2006). They analyzed the dynamics of peroxisomes in wild-type cells and then screened visually a subset of mutants for defects on the normal dynamics. The set of mutants studied needed to have two characteristics: (1) needed to be a

peroxisomal protein or have a "punctate" cytoplasmic localization, and (2) needed to have predicted coiled coil regions. Of the ~250 candidate mutants, only a strain deleted for *YMR163* exhibited a complete absence of peroxisomes in the bud, renaming the locus *INP2*. The defect was specific for peroxisome segregation, as vacuoles, mitochondria, and spindle were delivered fine in *inp2*Δ cells, and the actin cytoskeleton organization was unaffected. Moreover, Inp2p exhibited all the expected characteristics of a Myo2p receptor: 1) Inp2-GFP co-localized with a peroxisomal marker; 2) Inp2-TAP co-fractionates with peroxisomes, and in isolated peroxisomes subjected to disruption and fractionation, Inp2p-TAP behaves as an integral membrane protein; 3) Inp2-TAP protein level varies with the cell cycle; and lastly, 4) Inp2p interacts by two-hybrid and *in vitro* with the GTD of Myo2p. They also showed that Inp2-GFP labels mostly the peroxisomes in the bud and not the ones in the mother and, upon overexpression of *INP2*, mothers are now depleted of peroxisomes without affecting the distribution of other cargoes. Before this study, only two myosin V adaptors/receptors for organelles had been found (melanophilin and Vac17p), thus the identification of Inp2p strengthens the now well established idea that myosin cargoes are recognized and their transport regulated through the action of membrane receptors.

The same group subsequently identified the Inp2p binding region in Myo2p by scoring peroxisome inheritance in a collection of point mutants of Myo2p made in conserved residues (Fagarasanu *et al.*, 2009). They found that

the Inp2p binding region overlaps with the secretory vesicle region, and mutants in this region have decreased affinity for Inp2p. Just like cells overexpressing *INP2*, these new *myo2* point mutants have Inp2p labeling on all their peroxisomes yet they are not transported into the bud.

As mentioned above, Inp2p levels fluctuate with the cell cycle, suggesting that, just like Vac17p in vacuole inheritance regulation, synthesis and degradation of the receptor could result in the assembly and disassembly of the transport complex with Myo2p. In fact, *INP2* mRNA was found in a previous genome-wide screen for genes regulated in a cell cycle-dependent manner (Spellman *et al.*, 1998), nevertheless the protein levels are uncoupled from the mRNA levels in situations where peroxisome inheritance is disrupted. This indicates that there is a feedback signal coming from the delivery of peroxisomes into the bud towards the levels of Inp2p in the cell; in the absence of transport (e.g. peroxisome-specific *myo2* mutants) the levels of Inp2p increase and now labels all the peroxisomes in the mother, while when no retention mechanism is in place (e.g. *inp1*Δ), Inp2p levels are too low and unable to be visualized on any peroxisome under the scope. Although the main way to regulate peroxisome inheritance in budding yeast seems to be through the Inp1/2p interplay, other studies also implicate peroxisome biogenesis and fission in its inheritance. Pex3p, previously shown to play a role in peroxisome biogenesis from the ER, has recently shown to bind Myo2p in the yeast *Yarrowia lipolytica*, coupling biogenesis with motility (Munck *et al.*, 2009);

similarly, peroxisomes in cells lacking part of the scission machinery and also missing Inp2p failed to divide and segregate altogether, suggesting that motility may help division. Moreover, the fact that there is no clear homolog for Inp2p outside budding yeast's family supports the case for these other proteins to act as peroxisome-specific myosin receptors in other organisms, coupling biogenesis and division to segregation. Interestingly, Pex3p is widely conserved in evolution and it has also recently shown to bind directly to Inp1p (Munck *et al.*, 2009), positioning itself as a central player in peroxisome life, coupling biogenesis (Pex3p function in the ER) to retention (by binding to Inp1p) and motility (by binding to Myo2p).

Astral microtubules

Since fungi undergo closed mitosis, microtubules nucleated by the spindle pole body (SPB) are separated by the nuclear envelope, generating two distinct microtubule populations: nuclear and astral (or cytoplasmic) microtubules. Nuclear microtubules are involved in generating the spindle, required to separate the chromosomes prior to mitosis, and the astral microtubules are necessary to orient and position the spindle relative to the axis of division. Because the site of cytokinesis is fixed at the mother-bud junction, orientation of the spindle is critical for a successful division, and so yeast uses two partially parallel mechanisms to ensure correct spindle orientation and positioning. The first, or early, pathway is Myo2p-dependent and its main function is to align the spindle along the axis of division, while the

second, or late, pathway is dynein-dependent and its main function is to pull on one side of the nucleus to get the aligned spindle through the neck and segregate the chromosomes. There are several good reviews on yeast's spindle orientation that discuss all aspects of it (Miller *et al.*, 2006; Moore *et al.*, 2009), so I'm going to summarize only the early, Myo2p-dependent pathway below.

A role for both cytoskeletal elements in spindle orientation and migration was described early in the '90s (Palmer *et al.*, 1992). Here, cells were synchronized before anaphase and then the effect of disrupting either actin or microtubules examined soon after release from the block. It was clear that you needed both elements, but the link between them remained elusive until studies from our lab in collaboration with Tim Huffaker's lab demonstrated that Myo2p, through Kar9p, coupled actin-dependent movement to microtubules (Yin *et al.*, 2000). *KAR9* was initially discovered as a mutant where the two nuclei of a zygote failed to migrate and fuse, a process known as karyogamy (hence the gene name; Kurihara *et al.*, 1994). It was later shown to also affect nuclear orientation during mitosis (Miller and Rose, 1998). Since it was known at the time that all nuclear movements are dependent on astral microtubules, it was a surprise to find that Kar9p localization was independent of microtubules but dependent on actin, and yet, *kar9* mutations specifically affected nuclear orientation and migration (Miller *et al.*, 1999). This conundrum positioned Kar9p as a very likely candidate to link the two cytoskeletons. With the finding in the lab that *tpm1* and selected *myo2*

conditional mutants rapidly affected spindle orientation, and the known interaction between Kar9p and the microtubule plus-tip tracking protein Bim1p, Kar9p became the ideal protein to link the two cytoskeletons. In fact, localization of GFP-Kar9p was found to be affected in only the *myo2* alleles that also showed spindle orientation defects (Yin *et al.*, 2000). In the same study, Kar9p was shown to interact *in vivo* and *in vitro* with the Myo2p GTD, and that interaction was lost in the mutated Myo2p-tails that affected spindle orientation and Kar9p localization. The interaction between Myo2p and Kar9p found in this study, together with the known interactions between Kar9p and Bim1p and between Bim1p with microtubules, provided the molecular linkage from actin cables to microtubules, explaining all the data from the previous decade suggesting a link between the cytoskeletal elements in yeast. The Huffaker lab went on to show that a Myo2-Bim1p fusion can bypass *KAR9* and that the movement of astral microtubules along actin cables is dependent on the velocity of Myo2p, clearly demonstrating that Kar9p's function is to act as a microtubule receptor and that the Bim1-Kar9p complex is directly transported by Myo2p along the actin cables (Hwang *et al.*, 2003). Still the mechanism of binding and release of Kar9p by Myo2p is unresolved, but current data suggest that the secretory vesicle and Kar9p binding sites overlap, implying that at least for these cargoes, a competition mechanism could regulate what cargo to bind. Likewise, the mechanism by which Kar9p is asymmetrically loaded unto the bud-ward SPB is unclear.

Kar9p asymmetry is very important as it ensures that only the old SPB gets translocated through the neck, with the newly duplicated SPB remaining in the mother. In part, the fact that the old SPB is the one inherited by the bud contributes to the asymmetry by generating a bias for Kar9p recruitment to the old over the new SPB. As the new SPB is formed it cannot organize astral microtubules, while the old SPB already has and keeps forming microtubules that can interact with the cortex and recruit Kar9p (Shaw *et al.*, 1997; Liakopoulos *et al.*, 2003; Cepeda-Garcia *et al.*, 2010). However, Kar9p asymmetry is not achieve solely on this temporal delay, as disruption of nuclear microtubules with nocodazole or tubulin mutants erase the old or new label from the SPBs and yet, upon microtubule reorganization, only one SPB becomes labeled by Kar9p irrespective of it being the old or new SPB (Pereira *et al.*, 2001; Cepeda-Garcia *et al.*, 2010). Both phosphorylation and sumoylation have been implicated in Kar9p asymmetry, however their mechanism of action still is unclear (Moore and Miller, 2007; Leisner *et al.*, 2008). Kar9p is phosphorylated by CDK, and phosphomimetic mutants partially affect its localization to the SPB. It was proposed that phosphorylation releases Kar9p from the SPB, either to leave the new SPB or to travel on a microtubule out of the old one. Likewise, it was shown that Kar9p is sumoylated *in vivo* and mutants unable to be sumoylated were slightly more symmetric, but the defect was exacerbated in a double phosphomimetic and SUMO-deficient Kar9p mutant.

Kar9p and Bim1p are yeast homologues of animal APC and EB1, respectively, and although not in the same cellular process, their complex formation seems to be conserved. Melanophilin, the Myosin-Va receptor in melanosomes, was found to track with microtubule ends in an EB1-dependent manner (Wu *et al.*, 2005). Melanophilin in turn recruits Myosin-Va to the microtubule ends where, the authors speculate, may serve to focus the transfer of melanosomes from microtubules to actin at the start of the actin-rich region of the cell near the cortex.

Mitochondrial membranes

The mitochondrion is most popular for being the site of aerobic respiration and for its role in cellular death (apoptosis). However, it also carries out many other essential processes (metabolism, ion homeostasis, etc.), in addition to having its own genome, and unlike the previous organelles discussed, a mitochondrion cannot be generated *de novo*, making its inheritance absolutely critical for the continuation of the organism. One of the earliest discoveries related to yeast's mitochondria was the isolation of *petite* mutants, cells that were deficient in respiration. The fact that this phenotype was hereditary lead to the equivocal notion that the mitochondria in yeast is not essential. These mutants have lost part, or in some cases all, of their mitochondrial DNA, and hence lack the capacity of aerobic oxidation, but still have mitochondria and their inheritance is essential. Below I will describe its inheritance cycle, and present the current models of how mitochondria are

transported into the growing bud.

Mitochondria are not discrete, isolated organelles, in fact they are a highly interconnected branched network. The overall morphology depends on the balance between fission and fusion and between forward and retrograde transport. Like other organelles, their inheritance is coordinated with the cell cycle, resulting in a predetermined, ordered set of movements that partition the mitochondrial population equally between the cells. As soon as bud emergence occurs, mitochondrial tubules align along the mother-bud axis and move bidirectionally, with some moving towards the bud while others away from it. Both of these movements are dependent on actin, although they are mediated by two distinct mechanisms. Once these tubules reach the bud or mother tips they become attached to the cortex and immobile until mitosis is completed, when they are released and distributed throughout the cell. From this cycle we can clearly distinguish three types of events required for a successful segregation of the mitochondria: forward movement, retrograde movement, and retention at the poles. All of these events are actin dependent but only the retrograde movement has a clear mechanistic insight on how actin is involved while forward movement and retention are still controversial.

The first indication that actin was involved in mitochondrial organization came from studies of actin mutants (Drubin *et al.*, 1993). Mitochondrial morphology was affected differentially by actin mutations, and in wild-type cells the mitochondrial tubules co-align extensively with actin cables.

Moreover, the actin alleles that affected mitochondria are the ones with mutations in the myosin binding site, and treatment of isolated mitochondria with ATP, or use of actin filaments coated with a subfragment of the myosin, inhibited the binding of the mitochondria to filamentous actin *in vitro*, suggesting the involvement of a myosin motor. Shortly after, Liza Pon's lab found an ATP-dependent motor activity on the mitochondrial surface by using isolated immobilized mitochondria in an actin binding and motility assay (Simon *et al.*, 1995). Moreover, the binding and sliding of actin filaments on the mitochondria required ATP hydrolysis, all consistent with the biochemical activities of a myosin motor. However, careful examination of mitochondrial dynamics by time-lapse microscopy revealed two confounding pieces of data: that mitochondria exhibit retrograde flow on actin cables while these cables are tracks for bud-directed anterograde movement, and that the velocity of mitochondria movement was not affected by mutations in Myo2p, or any of the other myosins in yeast (Boldogh *et al.*, 2004). Both types of movements were, however, dependent on actin cables, because destabilization of cables using a conditional formin mutant (*bni1-11 bnr1*Δ) abolished all mitochondrial movements (Fehrenbacher *et al.*, 2004). The investigators reasoned that the same actin track could allow both movements since actin cables exhibit retrograde flow and, if the mitochondria are attached tip-to-tip with the cable, as the cable grows away from the bud, the mitochondria will also move in the same direction. Consistent with this idea, they found that the velocity of mitochondria moving backwards on a cable was the same as the velocity of

grow of its associated cable. Thus, the investigators conclude convincingly that the retrograde movement of mitochondria is the result of stationary mitochondria being attached to a cable undergoing formin-driven polymerization away from the bud. But what about the forward movement?

In a continuation of their results with actin binding to isolated mitochondria (see above), the Pon lab carried out a proteomics approach to identify this ATP-sensitive actin binding activity (Boldogh *et al.*, 2001). They salt-washed isolated mitochondria to extract membrane associated proteins and used the extract to identify an ATP sensitive actin binding fraction that restores actin binding to salt-washed mitochondria. They then subjected this fraction to mass spec-based peptide sequencing and identified Arc15p, a subunit of the Arp2/3 complex (a complex of seven proteins involved in the polymerization of branched networks of actin). It is important to note that they did not find any of the other components of the complex in this fraction. Moreover, Arc15-GFP, while not showing defects in actin dynamics or localization to actin patches, affected mitochondrial morphology and inheritance, suggesting that its localization to the mitochondria is a novel function of Arc15p independent from its role in actin polymerization. Nevertheless, the authors interpreted the data as indicating that the Arp2/3 complex associates with mitochondria and, by virtue of its actin polymerization activity, drives the movement of the organelle. There's a host of questions that this study left unanswered such as: if Arp2p is all around the mitochondria,

and indeed they see 'actin clouds' surrounding them, why does the Arp2/3 activity only propel the organelle forward? Is the whole Arp2/3 complex at the mitochondria, or just Arp2p and Arc15p? If the Arp2/3 complex is the forward motor, when inactivated all the mitochondria should be in the mother or exhibiting retrograde flow. They report that in the *arp2* and *arc15* mutants all mitochondrial movements stop, yet 60% of budded cells have mitochondria in their buds. As a reconciliatory note, it is important to say that multiple genetic pathways have been uncovered that affect mitochondrial inheritance (genetic data indicate that *MMR1*, *YPT11*, and *GEM1*, each contributes to mitochondrial inheritance independently; Frederick *et al.*, 2008). Perhaps there are multiple mechanisms to move and segregate such an essential organelle as there would be considerable pressure in evolutionary terms to do so. Note that the Arp2/3 complex would propel the mitochondria but would not attach it to the cable, a different complex, termed the mitochore (as an analogy for the kinetochore), has been proposed to provide that activity in a transient manner, allowing for movement of the mitochondria along the actin cable tracks (Boldogh *et al.*, 2003).

An alternative model is that Myo2p provides both the actin binding and the motor activity seen in the early *in vitro* studies with isolated mitochondria. The first evidence for this model came from two papers from the Matsui lab where they found genetic and physical interactions between two proteins involved in mitochondrial inheritance, Mmr1p and Ypt11p, and Myo2p (Itoh *et*

al., 2002; Itoh *et al.*, 2004). In an effort to characterize these interactions, they generated conditional mutations in the Myo2p GTD that abolished each interaction separately. The *myo2-573* mutant is temperature sensitive for growth although, surprisingly, the authors state that polarized secretion, vacuole inheritance, and actin organization is normal, and the only affected cargo is the mitochondria. This mutant is synthetic lethal with *ypt11*Δ, a positive regulator of mitochondrial inheritance, and does not interact with Mmr1p, also involved in mitochondrial inheritance. Ypt11p was found to interact in a GTP-dependent manner with Myo2p, and its function in mitochondria required that interaction. When overexpressed, mitochondria accumulated in the bud, but mutations in Ypt11p that abolished its interaction with Myo2p, or *myo2-338* defective in binding Ypt11p, suppressed that phenotype. The authors favor the idea that Mmr1p is the mitochondrial receptor and that the *YPT11* and *MMR1* genes represent two independent pathways for mitochondrial inheritance, both of which require Myo2p functions. However, the localization of Ypt11p does not agree with this role since it does not localize with the organelle and instead, its localization depends on Myo2p. Normally, Ypt11p labels the ER with an enrichment in the bud, but in the *myo2-338* mutant that does not binds Ypt11p, the enrichment in buds is lost. As mentioned above, a third genetic pathway was recently discovered involving the Rho GTPase Gem1p, and this pathway would be Myo2p independent.

Perhaps the most convincing evidence for a role of Myo2p in the forward transport of mitochondria came from studies of isolated mitochondria, from specific tail mutants of Myo2p, and cells depleted for Myo2p or its essential light chain (Altmann *et al.*, 2008). Using promoter-shut off strains of *MYO2* and *MLC1*, the Westermann lab showed that in *in vitro* actin binding assays with isolated mitochondria, the ATP-sensitive binding of mitochondria to actin filaments was lost when expression of these genes was turned off. Likewise, pre-incubation of wild-type mitochondria with Myo2p antibodies (it was not clear if raised against the tail domain, the head domain, or the full-length protein) completely block binding to actin. Using a collection of strains carrying point mutations in the GTD, they found that only a subset of mutations in subdomain I (see above) caused mitochondria to clump or aggregate and blocked transfer into buds. They next isolated mitochondria from these mutant strains and found a considerable reduction in the percentage of mitochondria that bind actin. Also, time-lapse microscopy of these mutants using a mitochondrial marker revealed that anterograde movement was severely impaired after incubation at nonpermissive temperature (defined in this study as 3 hours at 37°C – note that these subdomain I mutants grow normally at all temperatures). The authors suggested that movement of a large, tubular organelle, containing complexes that bind actin throughout its whole length (the mitochore), will require a large number of motors, and therefore the velocity of the motor will not be the limiting factor. This statement explains why in a previous report a myosin

motor with shorter lever arms did not affect the velocity of moving mitochondria. Still, the identity of the Myo2p receptor on the mitochondria remains unclear. Since *ypt11*Δ or *mmr1*Δ by themselves do not greatly affect mitochondrial inheritance, it is unlikely that they act as Myo2p receptors; however studies using isolated mitochondria from these mutant strains have not been reported. In conclusion, although inheritance of mitochondria is fundamental for survival, it does not seem to be an essential Myo2p function, consistent with the idea that there are many independent pathways for mitochondrial inheritance.

Finally, the third event influencing the efficiency of mitochondrial inheritance is retention at the cell poles. This retention is restricted to the mother and bud tip and it is an active process that requires the delivery of mitochondria to these zones. It has been suggested that retention is an actin dependent process because in the *act1-159* mutant, retention at the mother tip is compromised (Yang *et al.*, 1999). The same lab later reported that retention at the bud tip was actin dependent because it needed Myo2p to walk down the cables and reach the bud tip to deliver a retention factor that works in a Ypt11p-dependent manner (Boldogh *et al.*, 2004). Considering the fact that in the previous paper (Yang *et al.*, 1999) they also found retention in the mother severely affected in *mmm1* and *mdm10* mutants (considered at the time part of the mitochore on the mitochondrial surface, now known to also form ER-mitochondria contact sites; Kornmann *et al.*, 2009), and that Ypt11p is

associated with the ER, it is tempting to think that retention at both poles is mediated by the ER and not by a cortical structure. However, in a recent screen for suppressors of a mitochondrial fission mutant, the cortical anchor Num1p was identified as having a role in adhesion of mitochondria to the mother cell tip (Cerveny *et al.*, 2007). Pulling forces on a tubule that is anchored on the opposite site helps mitochondria to divide when the fission machinery is partially defective. In *num1*Δ cells, mitochondrial division is partially affected, and when combined with mutations in components of the fission machinery, cells contain a single interconnected network of tubules. Moreover, in these double mutants, a high proportion of cells have all their mitochondria accumulated in the bud, suggesting that retention on the mother side requires *NUM1* function.

Processing bodies (P bodies)

P bodies, ribonucleoprotein particles (RNPs) that form distinct cytoplasmic foci, are the latest of the cargoes associated with Myo2p. They function in the degradation of mRNA and in the storage of silenced mRNA, allowing for the quick translation of a specific mRNA by activating it rather than going through the whole transcription process. They were first identified in animal cells, but have since also been discovered in yeast and plants. There are several examples of myosin-Vs associating with mRNA in animal cells, but in yeast it was thought that only Myo4p was involved in those processes. However, Myo2p was recently reported to also associate with a large number of

mRNAs, and that the motor domain is important for P bodies' function (Chang *et al.*, 2008).

Fractionation of wild-type cells by differential centrifugation showed that most Myo2p is present in the P2 (or P13) and P3 (or P100) fraction. Further separation by density gradients of these fractions showed that the P2 Myo2p co-migrates with vacuolar markers but the P3 Myo2p migrated at a much denser fraction than the vesicle marker Snc1p. Most of the P3 Myo2p co-migrated with the ribosomal protein Rpl3p in a fraction that was insensitive to ATP, but sensitive to high salt or EDTA washes, indicative of protein-protein (but not Myo2p-actin interaction) or protein-nucleic acid interactions. That profile was similar to the sedimentation profile of components of polysomes, however treatment with cycloheximide prior to fractionation did not release Myo2p from this denser fraction. The Myo2p in this fraction could also bind actin in an ATP dependent manner, indicating that it is a folded, functional Myo2p. Immunoprecipitation of Myo2p from this fraction and cRNA microarray analysis showed that a large number of mRNAs are associated with Myo2p. They went on to identify this Myo2-RNP complex as P bodies, and showed that Myo2p plays a role in the disassembly of these RNP particles. Although P bodies have not been reported to undergo long range transport, they have been seen associated with actin structures or microtubules in animal cells. In yeast, no directed movement was reported but nevertheless the Myo2p motor activity was required for disassembly of these particles, suggesting that Myo2p may

apply some force necessary for disassembly by trying to walk on actin while being attached to these particles. Moreover, use of an antibody against the GTD of Myo2p does not co-IP P bodies components while an antibody against the head domain does, suggesting that P bodies might bind Myo2p through the cargo binding GTD. Interestingly, very recently it was reported that Myosin-Va also associates with P bodies in HeLa cells (Lindsay and McCaffrey, 2011). Here, however, the authors propose that MyoVa actively moves RNP particles to P bodies for their incorporation, making these particles another putative cargo for myosin-Vs.

Secretory membranes

As mentioned at the beginning of the introduction, the ability of budding yeast to grow is dependent on the selective transport of enzymes and lipids to the site of bud emergence. The delivered enzymes will remodel the cell wall allowing for membrane expansion driven by the turgor pressure and the concomitant addition of new lipids coming from the fusion of secretory vesicles. In the absence of this polarized transport, the yeast cell cannot grow, making secretory membranes the only essential function of Myo2p. In addition to secretory vesicles, several other secretory membranes have been shown to be polarized and moved by Myo2p. Recent experiments from our lab and others have shown that a small amount of polarized secretion is enough for bud growth, possibly by acting in conjunction with localized recycling of components at sites of growth (Yamamoto *et al.*, 2010; Santiago-Tirado *et al.*,

2011). I will describe what is known of these late secretory membranes, their function, and what we knew about their transport before this study.

The late Golgi comprises the last one or two trans-cisternal membranes of the Golgi apparatus where cargo modifying enzymes still localize at, such as galactosyltransferase and sialyltransferase. The trans-Golgi network (TGN) is defined as the tubular network emanating from the late Golgi where minimal cargo modification occurs but instead is the major cargo sorting station, where both incoming and outgoing traffic occurs (Griffiths *et al.*, 1989). The TGN counterpart at the opposite Golgi face would be the ER-Golgi Intermediate Compartment (ERGIC), also consisting of a tubular network that makes contacts with the cis-Golgi cisterna (Hauri and Schweizer, 1992). In higher eukaryotes, the distinction between these organelles is very clear, however in budding yeast, perhaps mostly due to the fact that the Golgi compartments are not stacked together, generally there's no distinction between the ERGIC and the cis-Golgi and between the trans-Golgi and the TGN. In this thesis, however, I make a distinction between the late Golgi cisterna and the TGN based on the morphology of carriers labeled by either Sec7p or Ypt31p. When we looked at Sec7-GFP, it labels vesicular structures that vary in size, but are always globular in morphology. In contrast, GFP-Ypt31 labels vesiculotubular membranes that in many instances are seen moving as a tubule that later breaks up into smaller vesicles. These different compartments are basically the result of a linear conversion of secretory membranes as they mature on their

way to the plasma membrane: from late Golgi to TGN and finally to secretory vesicles. Previous to this work, only secretory vesicles have been shown to be a direct cargo of Myo2p by visualization of GFP-Sec4 in Myo2p mutants that move at different rates (Schott *et al.*, 2002), while the late Golgi and the TGN were expected to be since these compartments are delocalized in *myo2* mutants after long shifts to the restrictive temperature (Rossanese *et al.*, 2001; Casavola *et al.*, 2008; Lipatova *et al.*, 2008). How Myo2p associates with all these compartments is not known, although it has always been an attractive idea that it is through the action of Rab GTPases. Rabs have specific localizations in the cell and regulate membrane transport steps from or to the membranes they label. Consistent with this, recently it was found that the interaction between Myo2p and Ypt11p can bridge Myo2p to the late Golgi (Arai *et al.*, 2008). Likewise, Myo2p GTD also binds activated Ypt31/32p (the Rab GTPases associated with the TGN) and mutations that abolish that interaction are lethal for the cell (this thesis; Casavola *et al.*, 2008; Lipatova *et al.*, 2008), indicating that Rabs play an essential role in Myo2p function. However, Ypt11p does not co-localizes with Myo2p as expected for a Myo2p receptor. Moreover, *ypt11*Δ cells are viable and Golgi localization is not grossly affected, indicating that although Ypt11p may play a role in late Golgi inheritance, is not the main receptor. Noteworthy, there is no clear homolog for Ypt11p in higher eukaryotes and, although more closely related to exocytic Rabs by sequence, its function is not known. In fact, the effector domain of Ypt11p is almost identical to the effector domain of Ypt31/32p and, since most of the effects

seen in Golgi localization were in cells overexpressing *YPT11*, one can imagine a situation where the excess Ypt11p is displacing Ypt31/32p and is acting, under these conditions, as a Myo2p receptor.

Ypt31/32p are redundant Rabs involved in intra- and post-Golgi transport, as well as recycling of proteins from endosomes to the Golgi (Jedd *et al.*, 1997; Ortiz *et al.*, 2002; Chen *et al.*, 2005). Activation of Ypt31/32p is required for the generation of secretory vesicles and the subsequent recruitment of the Rab GEF Sec2p, a process thought to contribute to the activation of the secretory vesicle Rab Sec4p (Ortiz *et al.*, 2002). Ypt32p was found to co-IP with Myo2p from the membranous fraction and to interact by two-hybrid in a GTP-dependent manner (this thesis; Casavola *et al.*, 2008; Lipatova *et al.*, 2008). This interaction is essential since mutations in the Myo2p GTD that abolish Ypt31/32p binding are lethal but can be rescued, albeit very poorly, by fusing Ypt31p to the end of the mutated Myo2p (Lipatova *et al.*, 2008). The accumulation of unpolarized secretory vesicles in strains overexpressing GDP-locked Ypt32p or in the Ypt31/32-binding deficient *myo2* mutant cells suggest that Ypt31/32p play a role in the association of post-Golgi vesicles with, and their subsequent movement by, Myo2p. However, in *sec2* mutants incubated at the restrictive temperature, secretory vesicles are no longer transported (Walch-Solimena *et al.*, 1997). Likewise, even at the permissive temperature, overexpression of activated *YPT32* in *sec2-59* cells causes an accumulation of vesicles, and of Golgi membranes, in the mother cell

(Casavola *et al.*, 2008). These observations indicate that Ypt31/32p, although required, are not sufficient for recruitment of Myo2p to their membranes. Still, since Ypt31/32p compartments can convert into Sec4p positive compartments, it is not known if Myo2p associates with these membranes through the same receptor or uses specific ones for each compartment.

There is another compartment that contains both endosomal and Golgi markers, but unique in that it contains several chitin synthases, and was, therefore, called the chitosome (Leal-Morales *et al.*, 1988). Chitin is a fundamental component of the yeast cell wall, required at different places during specific stages of the cell cycle. Just before bud emergence, a chitin ring forms marking the site where the bud will emerge. This ring persists throughout bud expansion and then, right after cytokinesis, chitin is deposited from both sides of the ring forming a disc of chitin separating the two cells, called the primary septum. This structure is reinforced by addition of layers of glucan and mannan, the major cell wall sugar polymers that form the secondary septum. At this point, a chitinase partially digest the primary septum separating the two cells, leaving most of the chitin ring on the mother side, a feature known as the bud scar. The major chitin synthase is Chs3p, and it was found that its polarization was blocked by temperature shift in a *myo2-66* strain (Santos and Snyder, 1997). The interesting thing about this process, as summarized above, is the unique spatial and temporal regulation of the chitin synthases delivery. Although their transport depends on actin and

Myo2p, like other membranes of the secretory pathway, their delivery is regulated and the same enzyme is recycled back and forth many times (Chuang and Schekman, 1996; Santos and Snyder, 1997). A difference between the transport of the chitin synthases and other cargo to the plasma membrane is their dependence on a protein complex of Chs5p and Chs6p (Santos and Snyder, 1997; Ziman *et al.*, 1998). In the absence of Chs5p or Chs6p, the chitin synthases still localize to chitosomes, but now they are not transported to their sites of work. It was later found that Chs5p forms a large multiprotein complex with Chs6p or three Chs6p paralogs: Bch1p, Bud7p, and Bch2p (Sanchatjate and Schekman, 2006). This complex forms a coat around vesicles carrying special cargo such as the chitin synthases, and is involved in the recruitment and capture of these enzymes into the forming vesicle. This new coat has been named the exomer for its role in the exocytosis of regulated cargo (Wang *et al.*, 2006). Interestingly, the localization and function of this new coat was found to be dependent *in vivo* on active Arf1p and, at least *in vitro*, on the PIs phosphatidylinositol 4-phosphate (PI4P) or phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$; see more below). This novel branch of the secretory pathway represents a new secretory cargo for Myo2p and it is unclear if it depends on the same adaptor/receptors as other exocytic membranes. Recently, *sro7*Δ and *ypt31*Δ were found to uniquely affect transport of Chs3p, but not other constitutively secreted proteins (Zanolari *et al.*, 2011). Also, they found that Chs3p transport is exquisitely dependent on Sec4p function, since in *sec4-8* cells even at the permissive temperature, Chs3p localization was

impaired (however, I have found that many secretory markers, such as GFP-Ypt31, GFP-Snc1, or RFP-Snc2, are affected in this allele even at the permissive temperature). Not surprisingly, Chs3p localization is dependent on the exocyst, however, during bud emergence Chs3p is targeted to the bud neck while Sec8p, an exocyst component, is delivered to the bud tip. The fact that they have different itineraries suggests that exocyst components and Chs3p travel on different vesicles. Transport of exomer-dependent cargo, or of the late Golgi or the TGN, is not essential for viability under lab conditions, leaving transport of secretory vesicles as the only essential duty of Myo2p.

The first evidence that secretory vesicles are essential for cell growth came from the isolation of the original *secretory* mutant, *sec1-1* (Novick and Schekman, 1979). In that mutant, upon shift to the restrictive temperature, protein synthesis continues but cell growth is blocked. Concomitant with a cell growth block, secretory vesicles accumulate tremendously throughout the cell, and upon shift back to the permissive temperature, the vesicles disappear, accumulated cargo is secreted, and cell growth re-starts. Later with the analysis of actin and myosin-V mutants, that do not have a block in secretion and keep growing in a depolarized manner, it became clear that the highly asymmetric growth of yeast is dependent on the polarized delivery of secretory vesicles (Novick and Botstein, 1985; Johnston *et al.*, 1991; Govindan *et al.*, 1995; Schott *et al.*, 1999). With the generation of the tropomyosin mutants in the lab, we have shown that actin cables provide the tracks for the Myo2p-

dependent transport of secretory vesicles (Pruyne *et al.*, 1998). We have also shown, by using *myo2* GTD and neck mutants, that Myo2p directly moves the vesicles. However, how Myo2p associates with the vesicles, releases them at the destination, and is recycled back are still outstanding questions. The Rab GTPase Sec4p has always been an attractive candidate since it is the Rab associated with vesicles, has a role in coupling vesicle movement with tethering, and has exhibited genetic interactions with *myo2* mutations. Moreover, Rab27a (a GTPase related to Sec4p), the Rab associated with a specialized secretory organelle in melanocytes called the melanosome, was shown to be part of the receptor for Myosin-Va and required for the final movement of melanosomes to the cell cortex by the myosin motor (Hammer and Wu, 2002; Wu *et al.*, 2002).

Likewise, similarly to Rab GTPases, several examples in higher organisms indicate that PIs can act as receptors for molecular motors. The KIF1A family of kinesins uses a PI4,5P_2-specific PH domain to dock onto vesicles rich in that lipid (Klopfenstein *et al.*, 2002). Moreover, clustering of the monomeric inactive kinesin by binding lipid raft-like PI4,5P_2 domains on vesicles triggers the dimerization of the kinesin and conversion into an active, processive motor *in vivo* (Tomishige *et al.*, 2002; Klopfenstein and Vale, 2004). There are other examples of molecular motors associating with secretory membranes through direct or indirect binding of Rab GTPases and PIs (Seabra and Coudrier, 2004; Horiguchi *et al.*, 2006; Roland *et al.*, 2007). The main

focus of this dissertation is to explore the role that Rabs and PIs may have in the Myo2p-dependent transport of secretory vesicles in yeast, with the final aim of providing a better picture of the molecular mechanism that Myo2p, and most likely other myosin-Vs, uses to associate with secretory membranes.

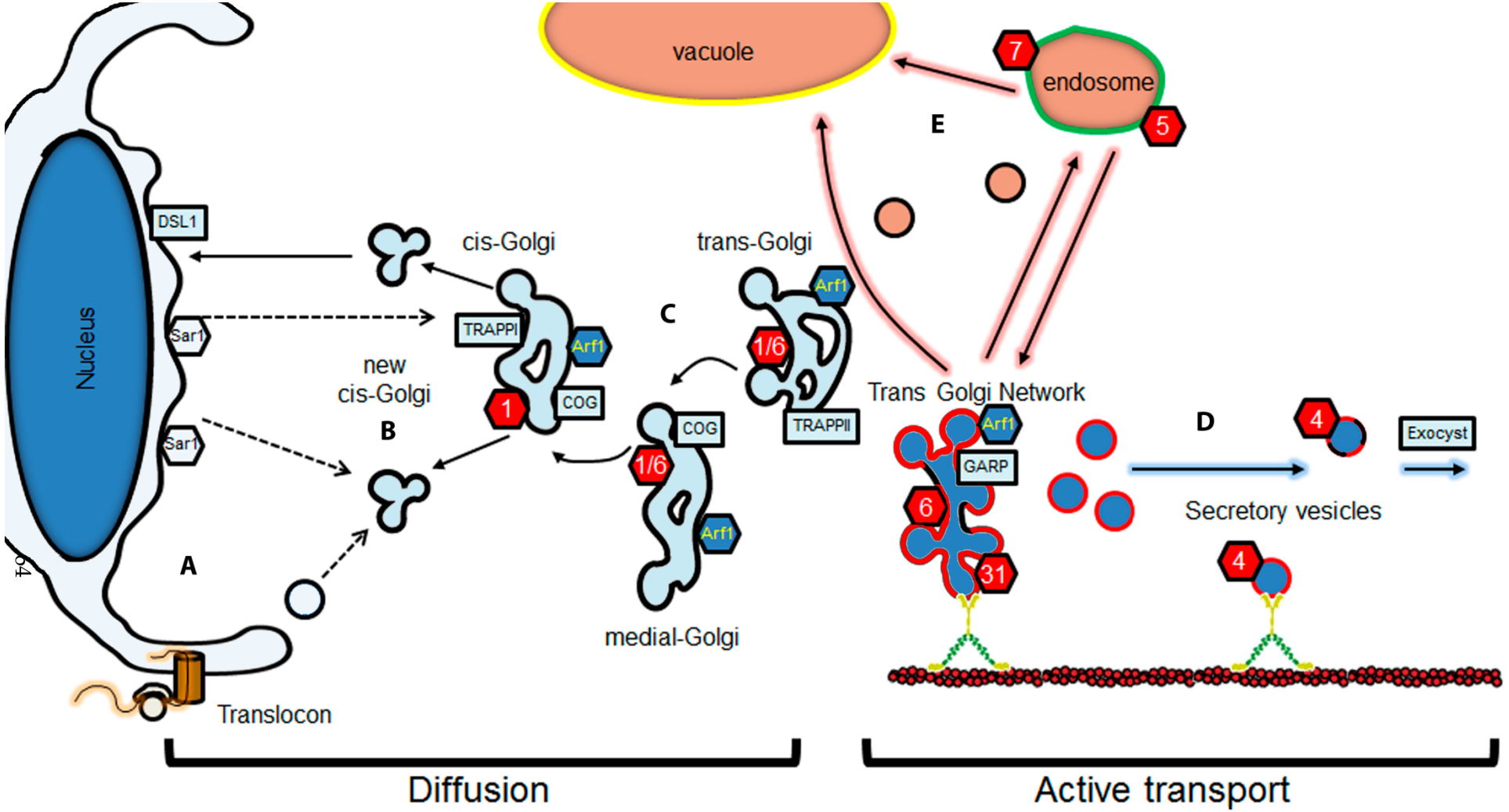
The secretory pathway and associated proteins

All eukaryotic cells contain a collection of distinct compartments separated from each other by a lipid bilayer. Each membrane-bound compartment, or organelle, carries out a specific assortment of chemical reactions that as a whole, makes cellular life possible. In order to carry out their functions, each organelle must be equipped with specific proteins, lipids, and metabolites necessary for each particular reaction. Moreover, in many instances, proteins have to be modified sequentially to reach a final, active complex, requiring communication between the organelles responsible for each modification. Some proteins have to be secreted outside the cell while others have to be embedded on the membrane of certain organelles, and all of this happens at the same time as material from the outside is been internalized and targeted to specific compartments for processing. Despite the obvious complexity of all these simultaneous fluxes of material, the cell handles it with exceptional accuracy. Although with some exceptions, all of these processes occur by intermediaries that can be vesicular or tubular in nature and transfer material from one donor to a recipient organelle, a process termed membrane trafficking. As mentioned above, in yeast membrane traffic is of utmost

importance since delivery of vesicles to the daughter cell provides lipids and enzymes that allow bud growth and later separation from the mother cell. Although there are many more obvious essential processes that occur by membrane traffic such as endocytosis or mitochondrial import, only the secretory pathway is relevant for this study. I will only, therefore, discuss how yeast regulates the secretory pathway, giving especial attention to the components that are at the interface between membrane traffic and Myo2p-dependent transport.

With his work, George Palade founded the field of the biosynthetic secretory pathway, which earns him the Nobel Prize in Physiology and Medicine in 1974. Palade and his colleagues developed an autoradiographic technique using the electron microscope to follow radiolabeled proteins in their journey from their site of synthesis to outside the cell. He cleverly chose a professional exocrine cell and fine-tuned both the *in vivo* and *in vitro* assays to be able to observe how the labeled proteins started at the ER, followed towards the Golgi in vesicles, and then onto the plasma membrane, always in vesicles. He showed that secretory cargo never mixes with the cytoplasm and that it moves between the different organelles in membrane-bound carriers. But how are all of these steps regulated? What controls the directionality? How do the carriers know where to go and fuse? The first clue came from the isolation in yeast of *secretory* (*sec*) mutants (Novick and Schekman, 1979; Novick *et al.*, 1980). Taking advantage of a special genetic background yeast strain from Susan

Henry's lab that became denser when starved for inositol, they were able to isolate mutants that blocked the secretory pathway at different steps by enriching for mutants that became denser. By looking at the ultrastructure of these and double mutants they were able to determine the order of secretory events reaching the same conclusions that Palade did a decade before, but now identifying the genes responsible for each step, making the genetic and molecular dissection of the secretory pathway possible (Novick *et al.*, 1981). The finding that Sec4p was related to *ras*, that like *ras* it bound GTP, and that it regulated the function of other late acting *sec* genes, offered the first mechanistic model for the regulation of the secretory pathway by Rab GTPases (Salminen and Novick, 1987; Goud *et al.*, 1988; Walworth *et al.*, 1989). Moreover, proteins involved in the retrieval of membrane and lumenal resident proteins were discovered, explaining how the cell could maintain the composition of its organelles despite a constant forward transport (Lewis and Pelham, 1990; Lewis *et al.*, 1990; Cosson *et al.*, 1997). Following the identification of the exocyst, a complex of eight proteins (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p) functioning at the very last step of the pathway, a molecular portrait started to emerge (TerBush *et al.*, 1996; Figure 1.5). After the discovery of all these proteins, two decades of research have shown how they work in concert to give both specificity and directionality to the pathway. Small GTPases of the Arf, Sar, and Rab families, together with SNAREs and tethering complexes, are the critical regulators of these processes by coupling carrier generation to transport, tethering, and fusion. I will



COPII vesicles

Clathrin coats

Myo2-transport along actin cables

COPI vesicles

Exomer and unknown coats

Tethers

Tethering complexes

Small GTPases: Sar1p Arf1p Rabs (Ypt1p, Ypt6p, Ypt7p, Ypt31p, Ypt5(1)p, Sec4p)

Figure 1.5 The yeast's secretory pathway. Membrane traffic is possible due to the interplay between coat proteins, tethering complexes, and small GTPases. (A) After MOS proteins are translocated into the lumen of the ER, they are packaged into COPII vesicles in a process dependent on Sar1-GTP. (B) COPII vesicles capture by incoming COPI vesicles or cis-Golgi membranes is mediated by the TRAPPI complex, and fusion occurs in a Ypt1-GTP dependent manner. (C) Intra-Golgi transport (or maturation) is dependent on the COG complex capture and Ypt1/6-dependent fusion of retrograde COPI vesicles. (D) Once the membranes mature into late Golgi, TRAPPII-activated Ypt31/32p recruit Sec2p, resulting in the recruitment and activation of Sec4p to secretory vesicles. This last step is coupled to Myo2p association with membranes. Traffic between the endosomal system and the TGN is also depicted. The colored lines represent the different phosphoinositide species: red PI4P, green PI3P, and yellow PI3,5P_2. Due to yeast's small size, vesicles in the early secretory pathway diffuse until captured by the appropriate tethering complexes, and no motor protein is required. In the later stages, however, vesicles must be bound by Myo2p for delivery to sites of growth.

summarize now the journey of a membrane or secreted (MOS) protein in yeast, highlighting how GTPases, coats, and tethers work together in the secretory pathway.

MOS proteins contain a specific ER-targeting sequence that while being translated is recognized by the signal recognition particle (SRP) complex. In yeast, the SRP is composed by the proteins encoded by *SRP14*, *SRP21*, *SRP68*, *SRP72*, *SEC65*, and *SRP54*, and by the RNA from *SCR1*. Upon binding to the signal sequence coming out of the ribosome, SRP stalls the translation of the protein until it is bound to the SRP receptor (SR) at the ER. The SR, encoded by *SRP101* and *SRP102*, is associated with the translocon (core channel formed by Sec61p, Sbh1p, and Sss1p) coupling translation of the protein with translocation into the ER lumen or membrane. As MOS is being extruded into the lumen, Sec11p cleaves the signal sequence, and the protein is either folded and released or inserted into the membrane. These MOS proteins have motifs that are recognized by adaptor proteins which tell the cell the location of its final destination. This process starts with Sec12p, an ER integral membrane protein with GEF activity for the small GTPase Sar1p, recruiting and activating the GTPase throughout the ER (in other fungi this process occurs at specific sites called tER and at ERES in mammals). Once activated, Sar1p inserts a helix into the membrane, inducing an initial membrane deformation that is stabilized by the recruitment of the Sec23/24 complex (inner coat), which are Sar1-GTP effectors. This complex acts as the cargo adaptors I mentioned, with

the Sec24p subunit (or one of its paralogues Lst1p or Iss1p) binding the cytoplasmic tails of specific membrane proteins while the Sec23p subunit recruits the outer coat components Sec13/31p. The outer coat can bind multiple Sec23p and collect them, together with their bound cargo, into the nascent vesicle. Polymerization of the outer coat drives the final deformation of the membrane and promotes vesicle fission by two parallel events: GTPase activating protein (GAP) activity on Sar1p and complete surrounding of the vesicle. The Sec23p subunit has a dormant GAP activity towards Sar1p that is activated by assembly of the outer coat, which presumably causes Sar1p to extract the helix from the outer leaflet, prompting a rearrangement of the membrane important for fission. At the same time, complete polymerization of the outer coat around the vesicle provides a driving force for membrane pinching (Jensen and Schekman, 2011). In mammals, these budded COPII vesicles fuse with each other, and with incoming retrograde transport, to form the ERGIC (see the *secretory membranes* section above), but in yeast they fuse directly with the membranes containing cis-Golgi enzymes, thus forming a new cis-Golgi cisterna (Cai *et al.*, 2007). Because COPII vesicles form everywhere on the yeast ER, and since the Golgi is not stacked but is scattered throughout, they diffuse until captured by cis-Golgi tethering complexes, and no motor protein is required. Also, unlike the mammalian case, complete uncoating of COPII vesicles doesn't happen until they are captured by the corresponding tethering factors (Cai *et al.*, 2007). Although inactivation of Sar1p occurs soon after vesicle formation, the inner coat may still localize to the vesicle by virtue

of cargo binding. This inner coat is recognized by two types of tethering factors, the long coiled coil Grh1p, and the multisubunit TRAPPI complex. TRAPPI also contains a GEF for the Rab GTPase associated with ER to Golgi transport, Ypt1p. Once captured, tethering factors may accelerate the final uncoating and coordinate fusion with the early Golgi membranes in a manner that depends on activation of Ypt1p. Once inside Golgi membranes, the MOS protein receives a number of modifications in a cisterna-specific manner. It is clear that in yeast this transport along the different Golgi cisterna occurs by cisternal maturation or progression, where COPI vesicles are constantly been generated in the later cisterna and fusing with earlier ones, delivering the required enzymes needed for the modifications (Losev *et al.*, 2006; Matsuura-Tokita *et al.*, 2006). When the membranes acquire the latest Golgi markers, specific effectors are recruited, turning the late Golgi into the TGN, where the MOS cargoes are sorted and packaged into carriers that will take them to their final destination. Again, because of the small size of the yeast cell, and the scattered nature of the Golgi cisterna, simple diffusion suffices for COPI vesicles to find and fuse with their target membranes. This retrograde traffic is also dependent on Ypt1p and the conserved oligomeric Golgi (COG) multisubunit tethering complex. COG is a Ypt1p effector, binds the Sec21p subunit of the COPI coat, and the cis-Golgi t-SNARE Sed5p, coupling tethering of specific vesicles with fusion with the correct membrane (Suvorova *et al.*, 2002). Ypt6p is a nonessential Rab that is, according to genetic analyses, partially redundant with Ypt1p in regulating intra-Golgi transport.

Overexpression of *YPT6* in *ypt1* mutants result in lethality, and *YPT1* overexpression can rescue some of the *ypt6*Δ phenotypes (Li and Warner, 1996, 1998). Subsequent analyses of null and conditional *ypt6* mutants showed defects in localization of TGN proteins, in modification of secretory proteins, and accumulation of 50nm vesicles of endosomal origin (Tsukada and Gallwitz, 1996; Bensen *et al.*, 2001; Luo and Gallwitz, 2003). In fact, another multisubunit tethering complex, the Golgi-associated retrograde proteins (GARP), is an effector of Ypt6p at the TGN, and it also binds the t-SNARE Tlg1p, thus coupling tethering at the TGN of endosome-derived vesicles with fusion (Conibear *et al.*, 2003). This function of retrieving TGN resident proteins from the endosome is unique to Ypt6p and can't be complemented by Ypt1p, although genetic arguments have also implicated the redundant Rabs Ypt31/32p in this pathway (Chen *et al.*, 2005; Furuta *et al.*, 2007), explaining why *YPT6* is nonessential.

Up to this point, the actual movement of the vesicles occurs by diffusion, however, because sites of growth are fixed, and to prevent mistargeting of cell wall modifying enzymes to the mother cell, all post-Golgi transport requires a motor (Myo2p) for targeting to its correct destination. How sorting at the TGN is coupled to Myo2p transport is not known, although just as in the earlier steps, there is some understanding regarding how Rab GTPases and tethering complexes control the directionality of the cargo in the final steps of the secretory pathway. As mentioned above, the TRAPPI complex couples capture

of COPII vesicles with Ypt1p activation at the cis-Golgi. Once these membranes mature into late Golgi, new subunits integrate into TRAPPI, switching its GEF activity from Ypt1p to Ypt32p, and is now termed TRAPPII (Morozova *et al.*, 2006). The joining of the new subunits might be due to the increase in PI4P in late Golgi membranes since many of the TRAPPII subunits exhibit genetic interactions with *pik1* mutants (see next section; Sciorra *et al.*, 2005). Once Ypt32p is activated it recruits Gyp1p, a GAP for Ypt1p, thereby inactivating Ypt1p in later Golgi cisterna (Rivera-Molina and Novick, 2009). Notably, there is a second coupling mechanism to define the Ypt1p/Ypt32p boundaries since an enzymatic GEF activity for Ypt32p can be pulled down with GST-Ypt1p-GTPγS, even if a TRAPPII-depleted cytosol is used (Wang and Ferro-Novick, 2002). In fact, there is a recent controversy regarding whether TRAPPII is a GEF for Ypt31/32p since in the crystal structure of TRAPPII, the Ypt1p binding site is still available (Yip *et al.*, 2010). Nevertheless, the newer membranes now rich in PI4P and active Ypt31/32p, are competent to recruit the GEF Sec2p (see next section), which activates the downstream Rab Sec4p. Sec2p requires a dual signal for localization: it binds active Ypt31/32p and PI4P, thus ensuring that activation of Sec4p will only occur at membranes destined to become secretory vesicles (Mizuno-Yamasaki *et al.*, 2010). Once Sec4p is activated, it recruits part of the exocyst complex to the vesicles surface, allowing for vesicle tethering only at sites where the remaining exocyst subunits are anchored. Sec2p also contributes to recruitment of exocyst components to the vesicles by binding Sec15p (Medkova *et al.*, 2006), the same subunit that Sec4p binds,

generating a microdomain containing Sec2p, active Sec4p, and the exocyst subcomplex. Once tethered at sites destined to grow, marked by the remaining exocyst components Sec3p and Exo70p, Sec4p is inactivated by the polarisome-recruited GAPs Msb3/4p (Tcheperegine *et al.*, 2005). The localization of these GAPs in turn depends indirectly (through the effects on the polarisome) on the GTPases Cdc42p and Rho1p, thus coupling the end of the active zone of one GTPase with the beginning of the next GTPase in line. Once Sec4p is inactivated, fusion occurs, and the vesicle-associated components are recycled for another round of vesicle generation, transport, and fusion. This sequence of post-Golgi events is thought to be coupled with Myo2p association with and release from secretory vesicles, however there is almost no mechanistic insight into how this occurs.

The interface between lipids and proteins in the secretory pathway

Together with the regulation imparted by Rab GTPases on the secretory pathway, lipids can also regulate diverse processes involved in membrane traffic. The first indication was the discovery that the *sec14* mutant, whose major phenotype was a block at Golgi exit, encoded a phosphatidylinositol transfer protein (PITP; Bankaitis *et al.*, 1990). It is now clear that Sec14p also regulates transport into the TGN (Curwin *et al.*, 2009), and that it is the founding member of a superclass of conserved proteins involved in lipid homeostasis. Moreover, mutations in the *sac1* gene were found as suppressors of both Sec14p inactivation and of actin defects, establishing a link between

lipid homeostasis and actin function (Cleves *et al.*, 1989). However, the first direct evidence for a role of phosphatidylinositol in membrane traffic was the demonstration that *sec14* mutants have reduced levels of PI4P and that by increasing these levels, you can rescue *sec14* cells, supporting a role for PI4P in exit from the Golgi (Hama *et al.*, 1999). At the same time it was shown that conditional mutations in the Golgi localized PI 4-kinase, Pik1p, block exit from the Golgi when PI4P levels are reduced upon shifting to the restrictive temperature (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999). It is now clear that PIs are a critical component of membrane traffic, with PI4P being the essential isoform for Golgi exit (and transport – this thesis). Yeast has the capacity to generate five PI isoforms, but only four play major roles in yeast's cell biology (Figure 1.6). Since the different isoforms can be either substrate or product of the PIs metabolizing enzymes, they can easily be interconverted in a compartment specific manner, resulting in a 4 digit code that can relay information to the cell (Di Paolo and De Camilli, 2006; Strahl and Thorner, 2007). That information can be read by lipid binding modules present in proteins, of which the most common is the pleckstrin homology (PH) domain (Figure 1.6). Moreover, by restricting the localization or the activity of the PIs kinases and phosphatases, the different PIs can serve as identification tags for the different organelles, in a manner similar to how Rab GTPases also identify specific membranes. The four isoforms and their metabolizing enzymes shown in Figure 1.6 have specific roles in distinct aspects of yeast membrane traffic and cytoskeletal organization, however only PI4P is relevant to this study. I will

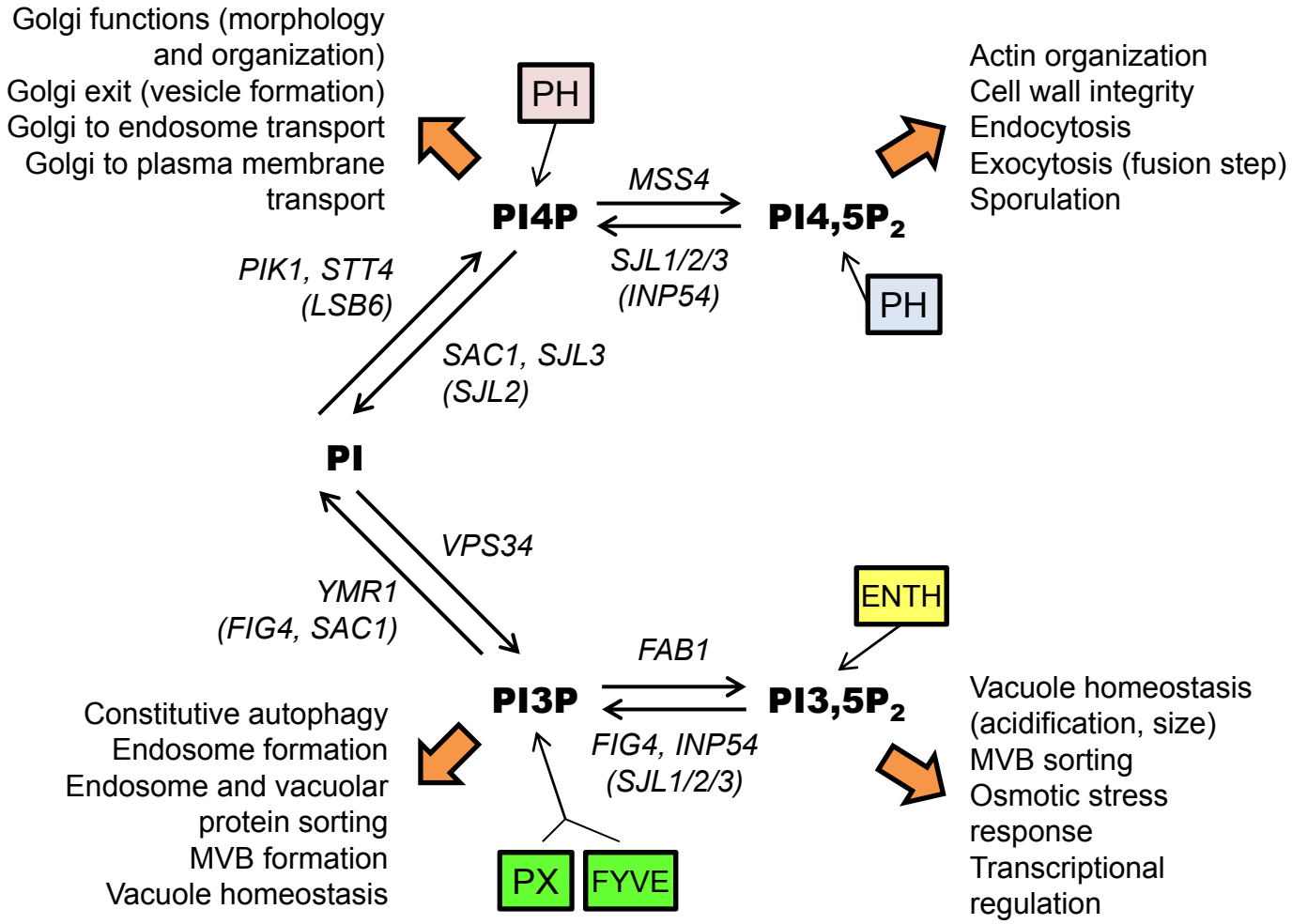


Figure 1.6 Interconversion of phosphoinositide isoforms in yeast and their binding modules. Phosphoinositides are substrates or products of different lipid kinases and phosphatases encoded by the genes below or on top the arrows, which restrict their localization to specific membranes. These isoforms can be recognized by specific protein motifs (shown in colored boxes) resulting in the regulation of specific processes on a phosphoinositides-dependent manner (colored arrows). The gene names in parenthesis denote proteins that can catalyze those reactions *in vitro* but not *in vivo*.

therefore summarize the evidence implicating PI4P in protein secretion and Golgi function, finishing with a statement about why we thought PI4P might be involved in Myo2p function.

1999 was a seminal year for the demonstration of a direct role for PI4P in the secretory pathway. It was first reported that *SAC1*, one of the *sec14*-bypass mutants, encodes a PI4P phosphatase, explaining why mutations in this gene had such diverse phenotypes (inositol auxotrophy, delocalization of the actin cytoskeleton, trafficking defects, etc.; Guo *et al.*, 1999). Following this discovery, it was demonstrated that *sec14* mutants have low PI4P levels, which in conjunction with the enzymatic activity of Sac1p, explained why inactivation of Sac1p rescued *sec14* mutants (Hama *et al.*, 1999). And finally, *pik1* was found in a screen for synthetic lethal mutations with GDI (guanine nucleotide dissociation inhibitor, negative regulators of Rab GTPases) overexpression, implicating PI4P in the last steps of the secretory pathway (Walch-Solimena and Novick, 1999). After the demonstration of PI4P role in yeast secretion, similar results were found in mammalian cells, and subsequently in plants, indicating that it is a conserved role and that any mechanistic insight found in yeast very likely will apply to other cells (Santiago-Tirado and Bretscher, 2011 in press).

Yeast contains three PI 4-kinases (Figure 1.6), each with specific localizations and functions (Strahl and Thorner, 2007). Pik1p is a soluble enzyme that localizes to the late Golgi and undergoes a regulated

nucleocytoplasmic shuttling (Strahl *et al.*, 2005; Demmel *et al.*, 2008a). Phenotypic analyses indicated that it regulates secretion, Golgi and vacuole morphology (most likely by its effects on secretion), and Golgi recycling. The other two enzymes are Stt4p and Lsb6p, which localize to the plasma and endosomal membranes and have no role in Golgi function. However, most of the cell's PI4P is generated by Pik1p and Stt4p at the Golgi or the plasma membrane, respectively, resulting in two distinct pools of PI4P. Moreover, many of the known PI4P effectors bind it weakly which cannot account for their exquisite localization to specific membranes, especially since at least two distinct pools of PI4P exist in the cell. It has therefore been proposed that PI4P, as well as other PIs, work cooperatively with other factors, such as GTPases, in a mechanism called coincidence detection. By using a dual recognition mechanism involving small GTPases and PI4P, membranes rich in these factors can recruit effectors to segregate, package, and transport cargo to specific locations (Santiago-Tirado and Bretscher, 2011, in press). Moreover, both Pik1p and Sac1p localization is dependent on growth conditions, and it has been suggested that the coordinated relocation of both enzymes would create a line of communication between secretory function and nutrient conditions (Demmel *et al.*, 2008a). Genetic interactions between these PI4P metabolizing genes and others involved in a variety of membrane transport steps are consistent with this idea. As mentioned above, mutations in nonessential subunits of the TRAPPII complex become essential under reduced PI4P levels. More interestingly, some of the mutations that show functional

relationship with PI4P also are involved in Myo2p's secretory function: *ypt31*Δ, *ypt31*Δ *ypt32*^{A141D}, *sec2-41*, and *sec4-8* (Walch-Solimena and Novick, 1999; Sciorra *et al.*, 2005; Demmel *et al.*, 2008b). Moreover, defects in Pik1p function are synthetic lethal with *myo2-66* itself, strongly suggesting that PI4P and Myo2p work in the same pathway (Walch-Solimena and Novick, 1999). In addition, the finding that PI4P is the main PI species at the TGN, where the secretory vesicles that Myo2p binds arise, prompted us to explore more carefully if PI4P plays a role in Myo2p association with secretory membranes. The current notion is that PIs work hand-in-hand with small GTPases to fine tune all the steps required in the complex process of membrane traffic. The Rab GTPases associated with the membranes transported by Myo2p are Ypt31/32p and Sec4p, which also exhibited genetic interactions with both *pik1* and *myo2*, therefore generating a functional triangle relating PI4P, Rab GTPases, and Myo2p to each other. As mentioned above, there is precedence for molecular motors working with Rab GTPases or PIs in transporting cargo, so the aim of my research was to explore if such a mechanism applies for Myo2p in its secretory functions.

Overview

We have learned a great deal about the mechanism of polarity establishment and organelle segregation in yeast by formins, Rab GTPases, and myosin-Vs function. These same mechanisms apply in other cells, including humans, making budding yeast a very attractive model to dissect the molecular

details about how these processes are regulated and coordinated with other processes in the cell. The myosin-V Myo2p is involved in the transport of secretory vesicles for bud growth and cell separation and in the segregation of organelles prior to cytokinesis. Although for some of the cargoes details are known regarding Myo2p association with them, for the only essential one, secretory vesicles, no information was available prior to this study. Additionally, once Myo2p associates with vesicles and targets them to sites of growth, how they are released and then how Myo2p is recycled back is completely obscure. Although these are very important questions, I decided to explore how Myo2p associates with secretory vesicles. Since unbiased, comprehensive screens have failed to give us any new leads, I followed a candidate approach, focusing on two factors that interact genetically with Myo2p, function on the same process, and localize to the same membrane that Myo2p move: the Rab GTPases Ypt31/32p, Sec4p, and the PI PI4P. Although we still lack a complete molecular portrait of how Myo2p associates with secretory membranes, my studies have added more pieces to the puzzle. Evidence has since surfaced from other systems that is in agreement with the findings discussed here, making our current working model most likely to be applicable to other more complex eukaryotes.

CHAPTER 2[1]

THE LIPID PHOSPHATIDYLINOSITOL 4-PHOSPHATE HAS A CRITICAL ROLE IN THE ASSOCIATION OF MYO2P WITH SECRETORY MEMBRANES

INTRODUCTION

As explained in the previous chapter, recently it has been shown that PIs play a role in determining the specificity of trafficking. By using chimeras of GFP with PI binding motifs like PH or FYVE domains (Figure 1.6), it became apparent that PI isoforms are distributed in an organelle-specific manner, with PI4P almost exclusively localized at the Golgi complex. Post-Golgi transport in yeast is essential for growth which requires the constant delivery of proteins and lipids into the bud, where cargo enzymes remodel the cell wall for bud expansion driven by turgor pressure and new membrane addition. This delivery of secretory vesicles is dependent on the presence of polarized actin cables that arise from the bud cortex and the neck, and extend into the mother cell (Pruyne *et al.*, 1998). These cables serve as tracks for the type V myosin Myo2p, which walk down these cables while bound to secretory membranes, transporting them to sites of growth (Pruyne *et al.*, 1998; Schott *et al.*, 2002). However, how Myo2p associates with secretory vesicles, its only essential cargo, is unclear. Earlier work has shown that PI4P performs an essential role in the secretory pathway regulating exit of cargo from the TGN (Hama *et al.*,

[1] Some of the experiments presented in this chapter were previously published in Santiago-Tirado *et al.*, 2011.

1999; Walch-Solimena and Novick, 1999). Cells depleted of Golgi PI4P fail to make secretory vesicles, and accumulate secretory cargo internally. Because of the defect in secretion, they stop growing at the restrictive temperature. Notably, this effect is specific for inactivation of Pik1p, the Golgi localized PI 4-kinase, and the inactivation of Stt4p or deletion of Lsb6p, the other PI 4-kinases, has distinct effects (Cutler *et al.*, 1997; Audhya *et al.*, 2000; Han *et al.*, 2002). We have described a series of conditional mutations in the cargo-binding GTD of Myo2p that very rapidly uncouple the motor from secretory vesicles at the restrictive temperature without significantly affecting secretion (Schott *et al.*, 1999). Since these mutants still secrete, they continue to grow but in an isotropic manner, giving rise to large round cells that cannot divide and eventually lyse. As Myo2p transports secretory vesicles, and Pik1p regulates exit of cargo from the Golgi through production of PI4P, we set out to explore whether PI4P might participate in the recognition mechanism by which Myo2p associates with nascent secretory vesicles.

MATERIALS AND METHODS

Yeast molecular techniques. Standard media and techniques for growing yeast were used (Sherman, 1991). Yeast transformations were performed using either the Frozen-EZ Yeast Transformation Kit (ZYMO Research, Orange, CA), following the manufacturer's instructions, or the LiAc/ssDNA/PEG method as described (Gietz and Woods, 2002). Sporulation of diploid strains was induced using a supplemented sporulation media (1% potassium acetate, 0.1% yeast

extract, 0.05% glucose, and 25% of the amino acids required by the strain). For protein extracts and immunoblots, the relevant strains were grown to an OD_{600} of 0.8-1.0 in 10mL of YPD or SD media, the culture chilled on ice, washed with ice-cold water and then resuspended in 4X volumes of protein extraction buffer (20 mM Tris-HCl pH 7.4, 0.2 mM EDTA, 0.1 M NaCl, 1 mM DTT) with 5% yeast protease inhibitors cocktail in DMSO (Sigma). Roughly the same volume of glass beads (Sigma) was added and the tubes vortexed for 1min 3 times with 1min incubation on ice between each vortex (or for 3min in the disrupter at 4°C, without pause). The samples were centrifuged 5min at max speed at 4°C to remove the cell wall and other membranes and the supernatant transferred to a new tube for protein concentration quantitation. Samples were resolved by SDS-PAGE, transferred into a PVDF membrane (Immobilon, MILLIPORE), and the relevant protein detected by ECL (GE Healthcare) or by use of an Odyssey system (LICOR).

Separation of PIs by Thin-layer chromatography and quantification. Lipid extraction and phosphoinositide analysis was performed in the Henry lab following their described protocol (Nuñez *et al.*, 2008), but the cells were instead labeled with 10µC/mL of [^{3}H]myo-inositol (Perkin-Elmer) for 20-30 minutes. A 30 minute chase with cold-inositol media followed before harvesting the cells by addition of TCA (5% final). Basically, between 50,000 and 100,000cpm were spotted of each sample and the plates developed using the solvent system propyl acetate:isopropanol:ethanol:6% ammonia (3:9:3:9) in

an equilibrated paper-lined twin trough chamber. After exposing to iodine to mark the positions of the standards, the plates were sprayed with EN3HANCE (Perkin-Elmer) and dried before exposed to film for 5 – 7 days at -80°C. After the film was developed, the PIs were marked and scrapped unto a scintillation vial. I tried to scrape similar sizes but variability was unavoidable. 5mL of scintillation fluid was added and the samples thoroughly vortexed prior to measuring in a scintillation counter.

Mutagenesis of PIK1 and SAC1 to generate catalytically inactive versions. Mutagenesis was carried out using Stratagene's QuikChange II XL Kit, following the standard recommendations. The different residues changed in Pik1p were chosen according to their known function as the PI 4-kinase catalytic domain is well characterized (Gehrmann and Heilmeyer, 1998). The same kit and procedure was used to generate phosphatase-dead Sac1p. Since at the time no mechanistic insight about Sac1p's 4-phosphatase activity existed, I made changes to mimic the amino acids in Sjl1p's Sac domain. This Sac domain lacks 4-phosphatase activity and it has been attributed to the fact that otherwise completely conserved residues in all Sac domains are altered in Sjl1p (Guo *et al.*, 1999). Moreover, two of the original cold sensitive *sac1* mutations map to this area and have been shown to lack 4-phosphatase activity *in vitro* (Nemoto et al., 2000). I therefore predicted that mutation of either cysteines 392 and 395 or aspartates 394 and 397 to alanines would abolish the enzymatic activity. These mutants were expressed to similar levels

as wild-type, however they could not complement a *sac1*Δ in our CUY30 background (where *SAC1* is essential), or the inositol auxotrophy and cold sensitivity of *sac1*Δ in the BY4741 background, supporting our prediction.

Generation of the Myo2p-PH domain fusions. Originally I intended to make a tandem PH^{FAPP1} domain to replace the GTD of Myo2p. Using the plasmid pCR2.1-PH^{FAPP1} (obtained from the Emr lab) as template, I generated by PCR a SpeI-MluI and MluI-NotI PH^{FAPP1} domain fragments and cloned them into a SpeI-NotI cutted plasmid containing the *ADH1* terminator sequences after the NotI site. Another round of PCR was performed to get the tandem PH domains followed by the *ADH1* terminator flanked by SpeI and PacI sites and cloned into the similarly cut plasmid pRS413-MYO2, replacing the Myo2p sequences after amino acid 1091 until the PacI site in the MYO2 3' UTR region with the PH domains and terminator sequences. However, subsequent sequencing, after the experiments reported in this chapter, showed a STOP codon in the junction between the PH domains, so in reality the fusion proteins contain only one PH domain instead of the GTD. I later remade the constructs and found similar results (the suppression of the alleles rescued by the single PH domain fusion was not as robust with the tandem PH). Mutagenesis of the PH domain was also carried out with Stratagene's QuikChange II XL Kit, targeting two positive-charged residues in the β1/β2 loop, known as the ligand binding site according to the PH domains that have been crystallized.

Microscopy. Micrographs were acquired with a spinning disc confocal microscopy system (3I Corp) using a DMI 6000B microscope (Leica) and a digital camera (QuantEM; Photometrics). Images were further analyzed and adjusted using Slidebook 5. The cells were prepared from log-phase cultures and mounted on agar slabs made on top of rectangular microscope slides. Basically, I pipette ~800 µl of the agarose medium along the center of the slide and cover it with a rectangular coverslip immediately to flatten the medium. I press gently on the two extremes to try and make it even, making sure not to press too hard as it will results in a slab too thin that will dry up very fast. The agarose medium will solidify after a few minutes and will be ready to use in ~5-10min. For most experiments I visualized the cells with the 63X objective.

PIP strips and protein-lipid blots. I followed the Emr lab protocol to prepare the PIP strips and blot them with recombinant proteins. Basically, I cut a supported-type nitrocellulose (it HAS to be a supported-type membrane, like the Hybond-C Extra from Amersham, otherwise it will wrinkle and become unusable after spotting the lipids) into the appropriate size and notch one corner for orientation. I used a 96-well plate to estimate the membrane size (depending how many PIPs and dilutions of each one you want to make, use the well-well distances as the lengths for each side of the membrane). I made the resuspension and spotting buffers in small glass tubes or vials (although eppi tubes also worked fine) fresh shortly before using. We obtained the free acid form of the PIs from CellSignals Inc., and I suspended them in a

methanol:chloroform (2:1 v/v) solution, making a 1mM stock kept under nitrogen at -20°C afterwards. Working in the cold room with all buffers and lipids on ice, I laid out all the membranes on aluminum foil and spotted one lipid at a time, making appropriate dilutions (usually 3 spots of 2-fold dilutions with 0.125mM final). Is important to work one lipid at a time and work fast, as the spot buffer will evaporate fast and will change the concentration of your spots. The Emr lab makes more dilutions and they do 3- to 5-fold serial series, so you can play with this regard. After drying the membranes at room temperature for at least 1 hour in a dark place, or overnight in the cold room inside a box (I don't know the reason for this, are lipid light-sensitive?), I cut the membranes into the individual strips and stored them in foil at 4°C until used. To block, I incubated the PIP strip for 1hr at room temperature in 5% non-fat dry milk in TBS with 0.1% Tween20 (TBS-T). I added the protein of interest at a standard concentration of 5µg/mL in 0.5% fatty-acid free BSA (Sigma – labeled "for special applications") in TBS-T and incubated overnight at 4°C. I found out that 5µg/mL is a good starting point, but this can vary from protein to protein. In my particular case, the Myo2p-tail showed very different specificities depending on the concentration. I washed 5 times for 5 minutes each with TBS-T and then incubated with the primary antibody (usually anti-GST) overnight at 4°C in a solution of 0.5% fatty-acid free BSA in TBS-T. I found out that, if in a hurry for results, with our GST antibody (Box 150) 1hr incubation at room temperature gave acceptable results. After washing like before, I incubated for 1hr at room temperature with the secondary antibody in

5% non-fat dry milk in TBS-T. After the final washes, the bound proteins were detected by ECL.

Liposome floatation assays. I adopted the protocol from the Fromme lab where they use fluorescent liposomes that mimic the Golgi in composition: 24%mol DOPC, 6%mol POPC, 7%mol DOPE, 3%mol POPE, 1%mol DOPS, 2%mol POPS, 1%mol DOPA, 2%mol POPA, 25%mol liver or soy PI, 5%mol PI4P, 2%mol CDP-DAG, 4%mol PO-DAG, 2%mol DO-DAG, 5%mol Ceramide (C18), and 10%mol Cholesterol (in MeOH). The liposomes are made fluorescent by the addition of DiR (in EtOH; Invitrogen) to 1%mol final. They were hydrated in 20mM HEPES, pH 8.0, 150mM KOAc (HK buffer), sonicated, freeze-thawed, and extruded through a 400nm pore filter (although I later used 100nm or 200nm filters; Avanti Lipids). The resulting suspension was adjusted to 1mM or 5mM and 20µL were used per reaction with 1µg of the appropriate recombinant protein. Typically, 100pmol of the protein of interest and 20µL of the liposome suspension were incubated at room temperature for 1hr in a total volume of 75µL, although I varied this amount for the Myo2p-cctail. 50µL of the 2.5M sucrose-HK buffer was added to the binding reaction and mixed well by pipetting up and down several times. The resulting mixture will be 1M sucrose. 100µL are transferred to a Beckman 7x20mm PC Ultracentrifuge tube (the remainder ~25µL is the input sample) and is carefully overlaid with 100µL of the 0.75M sucrose-HK buffer. Practice pipetting smoothly in a separate tube to avoid any burst when pipetting and introducing bubbles into

the gradient. Lastly, as before, overlay with 20µL of HK buffer. If the step gradient is made correctly, if you look at the tube against light you should be able to see the phase boundaries between the sucrose phases. The tubes are then loaded into a TLA100 rotor and ultracentrifuged for 20min at 20°C 100krpm with the slowest acceleration and deceleration (set to 5 in our ultracentrifuge). After the spin, the top 30µL (2 x 15µL, using the p20 tips) from the gradient are carefully withdrawn as the float fraction. Sometimes, if using very strong lipid binders, all the liposomes will aggregate in one spot on the outside top side of the tube, so take your 30µL sample from that area. In order to load the same amount of liposomes for each float sample, we quantify the fluorescent lipids by mixing 48µL of 0.1% triton X-100 with 2µL of the float fraction in a 96-well plate and measuring the fluorescence on an Odyssey system. Normalized float fractions are prepared in 5X sample buffer and input samples in 2X sample buffer. I routinely loaded 10µL of each input, and 15µL of the float fraction with the lowest liposome recovery, normalizing the rest to this fraction.

Calcofluor white plate assays. I prepared the plates soon before the day of the experiment as I found out that calcofluor white (CFW; now called fluorescent brightener, Sigma's F-3543) tends to precipitate (forming crystals on the medium) after prolong storage. As a precaution to prevent possible inactivation of CFW by light, I wrapped the CFW solutions in aluminum foil and kept the plates in the dark. All my experiments were done in YPD or YPGal plates

containing 20µg/mL of CFW as the wild-type strain grew relatively fine at this concentration. In minimal media (SD or SGal) with the same CFW concentration, the growth of the *myo2* mutants was not affected.

RESULTS

Two *myo2* alleles are especially sensitive to Golgi PI4P levels

With all the current evidence for PI4P as an important factor for post-Golgi secretion we wanted to explore its role in yeast's Myo2p-dependent polarized transport. If PI4P is involved in the association of Myo2p with secretory vesicles we reasoned that some of our mutants might be sensitive to alterations in its cellular levels. There was already some precedence for this idea as Daniel Schott, a previous student in the lab, suggested that was true based on some preliminary work with *myo2-17* and *myo2-18* and *sac1*Δ, however he never showed Tony any data. To verify that observation and to test our hypothesis, I started by overexpressing yeast's PI 4-kinases to determine if that could suppress the growth defects of any of the seven conditional GTD *myo2* mutants. I cloned *PIK1*, *STT4*, or *LSB6* behind the *GAL1/10* promoter and, upon induction by galactose, I found that one of the seven *myo2* alleles, *myo2-12,* could be partially suppressed only by *PIK1* overexpression (Figure 2.1A and Table 1). Under these conditions, the total level of cellular PI4P rises about 1.5 to 2.0-fold as measured by thin layer chromatographic separation of radioactive PIs (Figure 2.1B). This effect was specific for Pik1p as

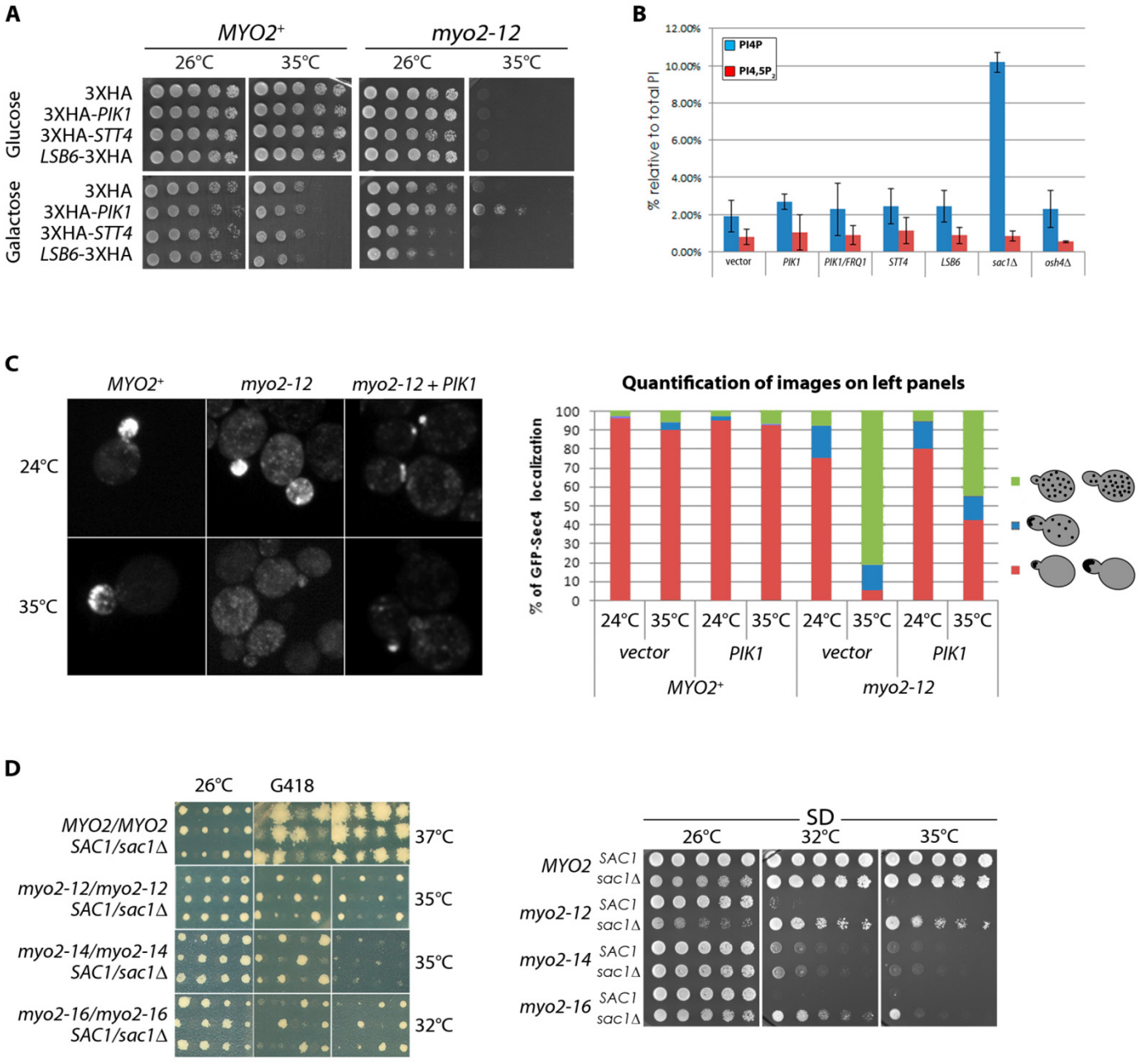


Figure 2.1 Increasing PI4P levels can suppress specific *myo2* alleles. (A) Growth of wild-type *MYO2* and *myo2-12* cells in glucose (no expression) or galactose (high expression) at the indicated temperatures and expressing the indicated genes. (B) Levels of PI4P and PI4,5P_2 determined in strains lacking *sac1* or *osh4* or overexpressing the indicated genes for 4hrs previous to labeling with [^{3}H]myo-inositol. PIPs were separated and quantified as described in Materials and Methods; error bars represent standard deviation of the sample. (C) GFP-Sec4 localization in the indicated strains at either room temperature or after 30-min incubation at 35°C with quantitation of GFP-Sec4 localization on the right panel. For quantification >100 small/medium budded cells were examined as wild-type cells polarize GFP-Sec4 to the bud tip. Since there is punctate GFP-Sec4p staining in the *myo2-12* mother cells even at the permissive temperature, a separate category was used to score these (blue bars). (D) Tetrad dissection of homozygous *myo2* cells deleted for one copy of *sac1*. G418 marks the *sac1*Δ spores. The panel on the right shows the growth of these *sac1*Δ spores in comparison to their *SAC1*$^+$ counterparts.

overexpression of *STT4* resulted in a similar PI4P increase but did not rescued growth. Notice that there is also a slight increase in the PI4,5P_2 levels, as reported previously for *PIK1* overexpression (Schorr *et al.*, 2001). Imaging of GFP-Sec4 (the Rab GTPase associated with secretory vesicles; Figure 1.5) showed an increase in polarized vesicles upon *PIK1* overexpression in *myo2-12* cells at the restrictive temperature (Figure 2.1C). In contrast, control or cells overexpressing the other PI-4 kinases showed a complete delocalization of GFP-Sec4 (Figure 2.1C and not shown). When measuring the levels of PI4P in these strains we used a *sac1*Δ strain as a positive control for elevated levels of PI4P (Figure 2.1B). Sac1p is one of the three lipid phosphatases that can dephosphorylate PI4P to PI. The other two, Sjl2p and Sjl3p (also known as Inp52p and Inp53p), function at the plasma membrane (Stefan *et al.*, 2005), while Sac1p functions at the ER and Golgi (Foti *et al.*, 2001; Tahirovic *et al.*, 2005). However, Sac1p is the main phosphatase since the level of PI4P in *sjl2*Δ*sjl3*Δ double mutants does not increase significantly (Stefan *et al.*, 2002) whereas in *sac1*Δ cells, the level of PI4P rises more than eight-fold (Hughes *et al.*, 2000; Foti *et al.*, 2001; Figure 2.1B). We therefore explored whether this method of acutely elevating PI4P levels would also suppress some of the conditional GTD *myo2* mutants, as had been suggested by Daniel.

My first approach was to cross our *myo2* mutant strains (made in the CUY30 background that was obtained from the Huffaker lab, originally from the Botstein lab) with the deletion consortium strain *sac1*Δ (BY4741). I tried

first the two alleles indicated by Daniel Schott to be rescued; however, sporulation and dissection of the mated strains yielded mixed results. Both diploids exhibited poor sporulation efficiency, as very few complete tetrads were obtained, but for *myo2-18* none of the double mutants grew at high temperatures. For *myo2-17*, some of the spores positive for both markers grew at high temperatures, but other double mutants did not. I subsequently mated all of the remaining alleles to the BY4741 *sac1*Δ strain and, after sporulation and dissection, found the same poor spore viability, with mixed results for *myo2-12*, *myo2-13*, and *myo2-16*; none of the others gave me signs of suppression. Because of the poor spore viability, I tried deletion of *SAC1* in CUY30 diploid strains as a second approach. To my surprise, sporulation and dissection of a heterozygous *SAC1*/*sac1*Δ in this background resulted in only two viable spores, all *SAC1*$^{+}$. I confirmed this result with two additional procedures: transforming the heterozygous diploid with a *URA3*-based plasmid containing *SAC1*$^{+}$ or with a *SAC1*$^{+}$ construct under the galactose promoter. Dissection of these strains yielded *sac1*Δ spores covered by the plasmids, however upon replication unto 5-FOA (to select against the *URA3* plasmid) or glucose plates (to shut off expression from the galactose promoter), these spores were inviable, indicating that in our CUY30 genetic background, *SAC1* is an essential gene, explaining the mixed results obtained above by crossing with the BY4741 *sac1*Δ cells. As a third approach I decided to generate the same *myo2* GTD mutants in the BY4742 background and then cross them with *sac1*Δ cells in the opposite mating type. This approach gave me very clear

suppression of *myo2-12*, *myo2-13*, and *myo2-16*, and some weak suppression of *myo2-20* (Figure 2.1D and Table 1). Further re-testing showed some variable suppression for allele *myo2-18*, however this allele, as well as *myo2-20*, are healthy in comparison with *myo2-12*, *myo2-13*, or *myo2-16*, and the change in their restrictive temperature is minimal (see Table 1). On the other hand, for *myo2-12*, *myo2-13*, and *myo2-16*, there is a large change in the restrictive temperature, bringing it almost as high as the wild-type parent strain, especially for *myo2-12* and *myo2-16*. Since *myo2-12* can also be partially suppressed by *PIK1* overexpression, and with additional data reported below, we consider *myo2-12* and *myo2-16* as two alleles particularly sensitive to alterations in the levels of Golgi PI4P.

The PIP phosphatase Sjl3p/Inp53p (Figure 1.6) has also been implicated in Golgi function, specifically in the AP-1-dependent pathway to the endosome (Ha *et al.*, 2003). However, it is unclear if it functions through its 4-phosphatase (Sac domain) or its 5-phosphatase domain. I nevertheless, tested if deletion of *SJL3* could also rescue some of our alleles. I deleted *SJL3* by the one step disruption method (transforming a linear piece of DNA containing the kanMX cassette flanked by regions of homology to *SJL3*) in the *myo2* diploids, and after confirming correct integration of the cassette, the strains were sporulated and dissected. None of the alleles showed suppression by *sjl3*Δ, consistent with the fact that a *sjl3* mutant does not accumulate PI4P (not shown). Likewise, Osh4p/Kes1p has been reported to be a negative regulator

of Pik1p function (Fairn *et al.*, 2007). In this study, a "*sec14* by-pass" mutant was crossed with the yeast deletion collection and the set of genetic interactions found overlapped significantly with that of a *pik1* allele, suggesting that PI4P is important for the *osh4/kes1Δ* suppression of a *sec14* mutant. Consistent with that, PI4P levels in *pik1-83* were restored to near wild-type levels at the restrictive temperature when *osh4/kes1* was inactivated. I measured PIP levels in *osh4/kes1*Δ and found a small, but reproducible, increase of 1.8X in PI4P levels (Figure 2.1B), without affecting other PIs. Because this increase was similar to that obtained by *PIK1* overexpression, I tested if *osh4/kes1*Δ could also suppress the two PI4P-sensitive alleles. I crossed the two sensitive alleles in the BY background with the *osh4/kes1*Δ strain from the deletion consortium, followed by sporulation and dissection. Picking several double mutants and testing alongside the single mutants demonstrated a weak suppression of only *myo2-12* (not shown), not as nice as that obtained by *PIK1* overexpression. No suppression was seen for the *myo2-16* allele. Although the suppression was not as good as that for *PIK1* overexpression, it is still consistent with the hypothesis that Golgi PI4P plays a role in Myo2p's secretory function.

One concern raised by all of these manipulations is the fact that *sac1*Δ cells, and all of our *myo2*^{ts} double mutants with PI4P-related genes, are very slow growing. Because many of our *myo2*^{ts} are sensitive to rich media and their restrictive temperature can be partially increased by growing on minimal

media, I tested if all of these effects were a result of slowing the growth rate. I did growth curves with different low amounts of cycloheximide (CHX) in both the BY and CUY background strains. I determined the amount needed to slow down but not inhibit growth, and tested if under these conditions I could get suppression of the same *myo2* alleles sensitive to rich media. I did not see any differences in growth for the wild-type control and the *myo2*^{ts} between media with or without CHX, indicating that the effects are not simply due to slowing down growth (not shown).

So far, my data indicate that increasing the levels of Golgi PI4P can restore the function of some of the *myo2*^{ts} mutants. The different extents of rescue seen between overexpression of the kinase and inactivation of the phosphatase might just be a result of the amount of PI4P accumulation achieved. I wondered if co-overexpression of *PIK1* with its Golgi anchor, *FRQ1*, could increase PI4P levels even more to suppress the other alleles rescued by *sac1*Δ. However, measurement of PIP levels in strains co-overexpressing these genes did not result in an additional increase in PI4P levels (Figure 2.1B). This is consistent with my previous findings, and that of others, suggesting that Golgi PI4P levels are tightly regulated, and an increased Pik1p levels is not equivalent to increased PI4P levels.

Because there are at least two distinct PI4P pools in the cell, and the PI4P phosphatases have been shown to act on multiple membranes (Audhya *et al.*, 2000; Foti *et al.*, 2001; Audhya and Emr, 2002), we wanted to more

carefully assess the location where Sac1p activity affects the *myo2*^{ts} mutants by using targeted Sac domain fusions (Stefan *et al.*, 2002). I targeted the Sac1p phosphatase domain to the plasma membrane by the PH domain of PLCδ (binds the high levels of PI4,5P_2 present there), or to the endosomes by the FYVE domain of EEA1 protein (that binds PI3P), or to the Golgi by the PH domain of FAPP1 (that binds specifically to the PI4P present at the Golgi). Targeting the phosphatase to these membranes had no effect on wild-type, but targeting to the Golgi reversed the ability of *sac1*Δ to rescue *myo2-12* and *myo2-16*, whereas targeting to the plasma membrane or endosome did not (Figure 2.2A). Again the suppression is dependent on PI4P, as expression of targeted phosphatase-dead *sac1* versions did not restore the temperature sensitivity of the *myo2* alleles (see Materials and Methods; Figure 2.2B). These data, together with the fact that only the Golgi-localized PI 4-kinase has effects on Myo2p, support the hypothesis that PI4P at the Golgi plays an important role in Myo2p's essential function of transporting membranes for cell growth.

A prediction of this hypothesis is that reduction of the PI4P levels should be deleterious to the *myo2*^{ts} mutants. A conditional allele of the PI 4-kinase, *pik1-139,* has been reported to have reduced levels of PI4P even at the permissive temperature, but not sufficiently low to affect secretion (Sciorra *et al.*, 2005). I therefore combined the two PI4P sensitive *myo2* alleles with the *pik1-139* mutation and after sporulation and dissection, found synthetic growth defects at otherwise permissive temperature for the single mutants

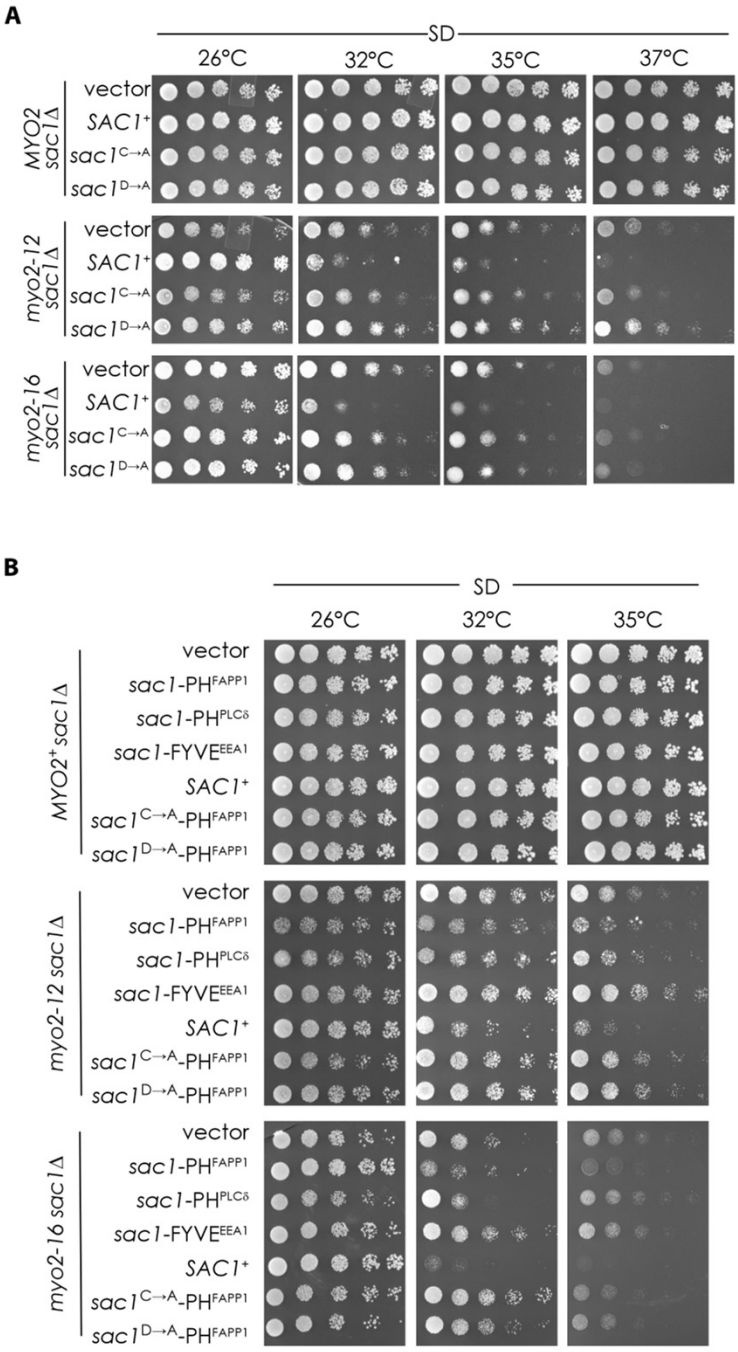


Figure 2.2 The 4-phosphatase activity of Sac1p at the Golgi is responsible for the effects on *myo2* alleles. (A) Growth of the two PI4P sensitive alleles in the absence of Sac1p or the presence of wild-type Sac1p or phosphatase-dead versions. (B) Growth characteristics of the same *myo2* tail mutants targeting the wild-type or mutant Sac1 phosphatase domain to different compartments in the cell. The targeting motifs are PH^{FAPP1}, to target to the Golgi, $PH^{PLC\delta}$, to target the plasma membrane, and $FYVE^{EEA1}$, to target the endosomes.

(Table 1). Taken together, my results showed that raising Golgi PI4P is not a general mechanism by which all *myo2* mutants can be suppressed, and the *myo2-12*, and to a lesser extent the *myo2-16* allele, are especially sensitive to alterations in Golgi's PI4P pool.

PI4P affects are specific for secretory membrane traffic and have no effect on other Myo2p functions

As mentioned in Chapter 1, Myo2p transports many different membranes (Figure 1.4). To explore the possibility that PI4P is affecting a general property of Myo2p rather than a cargo-specific one, I obtained vacuole-specific *myo2* mutants from the Weisman lab (Catlett *et al.*, 2000). Overexpression of *PIK1* has no effect on the inheritance of vacuoles in these mutants (Figure 2.3A). Likewise, *myo2-12* is defective in binding Kar9p and therefore has a defect in spindle orientation that cannot be corrected by *PIK1* overexpression (Figure 2.3B). These experiments, together with the previous findings, strongly suggest that PI4P's role in Myo2p function is specific for the transport of secretory membranes. Moreover, in our initial characterization of the *myo2* GTD mutants it was found that they have delocalized chitin staining when grown at the restrictive temperature (Schott *et al.*, 1999). Chitin is a minor but essential component of the yeast's cell wall that can be visualized with the dye CFW. The enzyme that generates chitin is transported in specialized vesicles at the very beginning and end of the cell cycle (see Chapter 1), restricting the detectable pool of chitin to emerging buds, large budded necks, or bud scars.

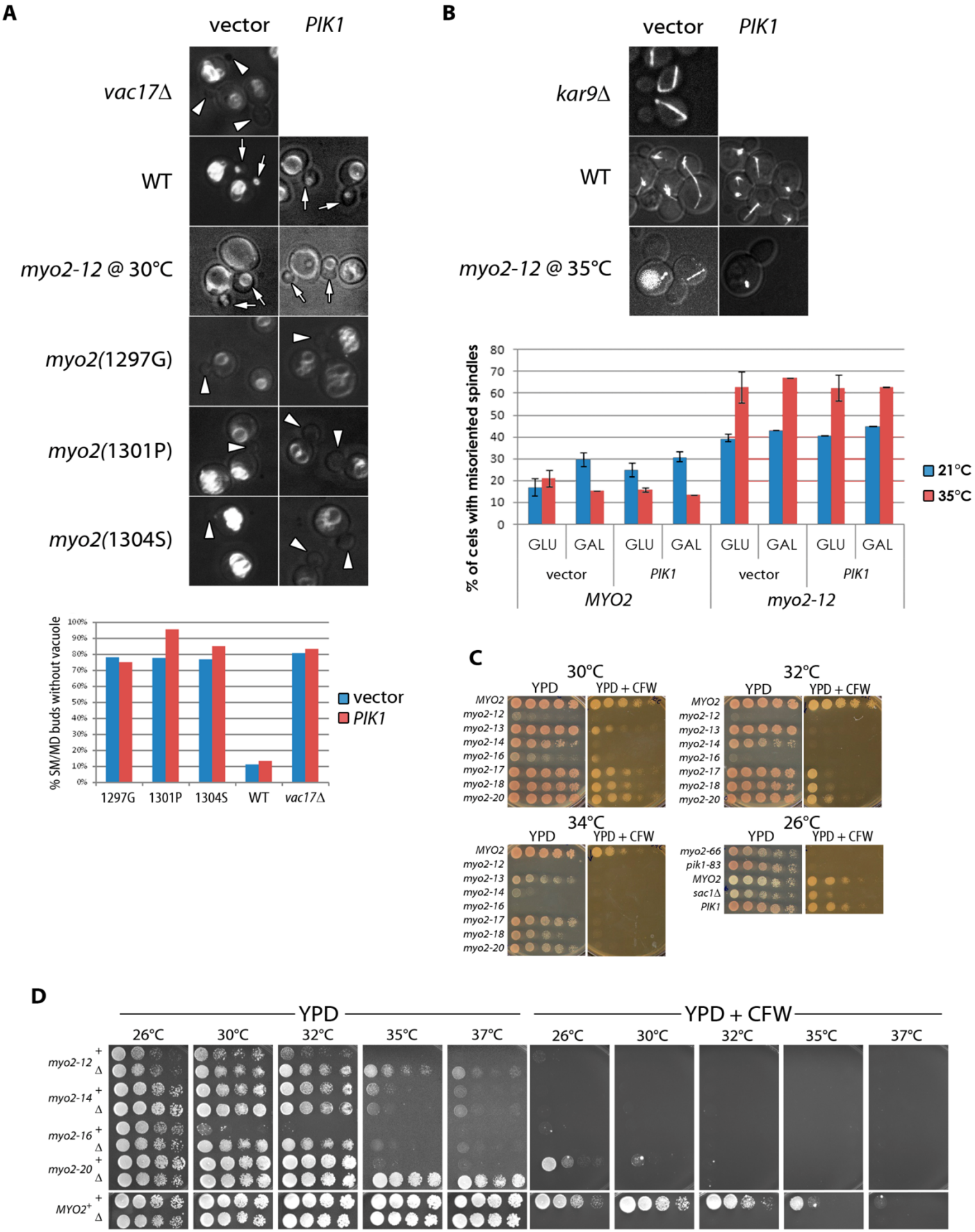

A
vector
PIK1
vac17Δ
WT
myo2-12 @ 30°C
myo2(1297G)
myo2(1301P)
myo2(1304S)
% SM/MD buds without vacuole
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1297G
1301P
1304S
WT
vac17Δ
vector
PIK1
B
vector
PIK1
kar9Δ
WT
myo2-12 @ 35°C
% of cels with misoriented spindles
80
70
60
50
40
30
20
10
0
GLU
GAL
GLU
GAL
GLU
GAL
GLU
GAL
vector
PIK1
vector
PIK1
MYO2
myo2-12
21°C
35°C
C
30°C
YPD
YPD + CFW
MYO2
myo2-12
myo2-13
myo2-14
myo2-16
myo2-17
myo2-18
myo2-20
32°C
YPD
YPD + CFW
MYO2
myo2-12
myo2-13
myo2-14
myo2-16
myo2-17
myo2-18
myo2-20
34°C
YPD
YPD + CFW
MYO2
myo2-12
myo2-13
myo2-14
myo2-16
myo2-17
myo2-18
myo2-20
26°C
YPD
YPD + CFW
myo2-66
pik1-83
MYO2
sac1Δ
PIK1
D
YPD
YPD + CFW
26°C
30°C
32°C
35°C
37°C
26°C
30°C
32°C
35°C
37°C
myo2-12 +/Δ
myo2-14 +/Δ
myo2-16 +/Δ
myo2-20 +/Δ
MYO2+ +/Δ

Figure 2.3 PI4P effects are specific for Myo2p secretory function. (A) Strains carrying versions of Myo2p defective in vacuole inheritance, or *myo2-12*, or wild-type Myo2p, were stained with FM4-64 and vacuole inheritance was quantified with or without overexpression of *PIK1*. Cells lacking *vac17* were used as a control as these cells do not inherit vacuoles. Arrowheads and arrows indicate buds without or with vacuoles, respectively. All cells were grown and counted at room temperature. (B) Similarly, spindle orientation was quantified in *myo2-12* or wild-type Myo2p cells expressing GFP-Tub1. Cells lacking *kar9* were used as control as they lack the actin-dependent pathway to orient the spindle. All temperature shifts were done for 15-min in a 35°C water bath, followed by a quick formaldehyde fixation. (C) Growth of cells showing that defects in PI4P or in Myo2-secretory function results in sensitivity to the cell wall drug calcofluor white (CFW). (D) Elevation of PI4P restores the temperature sensitivity of selected *myo2* alleles but not the sensitivity to CFW. The "+" and "Δ" signs denote the presence or absence of a copy of *SAC1*$^{+}$.

A diffusive chitin staining means that the chitin synthases have been incorrectly delivered along the whole plasma membrane, indicative of a loss of polarized growth. Consistent with this, I found that all the *myo2*^{ts}, and especially *myo2-12* and *myo2-16*, are hypersensitive to CFW (Figure 2.3C). Similarly, cells with high PI4P levels, as expected for *sac1*Δ, are also hypersensitive to CFW (Figure 2.3C). This last observation was previously reported and suggested to be the result of excessive forward transport (Schorr *et al.*, 2001), a theory in agreement with our hypothesis that PI4P promotes Myo2p association with secretory membranes and therefore, stimulates their transport. However, I found out that *PIK1* overexpression (not shown) or *sac1*Δ could not restore sensitivity to CFW to wild-type levels (Figure 2.3D). This could suggests that the reason *myo2*^{ts} mutants and cells with high PI4P are sensitive to CFW is distinct, or that a very thin balance is required and I am not attaining the right levels.

The essential role of PI4P in wild-type cells becomes more apparent in *myo2* mutants

As an additional way to examine the role of PI4P in secretion, we wondered if decreasing Golgi-localized PI4P, by overexpression of *SAC1*, or blocking the normal levels of PI4P, by overexpression of a ligand, affects polarized secretion in wild-type or *myo2*^{ts} cells. I obtained a multicopy plasmid containing *SAC1* under the galactose promoter from the Henry lab and constructed a similar plasmid containing the PI4P-specific FAPP1 PH domain.

However, induction of these constructs by galactose resulted in lethality even in the wild-type strain (Figure 2.4A). Even GAL-induced expression from a single copy plasmid was lethal, highlighting the importance of PI4P function. Consistently, overexpression of the targeted Sac domains resulted in lethality only when fused to the FAPP1 PH motif (Figure 2.4B). I nevertheless wanted to test the effect of moderate overexpression of *SAC1* on the *myo2* mutants, reasoning that in the PI4P sensitive *myo2* mutants the effects were going to be more pronounced. Because of the ease in inducing and repressing, and the rapid response of the promoter, I used truncated *GAL1* promoters previously reported to achieve reduced levels of expression (Mumberg *et al.*, 1994), to control the expression of *SAC1*. Induction from this weakened *GAL1* promoter had little effect on wild-type growth, but it lowered the restrictive temperature of all the *myo2* GTD mutants (Figure 2.4C). This effect was dependent on the phosphatase activity as expression of equivalent levels of Sac1p with mutations in the catalytic domain (see Materials and Methods) had no effect when overexpressed. Similarly, I wanted to test if the lethality of overexpressed FAPP1 PH domain was due to masking of PI4P and blocking of its functions. I obtained GFP-constructs of the related yeast homolog, Osh1p PH domain, both wild-type and with mutations (EE mutant) that abolished PI4P-binding (Levine and Munro, 2001). However, upon induction from the *GAL1* promoter both constructs inhibited growth of wild-type cells (Figure 2.4D). Although surprising, it is consistent with the previous finding that when overexpressed in COS cells, the EE mutant of OSBP (mammalian homolog of yeast Osh1p)

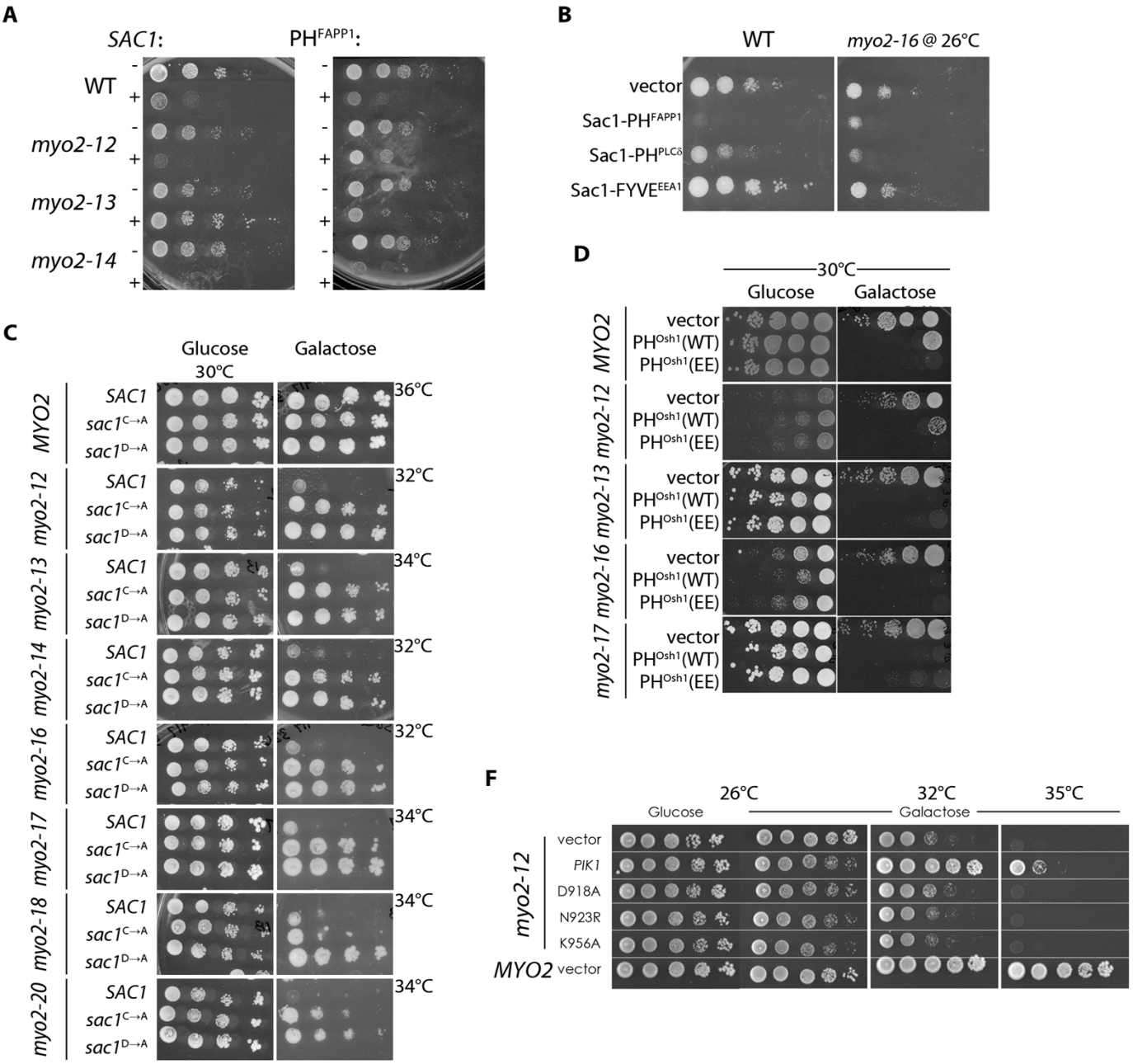

A
SAC1:
PH^FAPP1:
WT
myo2-12
myo2-13
myo2-14
B
WT
myo2-16 @ 26°C
vector
Sac1-PH^FAPP1
Sac1-PH^PLCδ
Sac1-FYVE^EEA1
C
Glucose
Galactose
30°C
MYO2
myo2-12
myo2-13
myo2-14
myo2-16
myo2-17
myo2-18
myo2-20
SAC1
sac1^C→A
sac1^D→A
36°C
32°C
34°C
D
30°C
Glucose
Galactose
MYO2
myo2-12
myo2-13
myo2-16
myo2-17
vector
PH^Osh1(WT)
PH^Osh1(EE)
E
30°C
37°C
Glucose
Galactose
MYO2
pik1-83
vector
PIK1
pik1(D918A)
pik1(N923R)
pik1(K956A)
vector
PIK1
PIK1/FRQ1
D918A
N923R
D936R
K956A
130
F
26°C
32°C
35°C
Glucose
Galactose
myo2-12
MYO2
vector
PIK1
D918A
N923R
K956A

Figure 2.4 PI4P has a critical role for growth that is more pronounced in the *myo2* mutants. (A) Growth assays showing that high overexpression of *SAC1* or PH^{FAPP1} is lethal in all strains, including wild-type. Only galactose plates are shown. The "–" and "+" signs denote absence or presence of the overexpressed protein. (B) Similarly, high overexpression of the targeted Sac1 domains is lethal when targeted to the Golgi in all strains. Some deleterious effect is also seen when targeted to the plasma membrane that is more apparent in the *myo2-16* strain. (C) When expressed from a truncated *GAL1* promoter, the reduced *SAC1* levels are no longer lethal in wild-type cells but all the *myo2* mutants are hypersensitive to it, reducing the restrictive temperature. This effect is due to the 4-phosphatase activity as overexpression of *sac1* mutants has no effect. (D) High overexpression of the Osh1p PH domain (WT), or a mutant version that cannot bind PI4P (EE), is lethal in all strains. This PH domain also binds Arf1p, regardless of PI4P binding. (E) The kinase-dead mutants of Pik1p cannot complement the conditional *pik1-83* strain, regardless of being expressed to similar levels as wild-type (western blot at the right side; the line denotes 130kDa). Notice also that overexpression of *pik1* kinase-dead versions is dominant negative in cells with already low levels of PI4P. (F) The suppression of *myo2-12* by *PIK1* overexpression is dependent on PI4P, as kinase-dead version cannot rescue growth.

has a dominant negative effect displacing AP-1 from TGN membranes (Levine and Munro, 2002). Because both the FAPP1 and Osh1p PH domains also bind active Arf1p, one can imagine a situation where the mutant PH domain, although not binding PI4P, can still bind Arf1p and can block its function, resulting in a dominant negative effect. At the time of these experiments I was not aware of other PI4P-specific PH domains of common use, however currently there are other PI4P-binding motifs that do not bind Arf1p and could represent a better tool to test the effects of disruption of PI4P signaling by specifically masking it (Roy and Levine, 2004).

In a similar manner, I wanted to test if the *PIK1* overexpression effects were due to its lipid product or were indirect through binding of Pik1p to other factors. The catalytic domain of PI 4-kinases is well characterized and kinase-dead mutants have been previously reported (Godi *et al.*, 1999; Strahl *et al.*, 2005). I generated point mutations that disrupted the phosphotransfer, or the Mg^{+2} and or inositol binding, abolishing the kinase activity of Pik1p. These kinases were expressed roughly to the same levels as wild-type (Figure2.4E), however they could not complement a *pik1*^{ts} mutant and were dominant negative in cells already defective in PI4P levels (Figure 2.4E). Moreover, kinase-dead *pik1* mutants could not suppress *myo2-12*, and instead very frequently I would find that it makes the strain even more slow-growing, indicating that the rescue is mediated through PI4P, and that PI4P's role is absolutely essential in strains with already low levels of PI4P, and more

important in strains with defects in Myo2p secretory function (Figure 2.4F).

Transport of secretory membranes by Myo2p is dependent on PI4P

One of the strongest pieces of evidence for a role of PI4P in Myo2p-dependent secretory membrane transport was the observation by post-doc Aster Legesse-Miller that the PH domain from FAPP1, specific for Golgi PI4P, is transported into the bud on tubulo-vesicular membranes in a Myo2p-dependent manner, very reminiscent of GFP-Ypt31p labeled membranes (see Chapter 3). I confirmed this finding and also noticed that in cells exhibiting rapid movement of this reporter, GFP-PH^{FAPP1} was always polarized to the growing bud or the neck region of large budded cells, the same localization pattern as Myo2p (Figure 2.5A). This PI4P reporter, as mentioned above, is limited to the Golgi pool of PI4P as it needs to bind active Arf1p, a protein generally thought to be absent from secretory vesicles. In order to test if secretory vesicles also are rich in PI4P we used the PH domain from yeast Osh2p, an unbiased reporter that recognizes all pools of PI4P (Roy and Levine, 2004). Imaging of mCherry-PH^{Osh2p} showed plasma membrane staining only at growth sites and numerous cytoplasmic puncta of variable sizes (Figure 2.5B). Many of the puncta showed directed transport to the bud by both tubular and vesicular profiles. Moreover, if we co-image with GFP-Sec4, mCherry-PH^{Osh2p}-labeled structures overlap with GFP-Sec4 enrichments at the bud tip and bud neck, which are sites of active growth (Figure 2.5C). However, very few instances of co-localization in the mother cells were seen in wild-type cells,

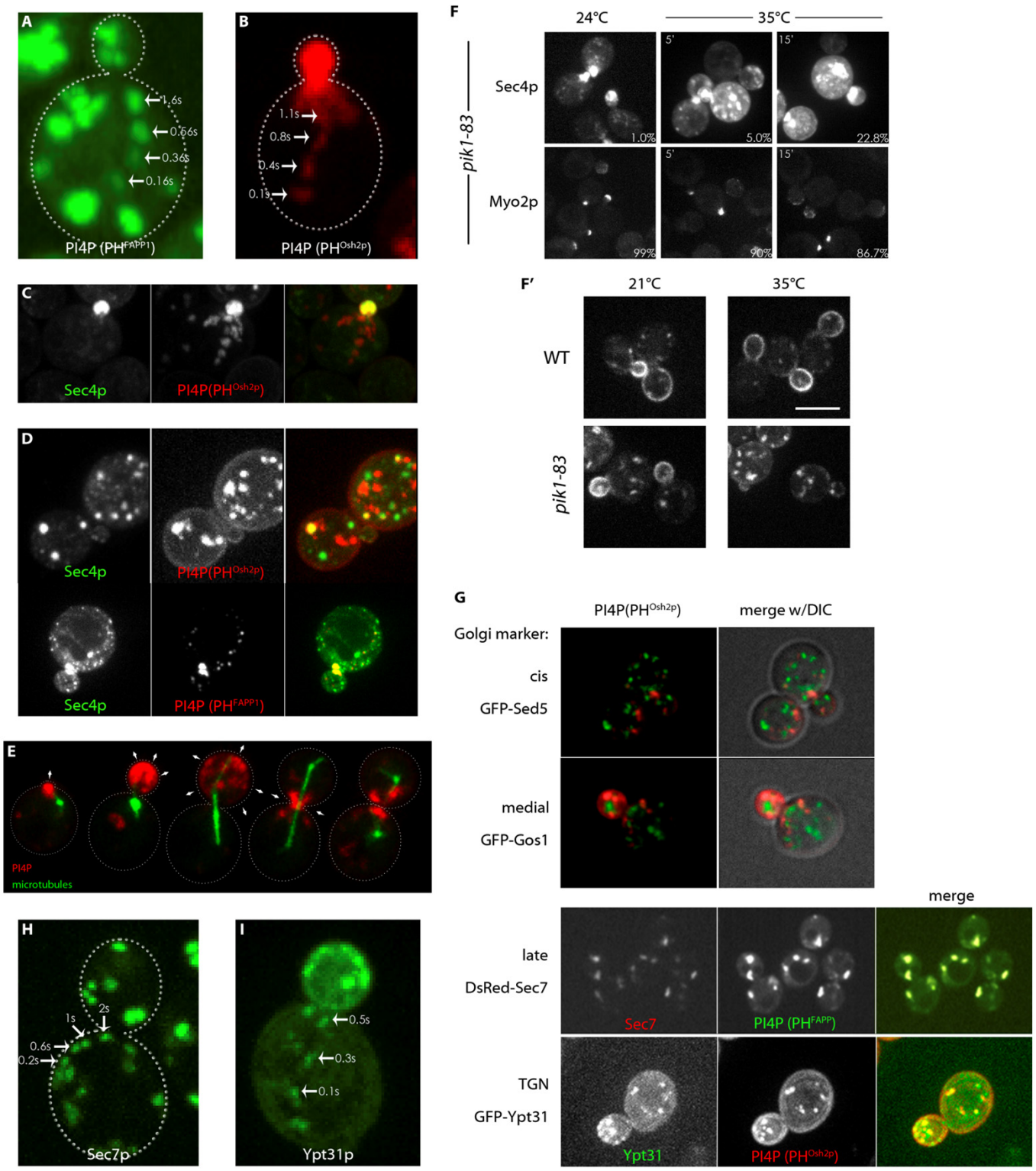

A
1.6s
0.66s
0.36s
0.16s
PI4P (PHFAPP1)
B
1.1s
0.8s
0.4s
0.1s
PI4P (PHOsh2p)
C
Sec4p
PI4P(PHOsh2p)
D
Sec4p
PI4P(PHOsh2p)
Sec4p
PI4P (PHFAPP1)
E
PI4P
microtubules
H
1s
2s
0.6s
0.2s
Sec7p
I
0.5s
0.3s
0.1s
Ypt31p
F
24°C
35°C
pik1-83
Sec4p
5'
15'
1.0%
5.0%
22.8%
Myo2p
5'
15'
99%
90%
86.7%
F'
21°C
35°C
WT
pik1-83
G
PI4P(PHOsh2p)
merge w/DIC
Golgi marker:
cis
GFP-Sed5
medial
GFP-Gos1
merge
late
DsRed-Sec7
Sec7
PI4P (PHFAPP)
TGN
GFP-Ypt31
Ypt31
PI4P (PHOsh2p)

Figure 2.5 PI4P is present in compartments of the late secretory pathway and is required for their transport. (A) Frames from a movie were superimposed to show vesicular-like structures labeled by the GFP-PH^{FAPP1} PI4P reporter moving rapidly towards the bud. Times in the movie of each frame are indicated. (B) Same as in (A), but showing the mCherry-PH^{Osh2p} PI4P reporter localizing to the plasma membrane and to mobile cytoplasmic puncta. (C) mChe-PH^{Osh2p} and GFP-Sec4 co-localizes at sites of growth. (D) mChe-PH^{Osh2p} and GFP-Sec4 overlap in some vesicular structures on the mother cell can be seen under conditions that affect vesicular transport, in this case *myo2-12* at the restrictive temperature (upper panels) or a strain with a mutation in Myo2p that abolishes Rab-binding (see text; bottom panels). (E) Using the mChe-PH^{Osh2p} reporter, PI4P enrichments at sites of growth can be clearly seen when following a cell during the cell cycle. Microtubules are labeled in green and the white arrows denote the direction of growth. (F) Using the *pik1-83* strain to rapidly deplete Golgi PI4P, we can see an uncoupling of Myo2p from Sec4p (upper panels) and a depolarization of Snc1 (lower panels) as soon as 5min after shifting to the restrictive temperature. The percentages in each panel denotes the number of cells with delocalized GFP-Sec4 or polarized Myo2p. (G) Early Golgi membranes, labeled with GFP-Sed5 (a cis-marker) or GFP-Gos1 (a medial marker) do not contain detectable PI4P and are not moved by Myo2p. Later Golgi cisternae, labeled by Sec7p or Ypt31p, are enriched in PI4P and are moved by Myo2p (H and I, respectively). H and I were assembled as in (A).

possibly because most of the mature secretory vesicles (which are labeled by GFP-Sec4) accumulate at growth sites. In order to test if vesicles formed in the mother are PI4P-rich, I decided to look in *myo2-12* cells shifted to the restrictive temperature, where the secretory vesicles are not transported and accumulate in the mother cell. Under these conditions most of the PI4P and Sec4p puncta were still distinct, but a significant number now co-localized (Figure 2.5D). We concluded that at least some Sec4p-containing secretory vesicles have detectable PI4P. This may be an underestimate as it is limited by our ability to image low levels of PI4P in small secretory vesicles. Moreover, when we image PI4P throughout a cell cycle, is clear that it is polarized, accumulating transiently at sites of growth and exhibiting the same behavior as polarized proteins that depend on vesicular trafficking (Figure 2.5E). Consistently, mobility and polarization of both reporters was abolished in the *myo2-12* mutant shifted to the restrictive temperature, indicating that indeed PI4P polarization is dependent on vesicular traffic and suggesting that the PI4P rich compartment is recognized and transported by Myo2p in a manner similar to that used for Sec4p-labeled secretory vesicles.

Indeed PI4P is a critical component of the secretory membrane transport machinery as judged by experiments in *pik1-83* cells expressing GFP-Sec4, or other secretory markers, and shifted to the restrictive temperature. Normally, Myo2p and other secretory markers co-localize at sites of growth, as is the case in *pik1-83* cells at room temperature (Figure 2.5F, top RT panel). Upon shifting

to the restrictive temperature of 35°C for just 5 minutes, Myo2p is still polarized, but Sec4p starts to become depolarized, labeling internal membranes that are not polarized (Figure 2.5F, top 35°C panel). Quantification of cells showing this uncoupling of motor from cargo increased in percentage at longer timepoints. Likewise, the polarized localization of GFP-Snc1p, the v-SNARE associated with secretory vesicles, also becomes affected after inactivating Pik1p (Figure 2.5F, bottom panels), indicating that the affect on GFP-Sec4 is not a consequence of it being lost from secretory membranes but of secretory membranes no longer being transported in a directed manner. Consistent with this, subcellular fractionation of *pik1-83* cells showed no significant change in the amount of transport vesicle-associated Sec4p before and after shift to the restrictive temperature in the same timeframe that results in a partial depolarization of GFP-Sec4 (not shown). However, these effects could be due to a lack of Sec4p activation by its GEF Sec2p. As explained in Chapter 1, Sec2p depends on a dual localization signal, including PI4P and the Rabs Ypt31/32p, and absence of any of them results in its partial mis-localization. To rule out this possibility, I generated a strain containing both the *pik1-83* allele and a constitutive active Sec4p version (Q79L mutation that abolishes intrinsic GTPase activity), and found that GFP-Snc1p still is partially depolarized immediately after Pik1p inactivation, indicating that the uncoupling of vesicles from Myo2p in the *pik1-83* strain is not due to changes in Sec4p activation but due to a reduction of PI4P on secretory membranes. Consistently, 8hrs after

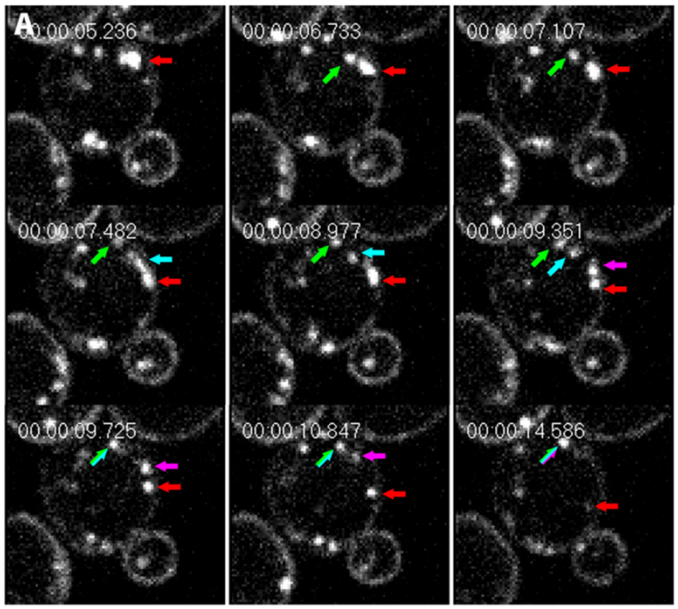
induction of *SAC1* overexpression, GFP-Sec4 is depolarized and cells are not growing (not shown), again indicating that PI4P is important for cell growth.

Since PI4P is necessary for the transport of secretory vesicles by Myo2p, I also tested if other Golgi membranes transported by Myo2p are enriched in PI4P. It has previously been reported that the late Golgi, labeled by Sec7p, is a Myo2p cargo (Rossanese *et al.*, 2001; Arai *et al.*, 2008). Sec7p is a GEF for Arf1p, which is the other localization determinant of the FAPP1 PH domain, and so it was expected that the GFP-PH^{FAPP1} compartment should precisely co-localize with RFP-Sec7p, and it does (Figure 2.5G). Consistent with these results, the Sec7p compartment also exhibits rapid directed movements towards the bud (Arai *et al.*, 2008; Figure 2.5H). The redundant pair of Rab proteins Ypt31/32p are enriched in the TGN (Jedd *et al.*, 1997), and have been found in our lab (Chapter 3) and by others to bind directly to the Myo2p GTD (Casavola *et al.*, 2008; Lipatova *et al.*, 2008). Imaging GFP-Ypt31p or Ypt32p in wild-type and *myo2* lever-arm mutants showed that this compartment too is transported by Myo2p (Daniel Schott, unpublished; Figure 2.5H). Consistently, co-imaging of GFP-Ypt31p with the PI4P reporter shows that most of these compartments are also rich in PI4P (Figure 2.5G). As expected, directed movements of these compartments were almost abolished in *pik1-83* cells at their restrictive temperature. On the other hand, membranes of the early secretory pathway that are not transported by Myo2p, were completely devoid of PI4P as judge by co-imaging of GFP-Sed5 (cis-Golgi) and GFP-Gos1 (medial-

Golgi) with mCherry-PH^{Osh2p} (Figure 2.5G). Thus, all the post-Golgi membranes, the Sec7p late Golgi, the Ypt31p/32p TGN, and the Sec4p-containing secretory vesicles, are all enriched in PI4P and transported by Myo2p in a PI4P-dependent manner. An additional, more direct way to prove that is the co-imaging of the PI4P reporter and secretory markers moving together in live cells. Although this proved difficult due to technical limitations, I obtained data showing that PI4P is enriched in membranes labeled by Sec7p or Ypt31p and, very likely, in ones labeled by Sec4p, moving in a polarized manner (Figure 2.6). In many instances I could see bigger PI4P structures moving as tubules or globular structures that at the same time gave rise to smaller vesicular profiles that could move and fuse with other PI4P-rich compartments (Figure 2.6A). Consistent with the transient nature of PI4P polarization (Figure 2.5E), in a few unbudded or very small budded cells with GFP-Sec4 caps, moving PI4P profiles will reach the cap and disappear rapidly (Figure 2.6B). Most frequently, in cells expressing these two markers, you see either red profiles or green profiles moving, but never a yellow structure (Figure 2.6C). On the other hand, co-imaging PI4P with Sec7-3XGFP or GFP-Ypt31 showed equally frequent examples of tubules or globular structures moving while having both markers or overlapping (Figure 2.6D, E; only showing GFP-Ypt31 examples).

Myo2p GTD does not bind PI4P directly *in vitro*

The data I have presented so far suggest a model where PI4P in secretory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00:00:07.107
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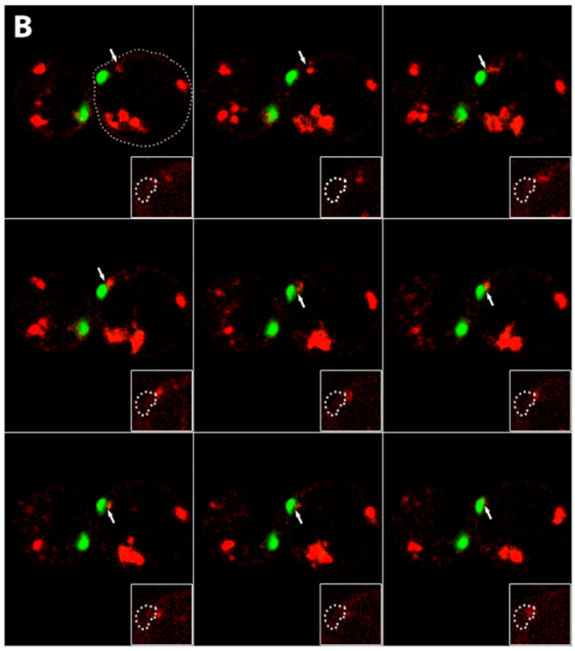
00:00:14.586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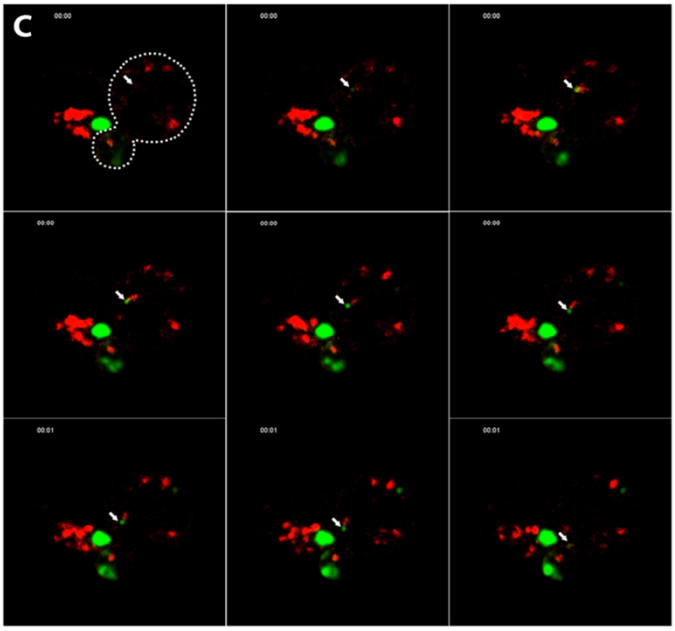
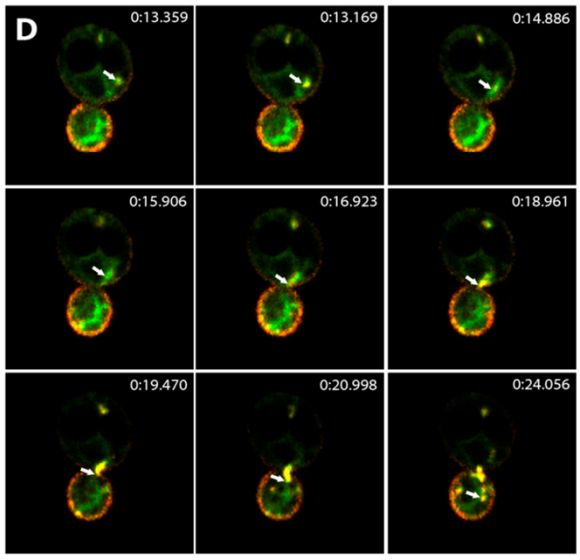

C


D
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0:15.906
0:16.923
0:18.961
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0:20.998
0:24.056

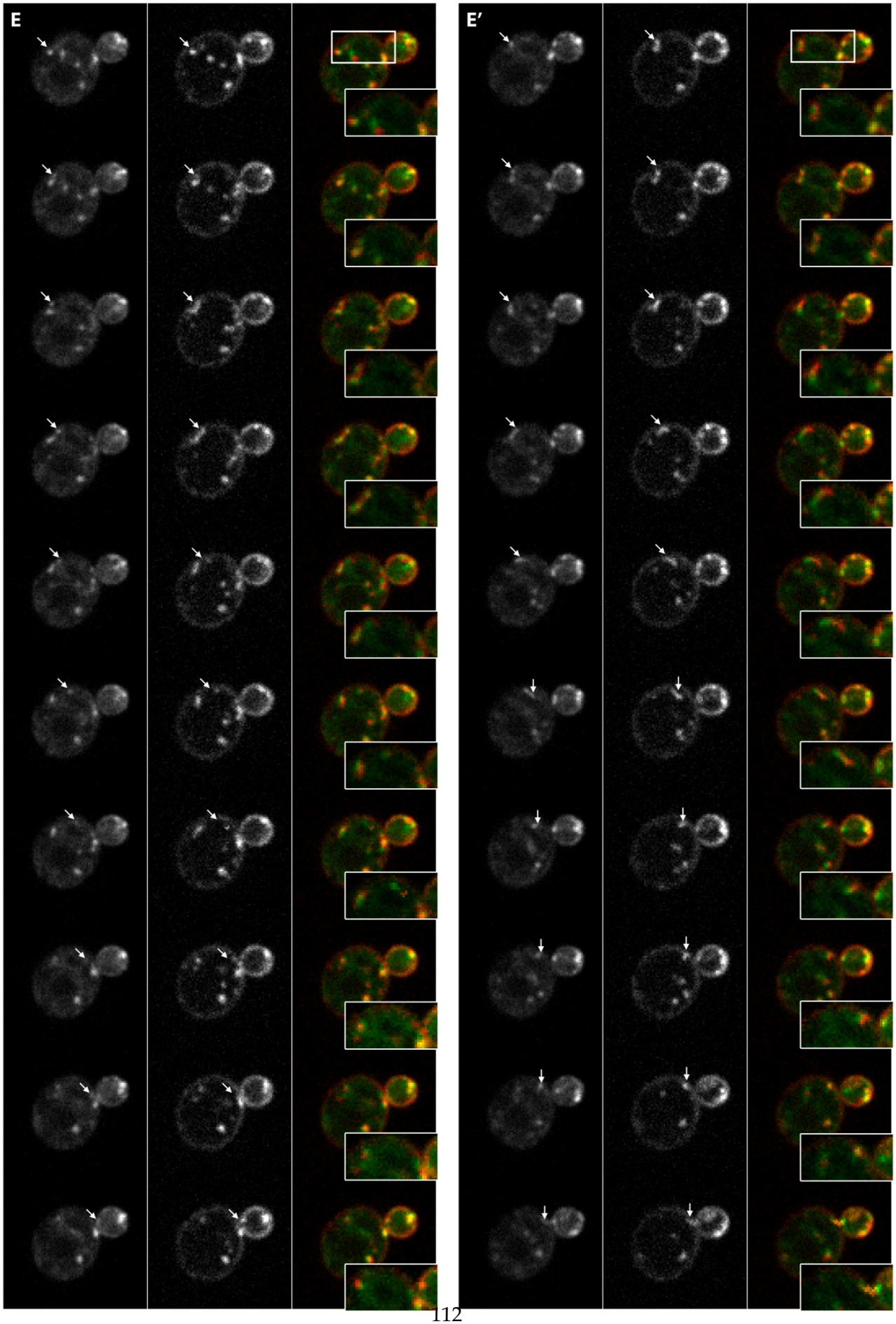

E
E′

Figure 2.6 PI4P is present on moving membranes labeled by late secretory markers. (A) mCherry-PH^{Osh2p} labels a tubule-vesicular structure that undergoes fusion and fission as it moves. The different colored arrows denote different examples of rapid movements. (B) Frames from a movies showing a mChe-PH^{Osh2p} PI4P-rich structure moving towards the GFP-Sec4 cap and disappearing rapidly upon reaching the site. In the inserts the GFP fluorescence was removed and outlined for better appreciation of the PI4P-labeling. (C) Frames from a movie showing a GFP-Sec4 vesicle forming and rapidly moving while being "followed" by a mChe-PH^{Osh2p} structure. (D) The bigger tubules labeled by GFP-Ypt31 are easier to see moving. In this movie the tubule, and the vesicles budding from it, is rich in PI4P as it moves towards the bud. (E and E') Examples of a vesicular and a tubular structure, with overlapping GFP-Ypt31 and mChe-PH^{Osh2p} labeling, moving along the same track. The first column shows the vesicular (E) example while the second column shows the tubular (E') example.

compartments is important for the recruitment of Myo2p to the membranes. Because some of our *myo2* alleles are especially sensitive to changes in PI4P levels, one could think that PI4P itself acts as a Myo2p receptor (or part of it). To test if Myo2p's compromised association with secretory vesicles in these alleles is in part due to defective PI4P binding, I explored this possibility using PIP strips and bacterially expressed Myo2p-tail constructs. However, the Myo2p-tail showed no specificity for any of the different PIs, binding most of them (except phosphatidylinositol) at very high Myo2p-tail concentrations while none of them at lower concentrations (Figure 2.7A). Although in these assays control proteins such as FAPP1 or PLCδ PH domains bind very strongly and specifically (Figure 2.7A and not shown), in many instances PIP strips do not recapitulate *in vivo* interactions. With the arrival of Dr. Chris Fromme to the Institute, I adopted their more physiological assay with liposomes of defined composition and tested if the Myo2p-tail will associate with them. This assay presents Myo2p with membranes (small unilamellar vesicles or SUV) of the same composition, curvature, and size as inside a living cell, and after some incubation time, a sucrose step-gradient is layered on top. Because the liposomes are filled with buffer, they will float to the top of the gradient upon ultracentrifugation, bringing with it proteins that stably bind lipids. I found no change in the amount of Myo2p-tail that floated with PI4P-containing SUV than without PI4P (Figure 2.7B). However, there was always a small amount floating with liposomes of 100, 200, or 400nm, but not with giant unilamellar liposomes (>1μm), indicating that either Myo2p-tail has affinity for curved

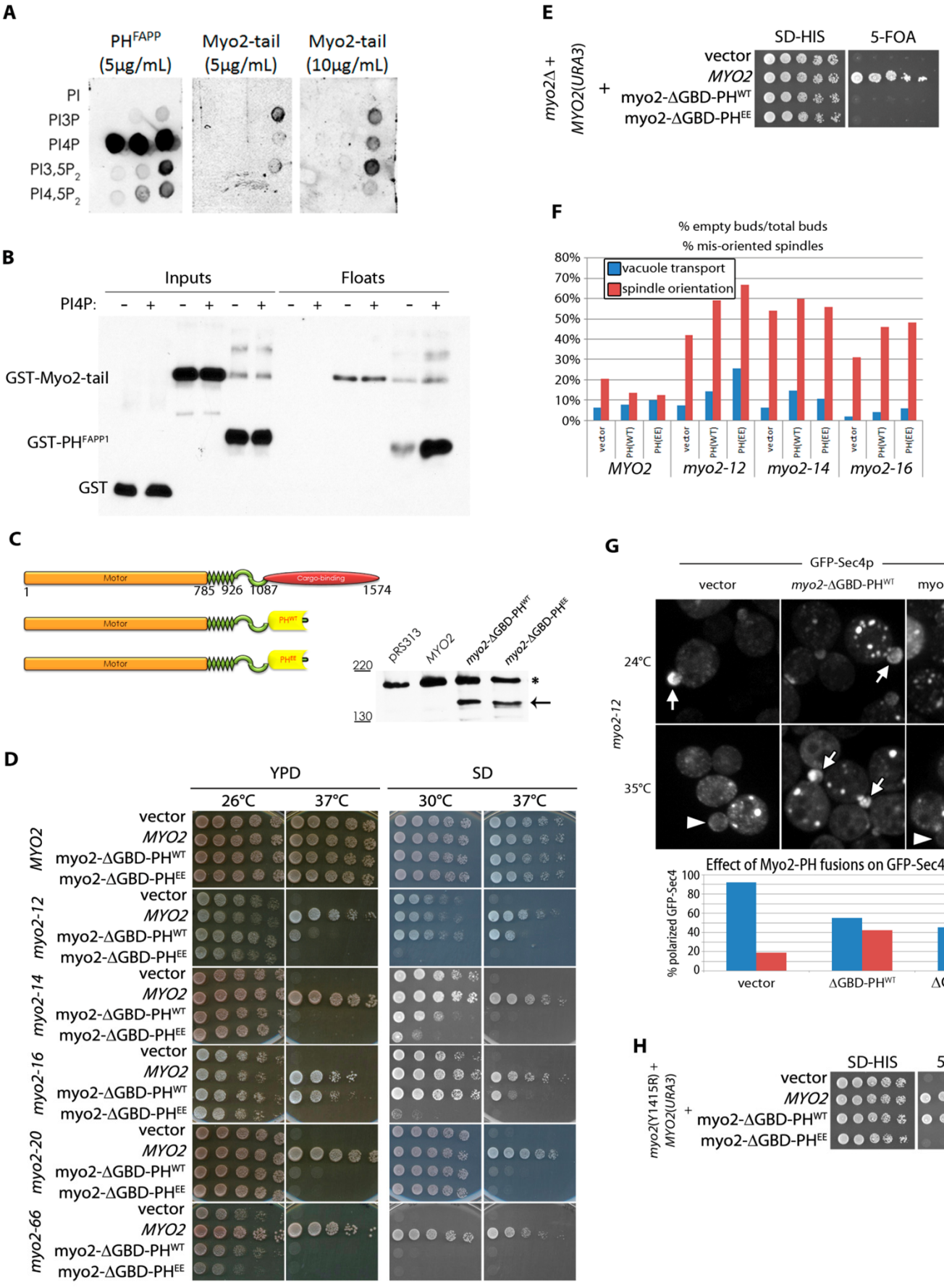

A
PH^FAPP (5µg/mL)
Myo2-tail (5µg/mL)
Myo2-tail (10µg/mL)
PI
PI3P
PI4P
PI3,5P2
PI4,5P2
B
Inputs
Floats
PI4P:
GST-Myo2-tail
GST-PH^FAPP1
GST
C
Motor
Cargo-binding
1
785 926 1087
1574
PH^WT
PH^EE
pRS313
MYO2
myo2-ΔGBD-PH^WT
myo2-ΔGBD-PH^EE
220
130
D
YPD
SD
26°C
37°C
30°C
37°C
vector
MYO2
myo2-ΔGBD-PH^WT
myo2-ΔGBD-PH^EE
myo2-12
myo2-14
myo2-16
myo2-20
myo2-66
E
myo2Δ + MYO2(URA3)
SD-HIS
5-FOA
vector
MYO2
myo2-ΔGBD-PH^WT
myo2-ΔGBD-PH^EE
F
% empty buds/total buds
% mis-oriented spindles
vacuole transport
spindle orientation
80%
70%
60%
50%
40%
30%
20%
10%
0%
vector
PH(WT)
PH(EE)
MYO2
myo2-12
myo2-14
myo2-16
G
GFP-Sec4p
vector
myo2-ΔGBD-PH^WT
myo2-ΔCBD-PH^EE
24°C
35°C
myo2-12
Effect of Myo2-PH fusions on GFP-Sec4 localization
% polarized GFP-Sec4
100
80
60
40
20
0
24°C
35°C
vector
ΔGBD-PH^WT
ΔGBD-PH^EE
H
myo2(Y1415R) + MYO2(URA3)
SD-HIS
5-FOA
vector
MYO2
myo2-ΔGBD-PH^WT
myo2-ΔGBD-PH^EE

Figure 2.7 An indirect interaction with PI4P is essential for the specific Myo2p association with secretory membranes. (A) PIP strips showing the specific association of PH^{FAPP1} with PI4P, but no recognition of any specific lipid by the Myo2p tail at low or high concentrations. (B) Liposome floatation assays showing the increased binding of PH^{FAPP1} to PI4P-containing liposomes, but no preference by Myo2 tail for either liposome. (C) Schematics of the Myo2-PH domain fusions and their similar expression levels by western blot (130 and 220 are kDa markers; the star denotes endogenous Myo2p and the arrow the PH domain fusions). (D) Growth of a subset of *myo2* mutants expressing either empty vector or the different Myo2p constructs showed in (C). The first column shows the growth in rich media with no selection for the plasmids, and the second column shows the dominant negative effect of the mutant PH domain fusion due to selection in minimal media. (E) Plasmid shuffle in a *myo2*Δ strain covered with a wild-type copy of *MYO2* in a *URA3*-based plasmid. The strain was transformed with the constructs showed in (C) and spotted in media lacking histidine or containing 5-FOA, to select against the *URA3* plasmid. (F) The Myo2-PH domain fusions are dominant negative for other Myo2p functions, like vacuole inheritance and spindle orientation. The blue bars represent the percentage of total buds without a vacuole and the red bars the percentage of budded cells with mis-oriented spindles. (G) The wild-type PH domain fusion can partially restore Sec4p localization, accounting for growth at high temperatures. Arrows and arrowheads indicate presence or absence of GFP-Sec4 enrichments, respectively. (H) Plasmid shuffle in a *myo2*Δ strain showing complementation of the otherwise lethal mutation Y1415R in Myo2p by the wild-type PH domain fusion.

membranes or very weakly associates with another component of the SUV (see Materials and Methods for lipid composition of the SUV). A very unlikely explanation could be that the PI4P-binding region is outside the tail domain, as it has been shown for other myosin classes that the lever arm or coiled coil region mediates interactions with PIs (Hokanson *et al.*, 2006; Feeser *et al.*, 2010; Komaba and Coluccio, 2010; Plantard *et al.*, 2010), however for a type V myosin that interaction could interfere with its translocation along actin.

Enhancing the interaction of Myo2p with PI4P can rescue *myo2* mutations and restore its association with secretory membranes

Instead, a more plausible explanation for the lack of *in vitro* binding is that the Myo2p GTD interacts with PI4P indirectly, through an adaptor protein. We reasoned that linking Myo2p directly to the normal levels of Golgi PI4P might bypass the need for such an adaptor protein. I made a construct in which the GTD of Myo2p was replaced by either the FAPP1 PH domain (Myo2-ΔGTD-PH^{WT}) or an equivalent construct with mutations (Q16E, R18E) that compromise the ability of the PH domain to bind PI4P (Myo2-ΔGTD-PH^{EE}; Figure 2.7C). Since Myo2p dimerizes, introduction of these constructs into strains with conditional *myo2* mutations is expected to generate a subpopulation of heterodimers – having one chain with the PH domain in place of the GTD, and one with the mutated GTD. The Myo2-ΔGTD-PH^{WT} completely suppressed the temperature sensitivity of *myo2-12* and *myo2-16*, the two alleles that are especially sensitive to reduced levels of PI4P (Figure 2.7D and

Table 1). Importantly, it also partially suppressed just those mutants that could also be suppressed by *sac1*Δ, but not those, like *myo2-14*, unaffected by elevation of PI4P (Figure 2.7D and Table 1). The fact that enhancing PI4P association only rescues a subset of mutants indicates that it is not a general non-specific mechanism to suppress conditional *myo2* mutants, but rather is restoring a defective interaction that only this subset of alleles exhibit. Consistent with that, the ability of Myo2-ΔGTD-PH^{WT} to rescue a specific class of *myo2* mutants does not extend to suppression of a *myo2*Δ, indicating that this fusion protein is not bypassing the essential function of Myo2p (Figure 2.7E). Moreover, since the Myo2-ΔGTD-PH^{EE} construct defective in lipid binding was unable to rescue any alleles, albeit being expressed at similar levels as the PH^{WT} fusion (Figure 2.7C,D), the suppression we observe is specifically due to an interaction with PI4P. This assay also demonstrates the essential role of PI4P in Myo2p secretory function, as expression of the mutated PH^{EE} fusion protein had a dominant-negative effect causing a decrease in the restrictive temperature of many *myo2* alleles (Figure 2.7D and Table 1). In part this deleterious effect could be due to the fact that the complementation of the essential function of *myo2-12* and *myo2-16* by the introduction of Myo2-ΔGTD-PH^{WT} did not extend to other functions of Myo2p; indeed it acted as a dominant negative for both spindle orientation and vacuole inheritance (Figure 2.7F). Thus, it specifically rescues the transport of compartments of the secretory pathway. Interestingly, consistent with this dominant negative effect, expression of either Myo2-PH fusion construct caused a partial depolarization

of GFP-Sec4 in *myo2-12* cells at the permissive temperature (Figure 2.7G). The Myo2-ΔGTD-PH^{WT} was, nevertheless, necessary to sustain sufficient polarization of GFP-Sec4 at the restrictive temperature for growth, whereas the Myo2-ΔGTD-PH^{EE} did not (Figure 2.7D,G). Thus, while the *myo2-12* and *myo2-16* alleles disrupt association with secretory vesicles at the restrictive temperature, enhancing the connection of these mutant proteins with PI4P now allows for an association with membranes that contain GFP-Sec4.

As it will be discussed in Chapter 3, the Rab proteins Ypt31/32p and Sec4p provide an additional linkage between Myo2p tail and secretory membranes. Recently, residues in Myo2p required for binding Ypt31/32p were identified (Casavola *et al.*, 2008; Lipatova *et al.*, 2008). I found that these same residues are required for binding all three exocytic Rabs and when mutated render Myo2p non-functional (see Chapter 3 for more details). Remarkably, when I transformed the Myo2-ΔGTD-PH^{WT} construct into strains carrying one of these mutations, Y1415R, the strain can now grow, whereas with the lipid binding defective construct, Myo2-ΔCTD-PH^{EE}, it cannot (Figure 2.7H). Thus, a lethal mutation that abolishes the interaction with the Rab proteins can be bypassed by providing a domain to enhance the association with normal levels of PI4P. In these cells, as expected, the PI4P rich compartment is polarized, but so is GFP-Sec4 (Figure 2.7G). Thus, although Myo2-Y1415R and Myo2-ΔGTD-PH^{WT} cannot bind to Sec4p, Myo2-ΔGTD-PH^{WT} can nevertheless associate with the PI4P and Sec4p-containing compartment to allow for both

polarized membrane delivery and for Sec4p to perform its essential function in exocytosis.

Golgi PI4P becomes limiting upon Myo2 GTD overexpression

Earlier studies have shown that Myo2p tail overexpression is lethal by interfering with endogenous Myo2p, presumably by binding the secretory vesicle receptor and thereby uncoupling secretory compartments from the motor (Reck-Peterson *et al.*, 1999; Schott *et al.*, 1999). I have found that overexpression of the Rab-binding deficient GTD is not lethal, but it nevertheless interferes with the nonessential functions of Myo2p like vacuole inheritance (not shown). Therefore these experiments demonstrate the presence of a specific saturable receptor on secretory membranes. Although we believe there are multiple components of this receptor (see Discussion), we wondered if by also increasing PI4P (assuming it acts as a receptor) we could overcome the saturation and lethality caused by the overexpressed GTD. I co-transformed *GAL1*-driven plasmids containing *PIK1* and *MYO2* GTD and tested whether *PIK1* overexpression could suppress the lethality conferred by overexpression of the GTD. Consistent with our model, overexpression of *PIK1* could partially suppress the lethal phenotype of the Myo2p tail, whereas the other PI 4-kinase *STT4*, or the Myo2p-binding partner *SMY1*, could not (Figure 2.8A). These growth differences were not due to decreased expression of the GST-Myo2p-tail construct as they remained equivalent in all the strains (Figure 2.8B). Moreover, in cells expressing the Myo2-ΔGTD-PH^{WT} construct, where we

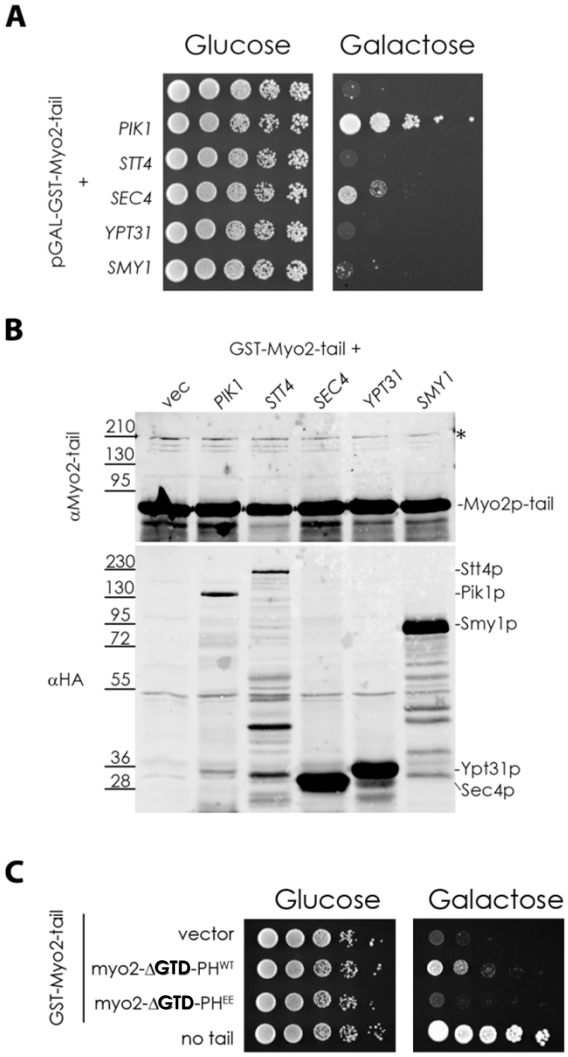


Figure 2.8 Golgi PI4P becomes limiting upon Myo2p GTD overexpression. (A) Cells co-overpressing the GST-Myo2-tail construct together with the indicated genes were spotted in glucose (repression) and galactose (expression). *PIK1* overexpression can partially suppress the lethality of the Myo2-tail. (B) Western blot showing the similar levels of GST-Myo2-tail expressed in the different strains used in (B). (C) Overexpression of the Myo2-tail in cells expressing the Myo2-PH domain fusions. Presence of the wild-type PH domain fusion partially block the lethality of the Myo2-tail.

presumably bypass the adaptor protein between Myo2p and PI4P, the GST-Myo2p-tail is also unable to completely inhibit growth (Figure 2.8C). These results indicate that PI4P becomes a limiting factor for the association of Myo2p with secretory vesicles in the presence of excess GTD, and under conditions where PI4P can be linked directly to Myo2p, the overexpressed GTD can no longer completely interfere with endogenous Myo2p. Disappointedly, however, overexpression of the GTD in *sac1*Δ cells, which accumulate much more PI4P than *PIK1* overexpression, still kills the cells (not shown). This result is complicated by the fact that *sac1*Δ cells are very sick, have defects in the actin cytoskeleton by themselves, and act in many more pathways than Pik1p. Perhaps the combination of all these defects with the overexpress GTD is too disruptive for the cell.

Possible linkers between Myo2p and PI4P

Recently, a PI4P-binding protein was identified in the fly that links Golgi PI4P to a myosin motor (Dippold *et al.*, 2009). This protein, GOLPH3, has homologues all the way from yeast to humans, although it is unclear if they perform exactly the same functions. Binding of GOLPH3 to PI4P is important for its localization to the TGN, where its recruits the PDZ-containing unconventional myosin MYO18a, and knockdown of MYO18a has a phenotype similar to loss of GOLPH3. The authors suggest that the PI4P-GOLPH3-MYO18a linkages provide pulling forces to maintain the shape of the TGN and for the efficient budding of vesicles or extraction of tubules. More importantly,

GOLPH3 can complement the phenotype of a deletion of its homologue in yeast, *VPS74*, indicating that at least they share a conserved function (Wood *et al.*, 2009). Although unlikely, since *VPS74* is a dispensable gene, I tested if it could act as a linker between Myo2p and PI4P by looking at its overexpression effects. The thinking was that if the reason *myo2-12* and *myo2-16* are PI4P sensitive is because of a partially defective binding to Vps74p, increasing the amount of that protein might compensate and rescue the temperature sensitivity. I constructed *GAL1*-induced plasmids containing *VPS74* tagged at its C-terminus with 3XHA, and a mutant *vps74* version that cannot binds PI4P (as reported by Dippold *et al.*, 2009; Wood *et al.*, 2009). Both versions were expressed similarly but failed to rescue any of the *myo2*^{ts} mutants (Figure 2.9A). I found out later at a cell biology meeting that tagging either end of *VPS74* renders it nonfunctional, a possibility that, given the nonessential nature of *VPS74*, I never tested. However unlikely, the involvement of Vps74p in bridging Myo2p to PI4P remains, because of functionality issues, an open question.

Another candidate to serve as a linker between Myo2p and PI4P is the Sec4p GEF Sec2p. Of all the late secretory genes, *SEC2* is the only one that has been reported to accumulate vesicles randomly throughout the cell, indicative of a problem with both transport and fusion (Walch-Solimena *et al.*, 1997). Sec2p associates with vesicles in a way that depends on the Rab Ypt31/32p and, recently shown, PI4P binding (Ortiz *et al.*, 2002; Mizuno-

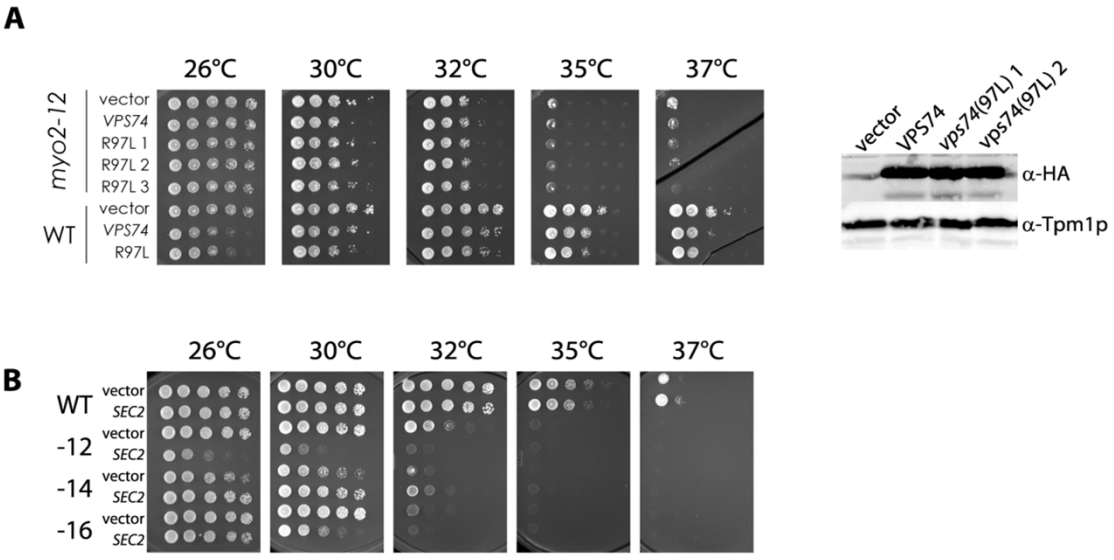


Figure 2.9 Effects of the overexpression of possible Myo2p-PI4P bridging proteins. Overexpression of the PI4P-binding proteins Vps74p (A) or Sec2p (B) have no effect on the growth of *myo2* mutants (selected ones shown). Expression levels of the Vps74p constructs are shown to the right, as well as Tpm1p levels as loading control.

Yamasaki *et al.*, 2010). These observations strongly suggest that Sec2p may play a role in linking Myo2p to secretory membranes, and the fact that it is a PI4P effector, makes it a likely candidate to serve as the bridge between Myo2p and PI4P. Following the same reasoning as for Vps74p, I obtained a *GAL1*-driven *SEC2* construct from the R. Collins lab and tested if its overexpression could rescue any of the *myo2*^{ts} mutants. I was unable to check expression levels, but after transformation of this construct, growth in galactose plates showed no differences in growth (Figure 2.9B). Nevertheless, I found an interesting two-hybrid interaction between Myo2p and Sec2p that prompted additional tests presented in Chapter 4.

Table 1 Summary of genetic interactions with *myo2* tail mutants

Allele	Restrictive temperature[1]	Overexpression of *PIK1*[2]	Combined with *pik1-139*	*Combined with sac1*Δ	Expression of *myo2*ΔCTD-PH^{WT} [3]	Expression of *myo2*ΔCTD-PH^{EE} [3]
MYO2^{+}	>37°C	NE	36°C	NE	NE	NE
myo2-12	33°C	35°C	30°C	37°C	>37°C	30°C
myo2-13	35°C	NE	NE	>37°C	37°C	33°C
myo2-14	35°C	NE	NE	NE	NE	33°C
myo2-16	32°C	NE	30°C	36°C	>37°C	30°C
myo2-17	37°C	NE	NE	NE	NE	NE
myo2-18	36°C	NE	NE	37°C	37°C	NE
myo2-20	37°C	NE	NE	NE	NE	NE

The highest temperatures showing growth are reported for strains with either wild-type *MYO2*, or *myo2* alleles with mutations in the C-terminal domain (Schott et al., 1999). NE, no effect.
[1]Restrictive temperature on synthetic complete medium. [2]*PIK1* was expressed behind the *GAL1* promoter. [3]The fusion constructs were expressed from the *MYO2* promoter.

DISCUSSION

Most of the receptor proteins that bridge Myo2p to its diverse cargoes have been identified, with the noticeable exception of the secretory compartments, which are its only essential cargoes. Not only are they the most

important cargo in yeast, but they are the one Myo2p function that is conserved throughout evolution. Metazoans undergo an open mitosis not requiring orientation of the nucleus, and other organelles are moved by microtubule motors, but secretory organelles still require myosin V-based transport to reach their destination. Given its importance, why is this receptor proving so elusive? One strategy used for delivery of organelles involves degradation of the organelle-specific receptor at its destination, as has been seen for Vac17p mediated delivery of the vacuole and suggested for correct peroxisome inheritance. This is well suited for organelles like vacuoles and peroxisomes that require a one-way trip at a specific point in the cell cycle. In contrast, compartments of the secretory pathway are transported throughout the cell cycle to specific locations, so the receptor is more likely to persist rather than be degraded. Moreover, there are at least three populations of vesicles, low density vesicles that go directly to the plasma membrane from the TGN, high density vesicles that form at the endosome, and exomer-dependent vesicles, with distinct contents and itineraries. All three are moved by Myo2p, thus it has to be able to recognize the type of vesicle is moving and when to deliver it. Therefore, it seems likely that the secretory compartment receptor is a multicomponent complex, with some basic core elements common to all types of secretory membranes, and with additional components unique to each vesicle. Several studies have suggested Rab proteins as secretory membrane-specific components involved in bridging to Myo2p. However, the interaction between a subset of exocytic Rabs and Myo2p is very weak both *in vitro* (with

purified proteins) and *in vivo* (by two-hybrid), is not specific for the activated form of the Rabs *in vitro*, and cannot account for the specificity in recognition of the different types of vesicles (see Chapter 3). In this chapter I have presented evidence to support the idea that additional components play a role in the association of Myo2p with secretory compartments, with the lipid PI4P being one of them.

This family of molecules, the PIs, is specifically located to certain membranes, providing an identification tag for the compartment. Because of their exquisite localization, efficiently regulated by PI kinases and phosphatases, these organelle-specific lipids could play a role in Myo2p's selectivity in cargo selection. We therefore focused on PI4P for two reasons: first, PI4P is greatly enriched at the TGN, where secretory vesicles originate; and second, mutations in *PIK1*, encoding the Golgi-localized PI 4-kinase, showed synthetic genetic interactions with the *myo2-66* allele, as do mutations in late *SEC* genes, including *SEC4*, the Rab GTPase associated with secretory vesicles, and *YPT31*, the other Rab GTPase in the secretory pathway, indicating that in some way, Myo2p, Pik1p, Ypt31p and Sec4p are functionally related. Consistently, we identified a subset of GTD *myo2* mutants that are especially sensitive to levels of Golgi PI4P, being rescued by increased levels of PI4P while being highly sensitive to its reduction. Moreover, in cells containing a wild-type Myo2p, use of the conditional *pik1-83* mutation to rapidly reduce Golgi PI4P levels, caused a rapid uncoupling of secretory vesicles from Myo2p when the

cells are shifted to the restrictive temperature, strongly implying a direct relationship between PI4P and the association of secretory vesicles with Myo2p.

Consistent with the specific localization of PI4P to late secretory compartments, we found that the PI4P effects are specific for the secretory functions of Myo2p and do not affect other cargoes. We show that the Sec7p compartment, generally believed to represent the late Golgi, is rich in PI4P and is actively transported towards growth sites. The Ypt31/32p compartment, which overlaps with the Sec7p compartment but additionally consists of distinct tubulo-vesicular structures, is also rich in PI4P and exhibits rapid polarized movements. Finally, the secretory vesicles labeled by Sec4p are known to be transported by Myo2p, and my localization experiments strongly suggest that they also contain PI4P, albeit in a diminished amount. Since all these compartments are moved by Myo2p and contain PI4P, we predicted that other earlier secretory membranes, not moved by Myo2p, were going to be devoid of detectable PI4P. Indeed, we used two PI4P reporters, GFP-PH^{FAPP1} that identifies PI4P together with Arf1p and the unbiased PI4P reporter GFP-PH^{Osh2p}, and found no overlap between these reporters and early secretory markers while they precisely coincide with the later compartments. This correlation suggested that PI4P may be necessary for the transport of these compartments by Myo2p, an idea consistent with the fact that upon depletion of Golgi PI4P, these compartments are no longer moved and become depolarized.

A more stringent test would be the ability to co-visualize markers for both PI4P and each secretory compartment during Myo2p-directed movement. This is very challenging due to the small size and rapid rate of movement and, more specifically, to the dim nature of the red markers. However, I was able to image both markers in cells containing versions of Myo2p with shorter lever arms that reduce the speed of movement. In these cells, timelapse imaging showed moving structures labeled by Ypt31p (and by Sec7p, not shown) also labeled, completely or partially overlapping, with the PH^{Osh2p} PI4P reporter. The several examples where they overlapped suggests that they are in different subdomains of the compartment, however I believe that because of their fast movement, and the delay of the microscope in changing lasers and filters, the images are not in perfect register. To prove that, I tried to change the fluorescent tags on these markers but was unsuccessful in getting functional fusions. However, since in the static compartments both markers co-localize perfectly (Figure 2.5G and 2.6D,E), I believe that they are both present in the moving structure. Although this is particularly clear for Sec7p and Ypt31p, even in the 0IQ-Myo2p mutant (the slowest of all IQ mutants) it was challenging to see a moving GFP-Sec4 profile labeled by the PI4P reporter. Nevertheless, the fact that when imaging only the mCherry-PH^{Osh2p} reporter we can see small vesicular, as well as tubular, structures rapidly moving towards sites of growth suggests that some vesicles have enough PI4P as to be imaged while moving (Figure 2.6A). Many of the tubular structures undergo size changes (maybe fission and fusion events) while they move towards growth

sites, and they are usually bigger than the GFP-Sec4 puncta. These vesiculo-tubular structures are reminiscent of the membranes seen by imaging GFP-Ypt31, supporting the findings with co-imaging of GFP-Ypt31 and PI4P. Secretory vesicles, however, with their small size may have amounts of PI4P that are frequently below the level of detection for the 10 frames per second required to document their movement. To alleviate this problem, we localized PI4P and Sec4p in the *myo2-12* conditional mutant at the restrictive temperature, where vesicles are uncoupled from Myo2p and do not exhibit rapid movements. Under these conditions, we could see a significant increase in the amount of membranes having both Sec4p and the PI4P reporter. However, not all Sec4p-vesicles have detectable PI4P even under these conditions, which could mean that the amount of PI4P diminishes as the compartment matures, to a point below detection. This conversion of PI4P, either by degradation or phosphorylation into another PI species, is consistent with our observation that PI4P-rich structures disappear once they join GFP-Sec4 rich sites (Figure 2.6B). This possibility could explain why we only see accumulations of PI4P at growth sites in rapidly growing cells, where the flux of incoming vesicles may exceeds the rate of PI4P conversion. The cell does not need secretory vesicles to deliver abundant PI4P to the plasma membrane as the PI 4-kinase Stt4p localizes there, where it provides the PI4P synthesis needed for production of the essential lipid $PI(4,5)P_2$ by plasma membrane localized Mss4p. Nevertheless, the strong accumulation of PI4P seen in small buds where secretory vesicles accumulate, and the fact that we sometimes see

small vesicular profiles moving with the mCherry-PH^{Osh2p} reporter, strongly suggests that PI4P is present during transport of secretory vesicles to their destination. Moreover, in rapidly growing cells, the PH^{Osh2p} PI4P reporter labels the plasma membrane only at sites of growth, and yet the Stt4p or Mss4p kinases are not polarized, indicating that the enrichment of PI4P is either due to the huge flux of incoming secretory vesicles to the site, or unlikely, to a specific protection of the PI4P generated in this area from dephosphorylation/phosphorylation. Furthermore, under special conditions of growth, the plasma membrane localized PI 4-kinase Stt4p is not essential (Cutler *et al.*, 1997; Foti *et al.*, 2001), and, although the cells grow very slowly, arriving secretory vesicles can supply a sufficient trickle of PI4P for $PI(4,5)P_2$ synthesis and cell viability, again supporting the concept that secretory vesicles must contain PI4P. Very recently, it was also shown in mammalian cells that vesicular traffic contributes to the production of $PI4,5P_2$ at the plasma membrane (Szentpetery *et al.*, 2010). It is not yet clear where, and by what phosphatase, secretory vesicle PI4P is normally hydrolyzed (or converted) and how this is integrated with exocytosis. However, these findings can now be used to explain how Myo2p release from the vesicle and vesicle tethering and fusion are coupled (see Chapter 5 for a more extended discussion about this idea).

How does Myo2p associate with PI4P? Since in our *in vitro* assays we failed to get a direct interaction between the Myo2p tail and PI4P, we

hypothesize that the binding to PI4P is indirect through an adaptor protein. We reasoned that if indeed the *myo2* alleles sensitive to low PI4P were partially defective in binding the PI4P adaptor protein, we could by-pass it by tethering Myo2p directly to PI4P. Instead of fusing a PI4P-binding module to the tail domain, we replaced the whole tail domain with the lipid-binding module to make the Myo2-ΔGTD-PH^{FAPP1} construct. Expression of this construct can restore viability to just those *myo2* alleles sensitive to low PI4P, indicating that PI4P is an important factor, but not the only one necessary for tethering Myo2p to secretory compartments. Importantly, the construct cannot suppress many of the other conditional *myo2* alleles, and cannot replace the function of wild-type Myo2p, thereby demonstrating the requirement for additional components. The Rab proteins Ypt11p and Ypt31/32p are known to interact with the tail of Myo2p, and we show that Sec4p also interacts directly (Chapter 3). Interestingly, a Myo2p-tail mutation that eliminates interactions with all these Rab proteins is lethal, thereby demonstrating that the Rab proteins are also part of the Myo2p-secretory membrane receptor. Surprisingly, the Rab requirement can also be by-passed by the Myo2-ΔGTD-PH^{FAPP1} construct, indicating that Rabs are not the only other component of the receptor. Although these results could be explained by saying that there is an additional essential function for Myo2p different from polarized transport, like mitochondrial inheritance or coordination of transport with exocytosis, I believe that there is an additional PI4P and Rab independent factor involved for many reasons. If we look at the co-overexpression experiments with Myo2p-tail and

SEC4 or *PIK1*, you only partially rescue, indicating that you still are titrating something down besides Sec4p or PI4P. Also, only three of the seven GTD *myo2* alleles exhibit synthetic lethality with mutations in Sec4p, and only two do with *pik1*^{ts}, yet they all uncouple Myo2p from vesicles. Therefore, we propose that PI4P collaborates in Myo2p secretory function by being part of a multicomponent receptor for secretory compartments. This model is further supported by the findings discussed in Chapter 3 regarding the Rab GTPases.

How general might be the requirement for PIs in tethering cargo to a molecular motor? In plants, the delivery of secretory vesicles necessary for pollen tube and root tip growth depends on PI4P, synthesized and regulated by the Pik1p and Sac1p homologs PI4Kβ1 and SAC7 (Preuss *et al.*, 2006; Thole *et al.*, 2008). Microtubule based kinesins also function with PIs. The nematode kinesin Unc104 has a PH domain that binds PI(4,5)P_2 directly to transport synaptic vesicles (Klopfenstein *et al.*, 2002; Klopfenstein and Vale, 2004), and special vesicles rich in PI(3,4,5)P_3 are delivered to the tips of neurites by the kinesin GAKIN (KIF13B) bridged through the PI(3,4,5)P_3-binding protein PIP3BP (Horiguchi *et al.*, 2006). As far as we are aware, Myo2p is the first myosin shown to require a phosphoinositide to associate with its cargo.

CHAPTER 3[2]

THE RAB PROTEINS YPT31/32P AND SEC4P PROVIDE A DIRECT LINKAGE BETWEEN THE MYO2P TAIL AND SECRETORY MEMBRANES.

INTRODUCTION

As described in Chapter 1, eukaryotic cells have multiple membrane-bound compartments, or organelles, that carry out specific biochemical reactions. The large number of organelles has enabled eukaryotic cells to perform many different types of functions and survive under many different environmental conditions. An inherent problem of this expansion in membrane compartments is how to maintain the fidelity of transport between organelles. Rab GTPases were first identified in yeast based on the similarity of Sec4p to Ras (Salminen and Novick, 1987), and subsequent work over almost a quarter century has showed them to be master regulators of membrane trafficking (Hutagalung and Novick, 2011). Specific members of the Rab family associate with each organelle as well as with the transport vesicles they form, and by binding effector proteins, can couple the transport carriers to their destination. Moreover, in order for the whole process to occur efficiently, other factors such as coat complexes and molecular motors have to work hand-in-hand with Rabs. In yeast, secretory membrane traffic is absolutely essential for growth and division. Secretory membranes are transported from their site

[2]Some of the experiments presented in this chapter were previously published in Santiago-Tirado *et al.*, 2011.

of origin to the growing bud or site of division by the myosin-V Myo2p. How Myo2p associates with these compartments is not known, but for several reasons it has always been an attractive idea that it is through the action of Sec4p: first, Sec4p is the Rab GTPase associated with secretory vesicles (Goud *et al.*, 1988); second, *SEC4* and *MYO2* interact genetically (Govindan *et al.*, 1995; Schott *et al.*, 1999); and lastly, they localize to the same places in the cell (Pruyne *et al.*, 1998; Schott *et al.*, 1999). With the direct demonstration in the lab that secretory vesicles (marked by GFP-Sec4) are moved by Myo2p (Schott *et al.*, 2002), a similar approach was used to find other compartments of the secretory pathway that might be transported into the bud in a Myo2p-dependent manner. The redundant Rabs Ypt31p and Ypt32p were also found to be polarized, labeling structures that rapidly move towards growth sites similarly to GFP-Sec4 (Daniel Schott, unpublished; this thesis). Ypt31p and Ypt32p are the Rabs associated with the TGN, another Myo2p cargo (Rossanese *et al.*, 2001). A third Rab, Ypt11p, was found to be a multicopy suppressor of *myo2-66* and to bind directly to the Myo2p tail (Itoh *et al.*, 2002). More recently, Ypt11p was also found to bind with high affinity to Ret2p, a component of the COPI coat complex, and thus proposed to be part of the Golgi receptor for Myo2p (Arai *et al.*, 2008). In light of these and other results in higher organism, the emerging idea is that myosin-Vs associate with their secretory cargo in a Rab GTPase-dependent manner (Seabra and Coudrier, 2004). In analogy to Ypt11p, and because of the evidence mentioned above, the three Rabs Sec4p, Ypt31p, and Ypt32p are excellent candidates to link

Myo2p to its secretory cargo. I therefore set out to extend Daniel's findings and explore the role these Rabs, and associated regulators, may have in Myo2p secretory function.

MATERIALS AND METHODS

Yeast molecular techniques and microscopy. As described in Chapter 2. For imaging of cells overexpressing the GST-Myo2p tail, the cultures were grown overnight in SRaff media (to de-repress the *GAL1* promoter), diluted in the morning in fresh SRaff, and galactose added to a final concentration of 2% when reaching an OD_{600} of ~0.8. When incubated at room temperature (most of the experiments), I induced for at least 4 hours before harvesting for imaging. On some occasions due to time constrains, I induced at 26°C or 30°C for at least 2 hours before imaging.

Yeast two-hybrid analyses. Yeast strains AH109 and Y187 were used to transform constructs with the activation and DNA binding domain fusion proteins, respectively. For some experiments, AH109 was co-transformed with both activation and DNA binding plasmids. The presence of both plasmids was selected in media lacking leucine and tryptophan (double drop-out or DD) while an interaction was tested by growing in media additionally lacking histidine (triple drop-out or TD). To compare the strength of the interactions on a plate, 3-amino-1,2,4-triazole (3-AT) was added to a final concentration of 2.5 – 10mM. Cells were grown to log-phase (OD_{600} 0.8 – 1.0) and adjusted in a 96-well plate

to an OD_{600} 0.5, and 10-fold serial dilutions were made. The cells were spotted on the appropriate plates using a multichannel pipette and, after drying, incubated at 30°C for 3 – 4 days. Additionally, for some of the two-hybrid pairs, the strength of the interaction was quantified by liquid β-galactosidase assays. For this assay, overnight cultures were diluted in the morning in 5mL of QSD media (Rossanese *et al.*, 2001), grown until log-phase and washed and concentrated in Z-buffer (60mM $Na_2HPO_4 \cdot 7H_2O$, 40mM $NaH_2PO_4 \cdot H_2O$, 10mM KCl, 1mM $MgSO_4$). The cells were permeabilized and disrupted by repeated cycles of freezing in liquid nitrogen and thawing at 37°C. ONPG was used as the substrate for the β-galactosidase. Different concentration factors were used, but for the pBridge vectors, due to the low expression levels of the fusion proteins, I found that resuspension of 5mL of culture in 300µL gave good results (~16.67X concentration factor). I added 0.7mL of Z buffer + β-ME (50mM) to 100µL of each sample and blank tube. I added 160µL of the pre-warmed ONPG stock (4mg/mL in Z-buffer) and immediately started the timer. I carried out the reaction at 30°C and stopped it (when the yellow color developed) with 0.4mL of 1M Na_2CO_3. The reactions were centrifuged for 10 min at 14,000 rpm to pellet cell debris and 1mL of the supernatant transferred to clean cuvettes for reading Absorbance at 420. β-Gal units (defined as the amount of β-galactosidase that hydrolyzes 1 mmol of ONPG to o-nitrophenol and D-galactose per min per cell) were calculated as follows: β-Gal units = $1{,}000 \times OD_{420} / (t \times V \times OD_{600})$ where t is elapsed time (in min) of the reaction, V is 0.1 ml x concentration factor, OD_{600} of the culture prior to processing, and

OD_{420} of the samples relative to the blank. I cloned the Myo2p-tail constructs in pGADT7, the Rab proteins lacking their prenylation sites in pBridge, and used a Smy1p construct generated in the Huffaker lab made in pAS2-1 (Yin *et al.*, 2000). I have to stress the differences between the pBridge and pAS2-1 vectors, since the fusion proteins they generate are expressed at very different levels. The pBridge vector has a truncated *ADH1* promoter and the expression levels of the BD-fusion proteins are very low, to the point that I was unable to check expression by western blot even after tagging the proteins with two different epitope tags (HA or myc). On the other hand, pAS2-1 has the full-length *ADH1* promoter, resulting in very high levels of the BD-fusion protein, to the point that for some proteins containing acidic domains or extended coiled coil regions, caused autoactivation of the reporter genes by themselves (the plasmid was discontinued and replaced by pGBKT7 by the company that created it; http://www.clontech.com/products/detail.asp?product_id=206489). I, however, never tested the interactions between the Myo2p tail and the Rab GTPases in the pGBKT7 vector.

Expression and purification of the Myo2p tail constructs. Purification of GST-Myo2p tail constructs was done as described (Legesse-Miller *et al.*, 2006), but using as a host the Rosetta2 bacterial strain (Novagen). For the expression of a GST-Myo2p tail construct containing the coiled coil region, a PCR fragment starting at amino acid 926 was cloned into pGEX-6P-3 (GE Healthcare). I followed the same procedure to induce as for the GTD construct, but

expression was induced with 0.4mM IPTG at room temperature for at least 8 hours. For most experiments, after purification, the protein was left on the beads and the amount of protein bound estimated from protein gels by comparing to known amounts of BSA. The yield of the GST-Myo2-ccGTD was always lower than that of the GST-Myo2-GTD.

Expression and purification of the HIS_6-Rab GTPases. I constructed bacterial expression plasmids by cloning PCR products containing the whole ORFs encoding Sec4p, Ypt32p, and Ypt1p, into the pQE series of vectors (Qiagen). These vectors generate a six histidine fusion protein that can be purified using Ni-NTA agarose or Talon resin. Purification was done as described (Du and Novick, 2001), but using as a host the bacterial strain BL21 containing the pREP4 plasmid (necessary to repress expression from pQE vectors until addition of IPTG).

Expression and purification of the Smy1p fragments. I cloned various constructs of Smy1p in the six histidine fusion plasmid pQE30 and followed the same conditions for induction and purification as the Rab GTPase fusion proteins. The smallest of these contained the globular tail domain of Smy1p that expresses very well and was soluble but did not bind the beads. A slightly larger construct containing part of the coiled coil did not expressed well. In all the experiments I ended up using the full-length Smy1p, although a proteolytic fragment of ~42kDa co-purifies with the full-length protein. This fragment, depending on the purification, was very prominent and most probably is the

head domain of Smy1p as it is of the expected size.

In vitro binding assays. The Rab GTPases were preloaded with GDP or GTP-γS by resuspending 125nM of the protein in binding buffer (25mM Tris, pH 7.4, 80mM NaCl, 1mM $MgCl_2$, 1mM DTT) containing 1mM EDTA and 1mM of the nucleotide and incubating at 30°C for 30-min. The reaction was terminated by the addition of $MgCl_2$ to 5mM final. The GST-Myo2p tail bound to the beads was added (~1mM, usually 10-20µL of slurry) and the total volume adjusted to 600µL with binding buffer. Reactions were incubated for 1hr at 4°C with rotation. To ensure mixing of the beads (and to lower the background binding without affecting binding to the GST-Myo2p tail), I started adding a very small amount of triton X-100 to the samples during the incubation step (some of the experiments reported here, however, were done without this step). Addition of concentrations above 0.1% triton X-100 abolished binding. Beads were washed four times with wash buffer (binding buffer except 5mM MgCl2, 10% glycerol, 0.1% triton X-100) by inverting the tubes 10 times and collecting the beads by spinning in the swinging-bucket centrifuge. After the final wash the beads were resuspended in the same volume of 2X protein sample buffer as that of slurry originally used. Bound Rabs were separated by SDS-PAGE and analyzed by western blotting with anti-HIS_6 antibodies diluted 1:6000 (Sigma). For the competition assays, the same procedure was used but adding increasing amounts of Sec4p (from zero to 125nM) while leaving Ypt32p constant at 125nM. The concentration of the antibodies used here was 1:4000

for the monoclonal anti-Sec4p (C1.2.3) and 1:2000 for the rabbit anti-Ypt31p.

RESULTS

Rab overexpression can suppress the secretory defect of *myo2* mutants

Budding yeast expresses 11 Rab GTPases divided very broadly into two branches by sequence similarity (Figure 3.1A). To see if the exocytic Rabs have a role in Myo2p-dependent transport of secretory membranes I tested if their overexpression had any effect on the growth of the GTD *myo2*^{ts} mutants. Initially, I cloned *YPT31* and *SEC4* in single copy plasmids under the *ADH1* constitutive promoter and found that this moderate elevation of Rab levels could suppress *myo2-12* and *myo2-16* (Figure 3.1B). I later constructed plasmids containing all the exocytic Rabs under the strong *GAL1* promoter and found that only *SEC4*, under these high level expression conditions, could rescue *myo2-12* and *myo2-16* (not shown). For some reason, although having no effect on wild-type cells, high overexpression of *YPT31* is deleterious for the *myo2* mutants. Subsequently, in separate experiments looking for multicopy suppressors of *myo2-12*, I also recovered *SEC4* from a genomic library (see Appendix II). To verify that the rescue was due to a restoration of vesicle transport I use GFP-Sec2 as a marker for secretory vesicles. Even at room temperature, GFP-Sec2 in *myo2-12* was mostly diffuse but enrichments in very small buds or at the neck in large buds were clear (Figure 3.1C; compare to wild-type cells where there is almost no cytoplasmic staining, requiring an

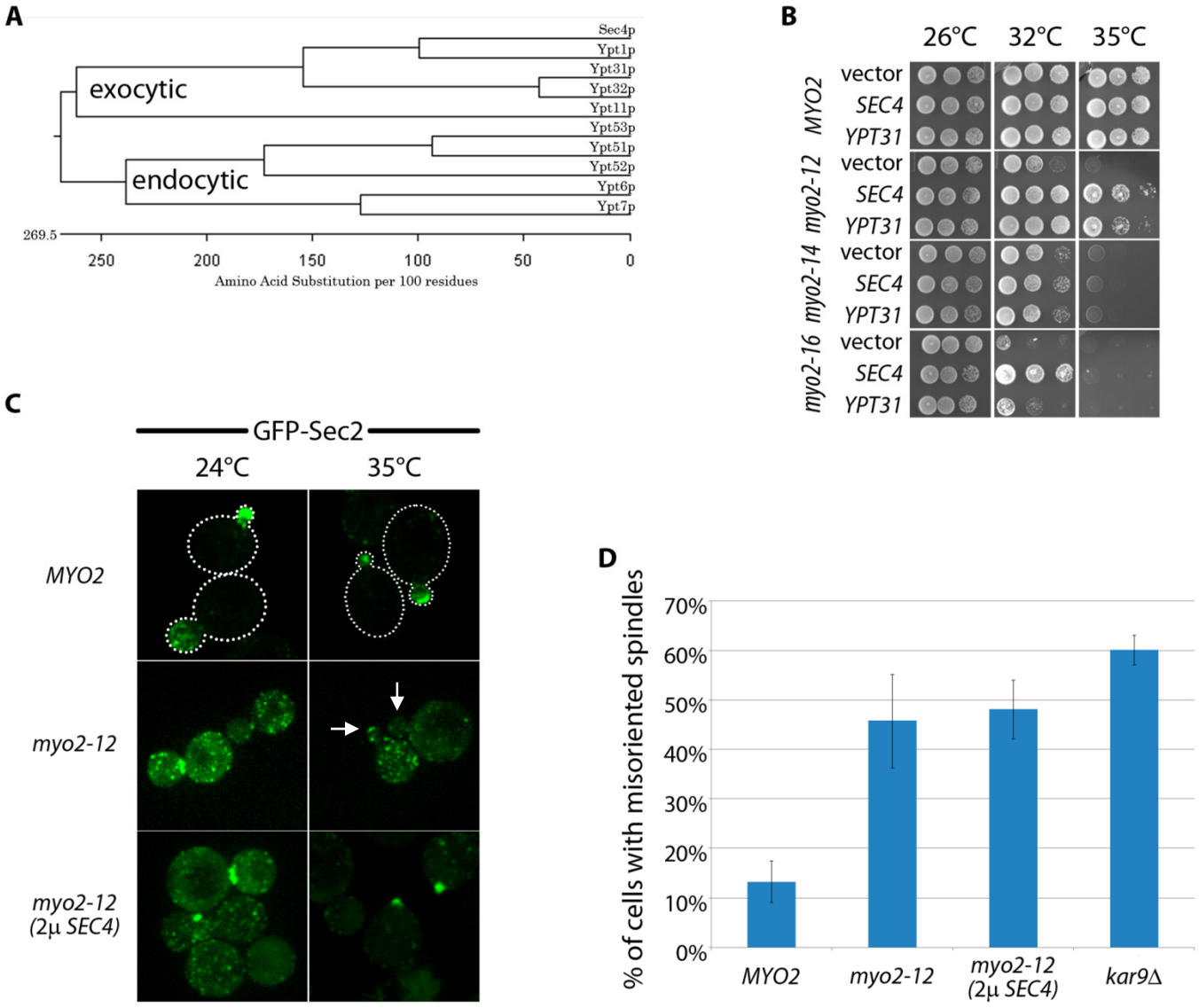


Figure 3.1 A subset of exocytic Rabs can rescue *myo2* defects. (A) Phylogenetic tree of 10 yeast Rabs (excluding Ypt10p that has no known function) by sequence alignment. They cluster into two major group, the exocytic and the endocytic. (B) Moderate overexpression of *SEC4* and *YPT31* can rescue the alleles sensitive to PI4P levels (*myo2-12* and *myo2-16*), but not the ones insensitive to PI4P levels (*myo2-14*). (C) Elevation of Sec4p levels can restore the polarization of vesicles in *myo2-12,* as judge by GFP-Sec2, but it cannot (D) correct the defect this strain has in orientation of the mitotic spindle. Arrows indicate small buds without GFP-Sec2 enrichments.

outline to see the cell). After 15min shift to 35°C, all small buds and necks in *myo2-12* were devoid of GFP-Sec2 enrichments, however, in *myo2-12* overexpressing *SEC4* at this temperature, GFP-Sec2 polarization was restored (Figure 3.1C). This result demonstrates that upon *SEC4* or *YPT31* moderate overexpression in *myo2-12*, vesicle polarization is restored, indicative of renewed association of Myo2-12p with secretory vesicles. The suppression of the temperature sensitivity was solely due to the restoration of the association with secretory vesicles, as *myo2-12* is also defective in spindle orientation and Rab overexpression did not corrected that defect (Figure 3.1D).

Sec4p and Ypt31p bind to Myo2p by the yeast two-hybrid system

Because previously Daniel Schott in the lab noticed that GFP-Ypt31 was transported in a Myo2p-dependent manner, and because the mammalian homolog of Ypt31p, Rab11, was shown to bind directly to the tail domain of a class V myosin, myosin-Vb (Lapierre *et al.*, 2001), we tested for a physical interaction between these Rabs and the Myo2p GTD by the yeast two-hybrid method. Using the GTD as the bait, we found an interaction with Ypt31p as well with the known Myo2p interactors Ypt11p (Itoh *et al.*, 2002) and Smy1p (Beningo *et al.*, 2000), but not with Sec4p (Figure 3.2A, AD-GTD panels). However, metazoan MyoVa (Myo2p homolog) binds melanosomes (a type of secretory granule) by binding melanophilin in an interaction requiring the coiled coil region of MyoVa (Wu *et al.*, 2002). Melanophilin in turn binds GTP-Rab27a (a Sec4p-related GTPase), which labels the melanosomes (Hume *et al.*,

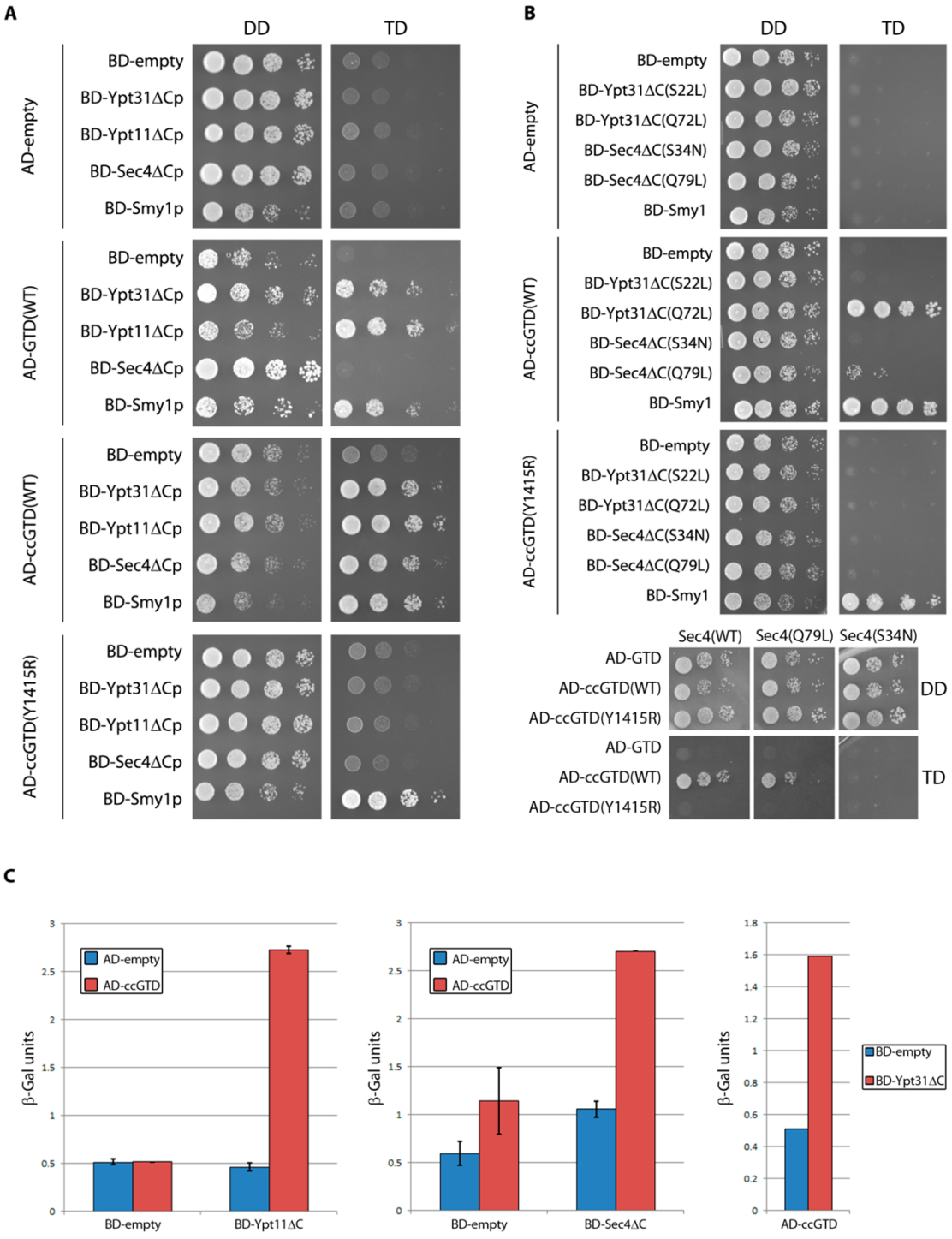


Figure 3.2 The exocytic Rabs interact with Myo2p by yeast 2-hybrid. (A) Yeast 2-hybrid interactions between the Myo2p-tail (AD-GTD), or coiled-coil region and tail (AD-ccGTD), or ccGTD with the Y1415R mutation, and the Rab GTPases and Smy1p. All Rab constructs were mutated in their C-terminal CXC motif (eg. Ypt31ΔCp) to eliminate prenylation. Growth on the control (DD) and test (TD) plates is shown. (B) The interaction with Myo2p is GTP-dependent as it does not interact with the GDP-locked mutants (S22L for Ypt31p and S34N for Sec4p), but Myo2p prefers wild-type Sec4p over dominant active (Q79L Sec4p). (C) Myo2p interaction with Ypt11p is 6X stronger than background, with Ypt31p is 3.1X, and with Sec4p is 2.4X, as measured by liquid β-galactosidase assays.

2001). I wanted to test if this interaction requiring the myosin's coiled coil was conserved in yeast. For this purpose I generated three two-hybrid constructs fusing the *GAL4* activation domain (AD) to the Myo2p coiled coil region (aa 926 – 1086), or to the coiled coil and GTD (926 – 1574; AD-ccGTD), or to the coiled coil and subdomain I of the GTD (926 – 1328). Testing these constructs with the activated Ypt31p gave a positive interaction only with the AD-ccGTD, as expected (Figure 3.2A,B). However, the AD-ccGTD also gave a positive interaction with both wild-type Sec4p and the dominant active Sec4p (Figure 3.2A,B). Interestingly, the interaction with Sec4p was stronger with the wild-type Sec4p than with the dominant active, while for Ypt31p it was the opposite case (Figure 3.2B). Shortly after these findings, it was reported that mammalian MyoVb interacts by two-hybrid with Rab8a (Sec4p homolog) in a way dependent on the coiled coil region immediately upstream of the GTD (Roland *et al.*, 2007). In the same system, the interaction between MyoVb and Rab11a (Ypt31p homolog) did not require the coiled coil region, very similar to what I found in yeast. These results support the hypothesis that Rab GTPases are required for the association of myosin-Vs with their target secretory membranes and that the Rab binding sites are not identical. Unfortunately, during the course of this investigation, the two-hybrid interactions between Myo2p and Ypt31/32p were published in two back-to-back papers (Casavola *et al.*, 2008; Lipatova *et al.*, 2008). Nevertheless, I carried out additional experiments that suggest that the association of Myo2p with secretory membranes requires at least one of these Rab GTPases. Both the Sec4p

vesicles and the Ypt31p compartment are no longer polarized in *myo2-12* after shifting to the restrictive temperature (Figure 2.1A, 2.5D, 2.7G, and unpublished observation). That means that Myo2-12p cannot associate with those compartments at high temperatures. I carried out the two-hybrid assay between Sec4p, Ypt31p, and Smy1p and the AD-ccGTD of wild-type Myo2p, or that of *myo2-12*, *myo2-14*, or *myo2-16*. The interaction with BD-Sec4 is greatly reduced in the *myo2* tail mutants even at room temperature, and is abolished at 37°C (Table 2). BD-Ypt31 interacts with all the Myo2p constructs at room temperature but the interaction is also lost at 37°C. Smy1p, on the other hand, interacts strongly with all Myo2p constructs at both temperatures except with *myo2-14*, which does not interact at either temperature. The interaction with BD-Ypt11 is not affected in any of the mutants at either temperature. These results support the hypothesis that the *myo2* GTD mutants dissociate from secretory membranes at high temperatures in part due to a defective binding to Rab GTPases. Furthermore, the fact that the interaction with the Rabs is differentially affected, with Sec4p binding greatly reduced even at room temperature, Ypt31p binding affected only at higher temperatures, and Ypt11p not affected at any temperature, confirms that Myo2p can differentiate between the exocytic Rabs. Interestingly, these results also explain why *SMY1* overexpression can rescue all the *myo2* mutants except *myo2-14* (Schott et al., 1999), since this allele has lost the interaction with Smy1p completely. This is not surprising, as *myo2-14* is a truncation, missing the last 39 amino acids, and this distal part of the GTD is precisely the region previously found to

interact with Smy1p by two-hybrid (Beningo *et al.*, 2000). The results of these two-hybrid analyses are summarized in Table 2. Consistent with the differential effect on Rab binding, measurement of the strength of the interactions by liquid β-galactosidase assays showed that *in vivo,* Myo2p binding to Ypt11p is the strongest, while Sec4p binding is the weakest (Figure 3.2C).

Table 2 Summary of two-hybrid interactions

	BD-Empty	BD-Ypt11ΔC		BD-Ypt31ΔC		BD-Sec4ΔC		BD-Smy1p	
		21°C	36°C	21°C	36°C	21°C	36°C	21°C	36°C
AD-Empty	–	–	–	–	–	–	–	–	–
AD-GTD	–	++	++	+	+	–[1]	–[1]	+++	+++
AD-ccGTD	–	++	++	++	++	++	++	+++	+++
AD-ccGTD (*myo2-12*)	–	++	++	+	–	+/–	–	++	++
AD-ccGTD (*myo2-14*)	–	++	++	+	–	+/–	–	–	–
AD-ccGTD (*myo2-16*)	–	++	++	+	–	+/–	–	++	++

"–" no growth in any media; "+" growth in low stringency media (TD) only; "++" growth in medium stringency media (TD + 2.5 – 5mM 3-AT); "+++" growth in high stringency media (TD + 10mM 3-AT or QD). [1]Not tested by the quantitative liquid β-galactosidase assays.

Recently, residues required for Ypt31/32p binding to the Myo2p tail were identified (Casavola *et al.*, 2008; Lipatova *et al.*, 2008). Many of these residues lie along helix 9 of the GTD, termed the vesicle-binding region. I tested two of these mutations, Q1412R and Y1415R, for two-hybrid interaction with the other Rab GTPases. Consistent with those reports, both mutations abolished Ypt31p binding, but they also abolished the interaction with Sec4p and Ypt11p, but not Smy1p (Figure 3.2A). When introduced into the chromosomal locus, these mutations render Myo2p non-functional, causing inviability. This

is another piece of evidence supporting our hypothesis that Rab binding is required for Myo2p association with secretory membranes, the only essential function known to Myo2p.

The Sec4p and Ypt31p binding to Myo2p is direct

An interaction by two-hybrid does not necessarily mean a direct physical interaction, as it can be mediated *in vivo* by a third protein bridging the AD to the BD. I therefore expressed and purified Myo2p tail constructs (ccGTD and GTD alone) from bacteria to test a direct interaction with recombinant proteins. Both Myo2p tail constructs exhibited Rab specificity by binding directly to HIS_6-Ypt31p and HIS_6-Sec4p, but not to HIS_6-Ypt1p (Figure 3.3A). Although HIS_6-Sec4p bound both Myo2p tail constructs, it preferred the ccGTD over the GTD. This is not inconsistent with the two-hybrid analysis since, to get a BD-Sec4 two-hybrid signal comparable to that of BD-Ypt31, you need the coiled-coil region present, but after an extended time of incubation (> week), colonies start growing with the Myo2p construct lacking the coiled coil. As explained in the Materials and Methods section, the expression levels of the BD-fusion proteins from the pBridge vectors are very low, and weak interactions require longer periods of incubation to be seen. However, I was unable to recapitulate the GTP-dependence seen by two-hybrid analysis with the *in vitro* binding assays. This could mean that additional proteins modulate the specificity of the tail *in vivo*, or simply that the only available pool of Rabs for Myo2p binding is the activated GTP-loaded, since presumably the GDP inactive Rabs are

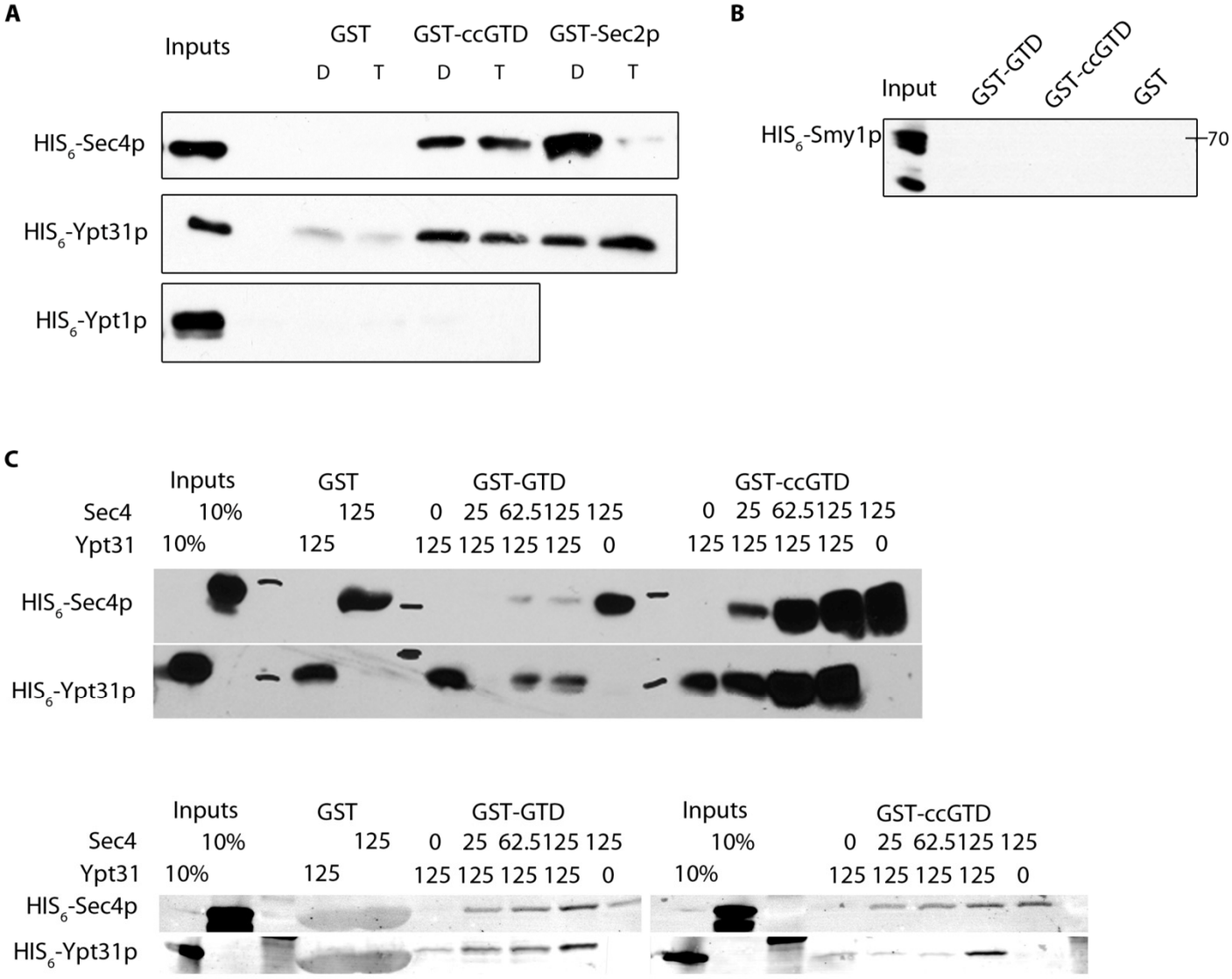


Figure 3.3 Myo2p interacts specifically with Sec4p and Ypt31p *in vitro*. (A) *In vitro* binding assays with GST, GST-Myo2p-ccGTD, or GST-Sec2p, bound to glutathione resin. The resin was incubated with the His_6-Rab GTPases that were pretreated with GDP (D) or GTP-γS (T), and after rigorous washes, the retained Rabs were detected by western blot and ECL. Myo2p bound, with similar affinities, Sec4p and Ypt31p independently of the nucleotide status while Sec2p preferred the GDP-Sec4p or the GTP-Ypt31p. No binding to the related Rab Ypt1p was detected. (B) *In vitro* binding assays between Myo2p and Smy1p. Resin with GST, GST fused to Myo2p-GTD only or to ccGTD, was incubated with His_6-Smy1p, and after rigorous washes, retained Smy1p was probed by western blot and ECL. No binding was detected. (C) Ypt31p and Sec4p exhibit cooperative binding to Myo2p. Two examples of competition assays where Ypt31p concentration was kept constant while Sec4p concentration was increased from none to the same levels as Ypt31p. Notice how much more Ypt31p or Sec4p binds Myo2p when the other Rab is present. The effect is more noticeable with the Myo2p construct containing the coiled coil region. The upper panels showed an experiment were the Rabs were detected by ECL while in the bottom one by the Odyssey infrared system.

extracted from membranes and are cytosolic in a complex with Rab GDI, and therefore inaccessible for Myo2p binding. I also tested the interaction of Myo2p with the kinesin-like protein Smy1p *in vitro*, as it has been suggested that the interaction is not direct (Hodges *et al.*, 2009). In agreement with these claims, I could never get a direct interaction with recombinant proteins between Smy1p and the Myo2p tail constructs (Figure 3.3B). This result is very strange, as the two-hybrid interaction between Myo2p GTD and Smy1p is one of the strongest, and genetic data indicate that an interaction between these two proteins is needed for them to affect each other function (Beningo *et al.*, 2000). This could simply reflect that the isolated interactions are very weak, and additional proteins may participate *in vivo* to stabilize the binding between Myo2p and Smy1p.

Since the helix 9 mutations abolish the interaction with all three Rabs, yet Sec4p-binding requires determinants in the coiled coil region, I wanted to carry out competition assays between Sec4p and Ypt31p for Myo2p binding. Since both Rabs are purified through HIS_6 tags, and they are of similar size, I couldn't use the HIS_6 antibody I used for the *in vitro* assays. I tested two uncharacterized antibodies we had in the lab against Sec4p or Ypt31p for cross-reactivity and found that, despite the high concentrations used *in vitro*, they are very specific. In the competition assay, I left HIS_6-Ypt31p constant at 125nM and added increased amounts of HIS_6-Sec4p, from 0 to 125nM. Both Rabs were preloaded with GTPγS and incubated with GST constructs

containing either the Myo2p GTD alone or the ccGTD. Surprisingly, I found that the presence of Sec4p enhanced the binding of Ypt31p to the Myo2p tail (Figure 3.3C). The enhancement varied between experiments, but it was always very clear for the ccGTD construct (Figure 3.3C). I never tried leaving Sec4p constant and increasing Ypt31p, but this result suggests that Sec4p and Ypt31p show cooperative binding to Myo2p. Although I have not explored this effect further, I speculate about its possible repercussions in the Discussion section.

Smy1p can modulate the binding of Myo2p and Rabs *in vivo* and *in vitro*

Because overexpression of Smy1p can restore the association of mutant Myo2p with secretory vesicles and yet we failed to see a direct interaction with purified proteins, we wondered if Smy1p could be acting in concert with other proteins in the binding to Myo2p. This could explain the clear and strong interaction between these two proteins *in vivo* as seen in the two-hybrid analysis (Figure 3.2A). If this is the case, we expect to see changes in the interactions between Myo2p and other proteins in the presence or absence of Smy1p. To test this hypothesis, we used the pBridge two-hybrid plasmid, which contains two multiple cloning sites, one for a BD-fusion protein, and one for an additional protein fused to a NLS. Use of pBridge allowed me to carry out a three-hybrid analysis between Myo2p and the different Rabs in the presence or absence of overexpressed, nuclear-targeted Smy1p. Expression of Smy1p in the nucleus clearly increased the strength of the Sec4p interaction

with AD-ccGTD (Figure 3.4A) while it had no effect on the interaction with AD-GTD (not shown). This was even more apparent in plates containing 3-AT, a competitive inhibitor of the *HIS3* reporter gene that makes the conditions more stringent for a two-hybrid interaction. The effect on Ypt31p was not apparent until we go to even higher concentrations of 3-AT, while I could not see any effects on Ypt11p. However, maybe the more striking difference is the effect Smy1p has on the two-hybrid interaction of the dominant active Sec4p. Previously, I found that the interaction with dominant active Sec4p was very weak compared to wild-type Sec4p, however expression of Smy1p now increases this interaction to levels similar to that of wild-type Sec4p alone (Figure 3.4A). Moreover, all of these effects seem to be through the Myo2p-Smy1p interaction, since the same constructs expressing a tail-less Smy1p, lacking the Myo2p-binding site, failed to enhance the interactions (Figure 3.4B). Additionally, the Smy1p effects are not due to an artifact of Smy1p itself binding the Rabs and bridging them to Myo2p, as the *myo2* helix 9 mutants still do not interact with the Rabs in the presence of Smy1p (Figure 3.4C) but Smy1p interacts with these helix 9 mutants (Figure 3.2A). These findings prompted us to see if purified HIS_6-Smy1p could have an effect on the *in vitro* binding assays between Myo2p tail constructs and the Rabs. Although addition of HIS_6-Smy1p to the *in vitro* binding assays did not affect the binding of GDP-loaded Sec4p or Ypt31p, it did increase the amount of GTP-loaded Rabs that bound to the Myo2p tail constructs (Figure 3.4D). This slight increase could be an underestimation and, if we take into consideration the very weak

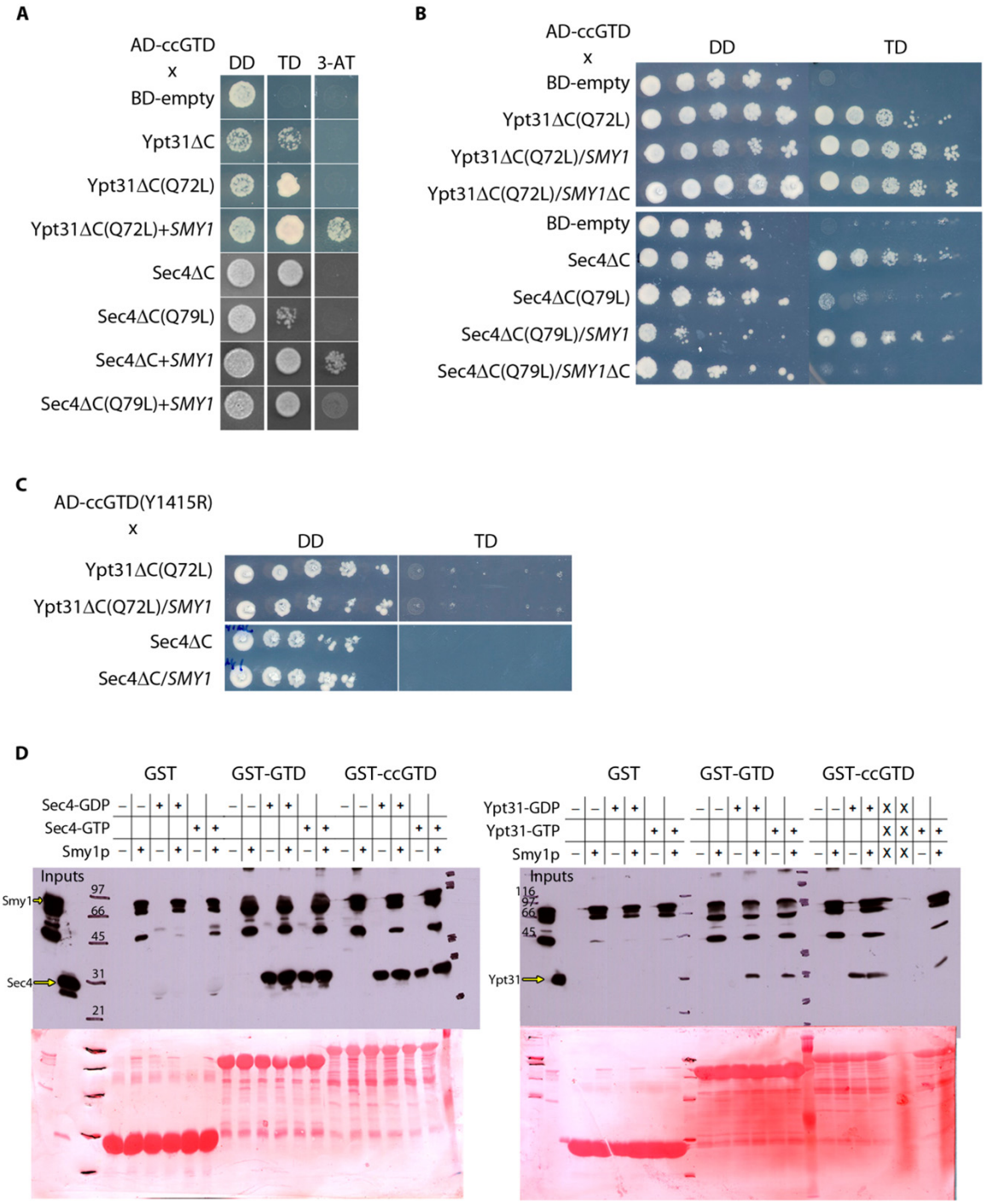


Figure 3.4 Smy1p can modulate Myo2p interactions with Sec4p and Ypt31p. (A) Three hybrid interactions between Myo2-ccGTD and Ypt31p or Sec4p in the absence or presence of nuclear-localized Smy1p. Notice how the presence of Smy1p enhances the interactions as judged by growth in higher stringency media (3-AT). The most prominent effect is seen with the dominant active Q79L Sec4p, which is very weak in the absence of Smy1p but can grow even in the high stringency media in the presence of Smy1p. (B) The enhancing effects of Smy1p depends on its Myo2p-binding tail region. Introduction of a tail-less Smy1p (SMY1ΔC) has no effect on the two-hybrid interactions. (C) Smy1p has no effect on the lack of interaction between the Rabs and the helix 9 mutant Y1415R, previously shown to be defective in Rab binding. (D) *In vitro*, presence of Smy1p slightly increases the amount of GTP-loaded Rabs that bind the Myo2p resin. The membrane stained with Ponceau S is shown below to appreciate the levels of GST or GST-Myo2p constructs on the resin.

interactions between Myo2p and Smy1p *in vitro*, could represent the small population of Myo2p acted on by Smy1p that will now bind preferentially to GTP-loaded Rabs.

Rab GTPases act as part of the essential Myo2p receptor

As mentioned in Chapter 2, overexpression of the Myo2p tail is dominant lethal presumably because it binds the secretory vesicle receptor and displaces the endogenous motor from it (Reck-Peterson *et al.*, 1999; Schott *et al.*, 1999). Previous work has shown, and I have confirmed, that the helix 9 mutations are nonfunctional and cannot perform the essential function of Myo2p (Casavola *et al.*, 2008; Lipatova *et al.*, 2008; see Figure 2.7H in Chapter 2). Since I found out that these mutations abolished the two-hybrid interactions with all three Rab GTPases, I wondered if a helix 9 mutant would still be lethal when overexpressed. Expression of wild-type GST-GTD inhibited growth as previously shown, but expression of the helix 9 mutants did not (Figure 3.5A). Although the Rab-binding deficient GTD were not lethal, they were expressed to similar levels, and could still affect nonessential functions of Myo2p, as I found it caused vacuole fragmentation and blocked inheritance of the organelle (not shown). Therefore these experiments clearly demonstrate the presence of a specific, Rab-dependent, saturable receptor on secretory membranes. To test this assumption, I co-overexpressed the Myo2p tail domain together with *SEC4* or *YPT31*, or with *SMY1*, reasoning that if indeed the Rabs are being titrated down, increasing their levels should suppress the lethality of the Myo2p tail.

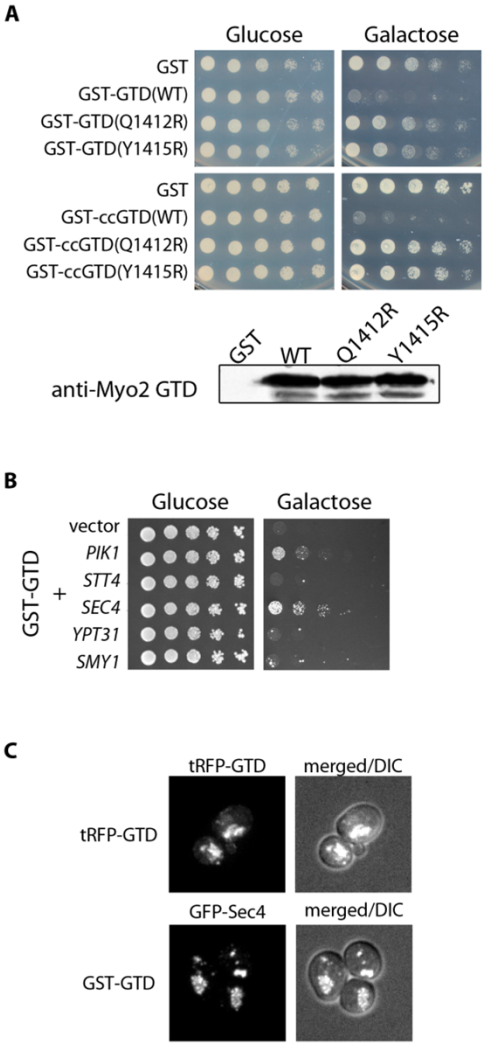


Figure 3.5 The Rab-binding site in Myo2p GTD is important for its dominant negative effect. (A) Mutation of two residues in helix 9 that abolish the interactions with the Rab GTPases renders the GTD nonfunctional for disruption of the essential function. Although being express at similar levels, they cannot inhibit growth as the wild-type GTD. (B) Co-overexpression of the Myo2p GTD with *SEC4* also suppresses the lethality, demonstrating that binding to Sec4p is, in part, one of the reasons it inhibits growth. (C) Overexpressed Myo2p GTD targets membranes and cause a patch of Sec4p to appear in the cell.

Although increased levels of Ypt31p or Smy1p do not suppressed the lethality, increased levels of Sec4p allowed partial growth of the strains expressing the Myo2p tail (Figure 3.5B). This indicates that Sec4p is at least one of the components being titrated by the overexpressed Myo2p tail. Furthermore, a fluorescent Myo2p tail, tagRFP-GTD, upon induction localizes to membranes that are also labeled by GFP-Sec4 and GFP-Ypt31 (Figure 3.5C and A1.2). The identity of these membranes is discussed further in Appendix I. These results, the targeting of tagRFP-GTD to GFP-Sec4-labeled membranes, the *in vivo* and *in vitro* binding of Sec4p to the Myo2p tail, and the fact that Rab-binding deficient Myo2p tails cannot block the essential function of Myo2p, is in agreement with our model that Sec4p is part of the secretory vesicle receptor for Myo2p.

DISCUSSION

As mentioned in the introduction, Rab GTPases have always been a candidate to link molecular motors to their membranous cargo. This is because they associate with specific membranes, and can cycle between an active form, where they recruit effectors to those same membranes, and an inactive form. In yeast, two Rab subgroups associate with secretory membranes, the redundant Ypt31p and Ypt32p, which represent the Rab11 group, and Sec4p, which belongs to the Rab8 group. These Rabs exhibit genetic interactions with *MYO2*, co-localize with Myo2p in the cell, and are moved by Myo2p, consistent with a role in bridging Myo2p to the membranes

they label. The evidence I present in this chapter support this idea, and importantly extend it to also include Smy1p as a factor that can modulate the association of Myo2p with secretory membranes through, at least in part, affecting Myo2p' ability to recognize and bind the Rabs Ypt31p and Sec4p.

This idea that Rabs can recruit a molecular motor to membranes is not new as there are a few examples in higher organism where Rabs play a critical role. However, this work demonstrates that this is evolutionary conserved and highlight other characteristic that may apply in animals, such as that the tail can contain different determinants to recognize the Rab that is binding, and that other factors could modulate this recognition. Most interesting is the fact that the two better studied examples in higher organism of Rab involvement in myosin-V recruitment to membranes involve homologs of Sec4p and Ypt31p. Rab27a is functionally similar, and lies in the same subgroup by sequence, as Sec4p. In the melanosome system, Rab27a is essential for the indirect recruitment of MyoVa to membranes (Wu *et al.*, 2002). Rab27a does that through its effector protein, melanophilin, which in turn binds directly to MyoVa in a way that requires specific sequences in both the coiled coil region and the GTD, thereby bridging the melanosomes to the motor. This general theme can be applied to other types of vesicles since in insulin-secreting cells, where Rab27a is associated with secretory granules, another Rab27a effector, Myrip or Slac2c, can recruit MyoVa to these membranes (Fukuda and Kuroda, 2002; Desnos *et al.*, 2003). The second example of a Rab involved in the

membrane recruitment of myosin-Vs is Rab11, the homolog of the redundant Ypt31/32p, in the endosomal system (Lapierre *et al.*, 2001; Roland *et al.*, 2007). Here specific isoforms of Rab11 can either bind directly to the GTD of MyoVb and MyoVc, or indirectly through adaptor proteins called Rab11FIPs, and recruit these myosin-Vs to endosomal tubules that need to be recycled back to the plasma membrane. Most interestingly, there are multiple Rab domains in the endocytic recycling pathway, and MyoVb can function in a subset of these, indicating MyoVb can recognize and differentiate these Rabs. One could then hypothesize that for every secretory membrane moved by a myosin-V, a Rab GTPase is involved, and in this work I identify the Rabs involved in the recruitment of Myo2p to TGN membranes and secretory vesicles.

In the previous chapter I discussed evidence supporting the presence of multiple components in the secretory vesicle receptor for Myo2p, with PI4P being part of it. The data presented in this chapter clearly support this model, extending it to include Ypt31p and Sec4p as additional components of the receptor. The fact that both the interaction with the Rabs and the association *in vivo* with the vesicles is temperature sensitive in the *myo2* mutants links the two processes together. Moreover, GFP-Ypt31-labeled structures are, in a general level, distinct from the GFP-Sec4-labeled vesicles, yet both are affected in the *myo2* mutants. Since Myo2p interacts with both it explains why both compartments are affected. The idea that different Rabs can use the same

molecular motor is consistent with the fact that Myo2p recognition of Ypt31/32p and Sec4p are not entirely equal. Although they both need determinants in helix 9 of the GTD, Sec4p binding also requires the coiled coil region of Myo2p. Moreover, binding of Ypt11p to the different *myo2* mutant GTDs is not affected at high temperatures while binding to the other Rabs is abolished. This is not unprecedented, as Myosin-Vb binds the endosomal Rab8a, Rab10, and Rab11a through nonoverlapping determinants in its tail domain (tail domain refers to the coiled coil and GTD region; Roland *et al.*, 2007; Roland *et al.*, 2011). Notably, Myosin-Vb shows similar structural requirements for Rab11a and Rab8a binding as Myo2p for Ypt31p and Sec4p, a strong suggestion that differential binding of Rabs is a conserved function of myosin-Vs.

A novel aspect of these studies is the finding that Smy1p, a kinesin-like protein, can modulate Myo2p function by selectively enhancing the interaction between Myo2p and Sec4p. *SMY1* was originally identified as a multicopy suppressor of *myo2-66*, the first Myo2p mutant (Lillie and Brown, 1992). It was shown to localize to growth sites, similarly to Myo2p, and that its localization depended on its association with Myo2p (Beningo *et al.*, 2000). This last statement is the authors' interpretation of the fact that a tail-less Smy1p that cannot interact with Myo2p by two-hybrid, is not polarized. However, it has been unclear how Smy1p localization is achieved, mainly for the lack of a direct interaction between the two with purified proteins. I also

failed to get an interaction with purified proteins (Figure 3.3B), however the strong two-hybrid signal between the two, together with the effects Smy1p has on Myo2p function and interaction with Rabs, strongly suggest that they interact directly. For these reasons we favor the idea that Smy1p is part of the receptor, and that it will only stably bind Myo2p in the context of the whole receptor complex.

Moreover, the finding that Sec4p and Ypt31p showed cooperative binding to Myo2p would only make physiological sense if they are together in a complex *in vivo*. In fact, cooperation between Ypt31p and Sec4p has been proposed to impart directionality to the secretory membranes in the later stages of secretion. Ypt31p recruits, together with the lipid PI4P, the Sec4p GEF Sec2p, resulting in the activation of the secretory vesicle Rab. If Myo2p binds both, but the presence of Ypt31p can enhance its binding to Sec4p, this cooperative binding could help in the transition from a Ypt31p-labeled membrane to one labeled by Sec4p. If we also consider that Smy1p promotes the binding of Myo2p to active Sec4p, the receptor complex not only would play a role in the recruitment of Myo2p but also serve as a regulator of membrane maturation. This could be important if some essential component is limiting and needs to reach a specific threshold for it to function correctly. In the absence of this component, the maturation would be delayed, resulting in a slow recruitment of motors and GTPases. However, upon reaching the necessary amount of components, the cooperative binding would rapidly promote maturation and

recruitment of motors and Rabs, resulting in its rapid transport towards sites of growth. Although more experiments are required to support this idea, it is an attractive model to couple transport carrier generation with motor recruitment, transport, and release.

Combining the results here with those presented in Chapter 2, a coincidence detector model for the recruitment of Myo2p to secretory membranes emerges. This model predicts that two signals are required for the efficient localization of a protein, and that either signal alone is not sufficient. The *myo2-12* and *myo2-16* alleles, found to be sensitive to PI4P levels, are also sensitive to Sec4p function (Schott *et al.*, 1999). Likewise, overexpression of *SEC4* as well as increased levels of PI4P is able to restore association of Myo2p tail mutants with secretory membranes. These results indicate that Myo2p requires interactions with both PI4P and Rabs, especially Sec4p, and these are specially affected in the *myo2-12* and *myo2-16* alleles. Nevertheless, our data indicates that additional factors, perhaps independent of PI4P and/or Rab GTPases, exist. Smy1p could be one of these additional factors that modulate the association of Myo2p with the Rab GTPases, however since Smy1p is not an essential gene, other factors should exist. Before these studies presented here no molecular mechanism was proposed for the association of Myo2p with secretory membranes. Now, with the interactions described here, we have more tools and pieces to start building a molecular portrait of this very elusive receptor.

CHAPTER 4

THE SEC4P ACTIVATOR SEC2P IS IN A COMPLEX WITH MYO2P

INTRODUCTION

As explained in the previous chapters, growth and division in yeast depends on the polarized delivery of secretory membranes to sites of growth. The actual transport of the membranes is in turn carried out by the essential myosin-V Myo2p. I have shown that Myo2p recognizes these secretory membranes by a mechanism involving PI4P and the Rab GTPase Sec4p. However, in our *in vitro* assays Myo2p does not bound PI4P directly and does not exhibited preference for active GTP-loaded Sec4p, which is the form of Sec4p that presumably localizes to the secretory membranes (inactive Sec4p is rapidly extracted from membranes by GDI). This suggested that additional factors must exist *in vivo* that bridge Myo2p to the PI4P-responsive process and that ensures Myo2p association with only active GTP-bound Sec4p. This is very important if we consider the existence of another pool of PI4P at the plasma membrane which, at steady state, is also the location of the biggest pool of Sec4p, presumably in an inactive GDP form since fusion of the vesicles has already occurred. This presents a huge problem for the cell, as these two factors are precisely the ones that recruit Myo2p to secretory membranes, therefore there has to be a mechanism allowing Myo2p to respond to these signals from the secretory apparatus and not from the plasma membrane.

Since our data support the existence of additional factors necessary for Myo2p recruitment to secretory vesicles, these very well could play a role in the recognition mechanism of Golgi PI4P and active Sec4p over plasma membrane PI4P and inactive Sec4p. With the recent demonstration that Sec2p, the GEF for Sec4p, is a PI4P effector (Mizuno-Yamasaki *et al.*, 2010), I became interested in the role Sec2p might have in coupling Myo2p with the Golgi PI4P and active Sec4p.

SEC2 was isolated as one of the original *sec* mutants that blocked secretion at the restrictive temperature (Novick *et al.*, 1980). However, it was also unique in the sense that vesicles accumulated randomly throughout the cell rather than at sites of growth, indicative of a problem with fusion and also with transport. After the gene was cloned, several alleles were sequenced and found all to be truncations of the C-terminal region (Nair *et al.*, 1990). Targeted mutations were made to the 759 amino acid protein to test how much of the gene was dispensable and the first 374 amino acids were found to be essential, while truncations between 374 and 508 renders the protein temperature sensitive (Figure 4.1A). Truncations after amino acid 508 were not temperature sensitive, indicating that the essential region is before amino acid 374, and the region between 374 and 508 was important for function at room temperature and essential at higher temperatures. By localizing Sec2p *in vivo* and determining its presence in different membranes by cellular fractionation, it was shown to be associated with secretory vesicles as they emerged from the

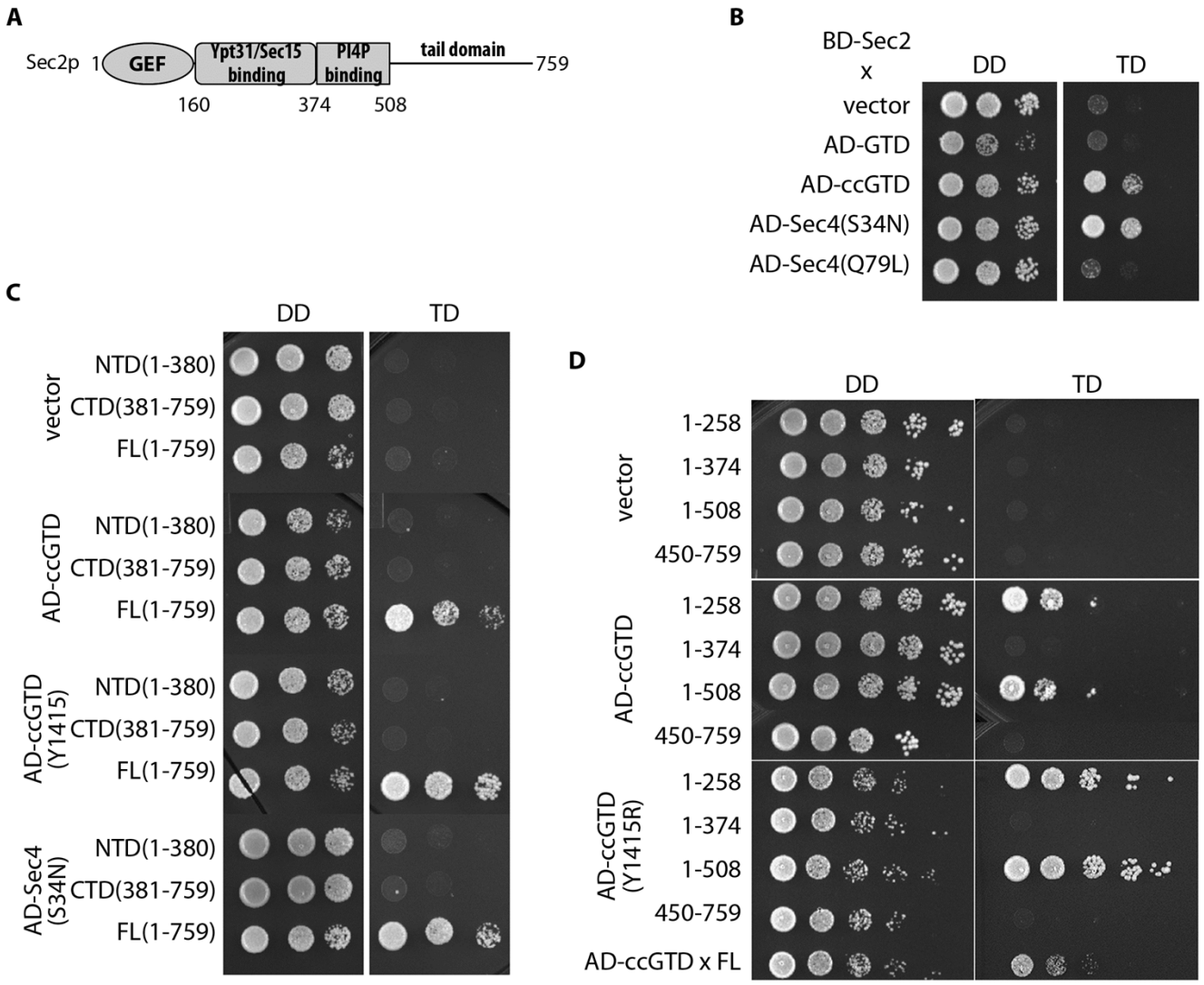


Figure 4.1 The Sec4p GEF Sec2p interacts with Myo2p by two-hybrid. (A) Schematic representation of the domain organization of Sec2p. The numbers represent the amino acid boundaries of each domain. (B) Full-length Sec2p interacts by two-hybrid with the Myo2p construct that contains the coiled coil region. The Sec4p constructs are used as controls, since Sec2p will only bind the GDP-locked mutant S34N, albeit weakly (GEFs prefer the nucleotide free GTPase). (C and D) Fragment analysis of Sec2p to map down the Myo2p interacting region. The smallest functional region that gave a positive interaction encompases the GEF domain and half of the Ypt31/Sec15 binding region. Notice also that the Rab-binding deficient Myo2p still interacts with Sec2p, indicating that is not through Sec4p.

TGN and remained with them until they dock at sites of cell growth (Walch-Solimena *et al.*, 1997). Because of its genetic interactions with *sec4-8*, the authors tested for a physical interaction by two-hybrid and found that Sec2p binds only to Sec4p mutants that resemble the GDP or nucleotide free state (Walch-Solimena *et al.*, 1997). This prompted them to check if Sec2p had GEF activity on Sec4p, which it does, and identified the catalytic region in the first 160 amino acids. Later, through overexpression-suppression screens, the two TGN Rabs Ypt31/32p were found to restore localization of the C-terminal truncation mutants, indicating that these Rabs could act as Sec2p receptors on membranes (Ortiz *et al.*, 2002). However, the binding site for Ypt31/32p was mapped between amino acids 161 and 374, a region that is present in all the truncated mutants, therefore Sec2p must have another localization determinant that can be compensated by *YPT31/32* overexpression. Sec2-GFP was also found to be transiently mislocalized in *smy1*Δ cells; however after several generations, Sec2-GFP appeared localized again (Elkind *et al.*, 2000). Nevertheless, although it is not the other determinant, *smy1*Δ is synthetic lethal with *sec2-41*, implying Smy1p function is related to Sec2p (Lillie and Brown, 1998). This other determinant was recently discovered and it turned out to be PI4P, with the binding site being three positive-charged patches in the region between 374 and 508, explaining why truncations after amino acid 508 did not have a strong phenotype and the protein localizes correctly (Mizuno-Yamasaki *et al.*, 2010). This finding positioned Sec2p as the perfect candidate to be the additional component in our model for several reasons: first, in the

sec2 mutants vesicles are not transported, demonstrating that upon Sec2p mislocalization the connection between vesicles and Myo2p is disrupted; second, Sec2p localizes to vesicles, where it links Ypt31p and Sec4p signaling, both of them GTPases that bind Myo2p; and lastly, by requiring PI4P and active Ypt31p for localization, it establishes a coincidence detection system specific for the Golgi that could provide the additional input necessary for Myo2p recognition of Golgi PI4P and active Sec4p. Moreover, it has been known that isolated Sec2p from yeast cells is in a >500kDa complex (Nair *et al.*, 1990), generated in part by homodimerization of Sec2p, as the GEF domain is a catalytic coiled coil (Dong *et al.*, 2007; Sato *et al.*, 2007). However, Sec2p dimerization cannot account for the high molecular weight of the complex, thus other components must be present that could include Smy1p or, perhaps, Myo2p. An initial experiment was presented at the end of Chapter 2 looking at the effects of overexpressing *SEC2*. Here I extend upon that experiment and present evidence that *in vivo* Sec2p is in a complex with Myo2p. I also explore the possibility of reconstitution of this complex *in vitro* using the liposome floatation assay used in Chapter 2.

MATERIALS AND METHODS

Yeast molecular techniques. As described in Chapter 2. To test the effects of the Myo2-PH domain fusion protein in the *sec2* mutants, I transformed the *myo2*-ΔGTD-PH^{WT} construct into *sec2-41*, a truncation at amino acid 397. For the overexpression or the two-hybrid analysis of full-length *SEC2* I used the

plasmids RCB2130 and RCB119, respectively, obtained from the Ruth Collins lab collection.

Two-hybrid analyses. For the full-length *SEC2* construct I used plasmid RCB119, while for the analysis of truncated versions I cloned the corresponding constructs into pGBKT7 (Clontech). All the experiments, except for the screen explained below, were done by co-transforming strain AH109 with both two-hybrid fusion constructs. To screen for Sec2p mutants defective in the Myo2p interaction, I transformed a $Sec2^{1-258}$ library (created by PCR mutagenesis) into strain pJ694-α by the LiAc method (Gietz and Woods, 2002) with the modification of resuspending the cell pellet into 10mL of minimal media (SD, no amino acids) and spreading different amounts on selective plates. I was aiming to get between 200 and 300 colonies per plate, as I needed them to be well isolated and big. I picked individual colonies and resuspended them in 150μL YPD in a 96-well plate, one colony per well. At this point, I tried different methods to mate these clones with the opposite mating type strain AH109 transformed with AD-ccGTD, AD-*SEC4*(S34N), or AD-*SEC15*. These included use of the 'frogger' to spot them onto plates with lawns of the appropriate strains (sterilizing the frogger between each transfer), to mate on plates, or adding these strains to each well and incubate for various times, to mate on liquid. In my experience, I found it worked best by mating in liquid (in the 96-well plate) overnight and then spotting on the selective plate using a multichannel pipette rather than the frogger.

Expression, purification, and in vitro binding assays of Sec2p constructs. I cloned the Sec2p constructs into pGEX-6P-3 (GE Healthcare). Most of the Sec2p constructs are very insoluble, requiring a host with extra codons for eukaryotic proteins (like Rosetta 2 cells) and growing and inducing at lower temperatures (20°C – 18°C). I routinely started growing an initial inoculum in 10mL of LB + AMP + CAM the night before at 37°C and dilute it the next morning into 500mL of SOB or TB media (remembering to add the salts and antibiotics to the media before using). When the culture reached an OD_{600} of 0.8 – 1.0 I cooled down the culture in a container with cold tap water for 5min and then added IPTG to 0.4mM final. I induced at room temperature for 8 – 10hrs or at 18°C, if using the refrigerated shaker, overnight or ~16hrs. After washing the pellet with cold PBS and transferring to smaller conical tubes, I either continued with the purification or froze the pellets in dry ice for indefinite storage at -80°C. For the purification I followed the standard lab procedure with 2 modifications: I used a higher concentration of DTT in buffer A (I have used 2mM, but the Novick lab reported use of 5mM in their papers) and added triton X-100 to 1% final after sonication and incubated for 10min on ice before spinning down the membranes. Also, if eluting the protein from the beads, I washed with 150mM NaCl in buffer B and eluted with 20mM GSH in 40mM Tris, pH 8.0, 150mM NaCl. After elution, the buffer was exchange to storage buffer (40mM Tris, pH 8.0, 200mM NaCl, 2mM DTT) using Amicon Ultra-0.5 mL Centrifugal Filters (Millipore) and then added the same volume of 80% glycerol. The protein was stored at -20°C for several months before

discarding. For the *in vitro* assays, different buffer conditions were tried due to the variable binding of Sec2p to Myo2p tail constructs. I always started with 125nM of the Myo2p tail construct and the standard binding buffer was 25mM Tris, pH 7.4, 120mM NaCl, 5mM $MgCl_2$, and 1mM DTT, with 5mg/mL BSA or 0.1% triton X-100 added in some experiments. After incubating for at least 1hr at 4°C, the beads were washed with the binding buffer plus 10% glycerol and 0.1% triton X-100 (in the experiments were BSA was used in the binding buffer, it was omitted in the wash buffer). After 3 or 4 washes, the beads were resuspended in the same volume of 2X sample buffer, boiled and loaded in an SDS-PAGE gel for separation and western blotting.

Supernatant depletion assays. I followed the recommendations made in (Pollard, 2010) to attempt estimation of the binding affinity between Myo2p and Sec2p. Assuming a binding constant over 1µM, I prepared binding reactions (same as in the *in vitro* binding assays with purified proteins) with 0.3µM cleaved Myo2-tail and varying concentrations of GST alone or GST-Sec2 constructs in a total volume of 400µL. I tried varying the amounts of GST-Sec2 in two different ways, first by increasing the amounts of slurry with protein bound, or by adding increasing amounts of eluted protein, followed by addition of a fixed volume of GSH-beads equilibrated with the binding buffer. Both ways resulted in the same problem of not been able to reach concentrations over 1 – 3 µM, since that would require addition of hundreds of µL of GST-Sec2 resin, or a substantial amount of the eluted protein added would not bind the

resin and would remain soluble. After incubating the reactions at 4°C with rotation for 30min, the beads were spun down at max speed for 5min and an aliquot of the supernatant taken into a new tube. I transferred enough supernatant to let me run several gels if necessary. I ran equivalent amounts of the supernatants on a protein gel to transfer, blot and quantify for Myo2p using the Odyssey system (LICOR).

Liposome floatation assays. As described in Chapter 2, but the composition of the liposomes was simplified to be 50%DOPC, 30%DOPE, 10%POPS, and 10% PI or PI4P (all in mol%). This change was made to follow the Novick lab's condition for Sec2p binding to PI4P and to increase PI4P availability when using multiple proteins in a reaction.

Co-immunoprecipitation of Myo2p. I diluted overnight cultures (5mL) into 15mL of SRaff to OD_{600} of 0.2. When in mid-log phase (OD_{600} ~0.8), galactose was added to 2% final, and the cells were grown for 45 minutes. I spun down, washed in cold PBS, quick froze the pellet in dry ice, and put at -80°C until ready to process the samples. I resuspended in 3 volumes of cold lysis buffer (1X PBS, 1mM EDTA, 4mM $MgCl_2$, and PI cocktail) and added half the volume of glass beads. I disrupted the cells by vortexing for 3min at 4°C. After spinning at max speed for 10min in the cold centrifuge, I took the supernatant as the lysate for the IP. I saved ~5% of lysate as input and divided the rest in half to treat with or without crosslinker. DSP (20mg/mL stock) was added to 1mg/mL final to one sample and DMSO to the other and incubated for 10min

on ice. The reaction was stopped by adding 1M Tris to 20mM final to quench the DSP. I added triton and BSA to 0.5% and 0.1% final, respectively, and adjusted the volume to 500µL with lysis buffer. Approximately 1µg of B74 (antisera to the Myo2p GTD) was added and incubated with rotation for ~3hrs at 4°C, followed by addition of ~20µL total of proteinA beads and further incubation for ~1.5hrs at 4°C. I washed the beads 3X with wash buffer (lysis buffer except 10% glycerol and 0.5% triton X-100) before resuspending in the 20µL of 2X sample buffer. The strains used to prepare the lysate expressed HA-tagged proteins, allowing for detection of co-immunoprecipitated proteins by western blot. I tried this protocol twice but always had problems with background proteins. This is by no means optimized for B74, as I never tried overnight incubation with the antibody or verified the immunoprecipitation efficiency of endogenous Myo2p by B74 in 3hrs. Because this antibody has never been used for immunoprecipitation it was going to require a lot of testing for optimization. To save time, I decided to go with the GST-pull downs as a more standard way to check *in vivo* association of Sec2p with Myo2p.

Pull-downs with GST-Myo2p-tail constructs. I used strains co-expressing the GST-ccGTD Myo2p construct and HA-tagged proteins of interest. Expression of both proteins was induced with galactose for 45min prior to processing with the glass beads methods as described above for the co-IPs. 25µL of equilibrated GSH-resin was added and incubated for at least 1hr withy rotation at 4°C. After washing the beads, they were resuspended in the same volume of

2X sample buffer. The amount of GST-Myo2p tail and HA-tagged protein were quantified using the Odyssey system (LICOR).

Sec2$^{1-258}$ mutagenesis. In order to get a wide-variety of mutations you can either start with a large template input and carry out very few PCR cycles or add $MnCl_2$ to the reactions after 10 cycles, when you had already amplified your template. I followed a modification of the first option mentioned based on the Cold Spring Harbor Lab protocols. I prepared a 10X mutagenic PCR buffer containing 70 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl, pH 8.3, and 0.1% tween, and a 10X dNTP mix with 2 mM dGTP, 2 mM dATP, 10 mM dCTP, and 10 mM dTTP. It is important not to combine these 10X solutions yet as it would result in the formation of a precipitate that will disrupt the PCR amplification. I used 30pmol of each primer and 20 fmol of the template DNA (~100ng of plasmid DNA) and combined all the components in a thin-walled PCR tube with water added first, to a total volume of 94µL. After all these components were mixed together, I added 5µl of 10mM $MnCl_2$ and 5 units (1µl) of Taq DNA polymerase, bringing the final volume to 100 µl. After mixing well by pipetting, I ran the PCR for 30 cycles of 1 minute at 94°C, 1 minute at 45°C, and 1 minute at 72°C, with no prolonged extension time at the end of the last cycle. The reason I did 30 cycles is because I was afraid, due to the small nature of the fragment (774 base pairs), my mutagenesis rate was going to be too low. To create a library, I set up three different ligations, with molar ratios of 1:1, 1:3, and 1:10 between the cut backbone (pGBKT7) and the PCR

product. After electroporation, I picked up 12 colonies (4 from each ligation) and determined by digestion that all had inserts. After sequencing, I obtained a combination of clones with 1 or 2 mutations up to ones with 7 mutations, with an average of 3 mutations per construct. After spreading all the transformation reactions, I pooled the transformants together by scrapping the colonies into 5mL LB+Amp per plate, recovering for 30min at 37°C, and then processing as a maxi-prep.

RESULTS

Sec2p fragments interact with the Myo2p coiled coil and GTD region

Originally, when I obtained the *SEC2* overexpression construct from the Collins lab (RCB2130), they also had available full-length *SEC2* in a two-hybrid vector (RCB119, see Materials and Methods). Overexpression of *SEC2* did not rescue any *myo2* mutants as hoped, however it did caused a deleterious effect in some of them (see Figure 2.9 in Chapter 2). This suggests that Sec2p may share binding partners with Myo2p, such as Ypt31p and Sec4p, that are being titrated down or that the overexpressed *SEC2* is promoting a pathway that negatively regulates Myo2p function. To test if these effects could be due to a direct interaction between the two, I used plasmid RCB119 (BD-*SEC2*) to see if I could get a two hybrid interaction with the Myo2p tail constructs. Surprisingly, I found an interaction with the AD-ccGTD construct, albeit very weak as it is abolished in the presence of 3-AT (competitive inhibitor of the

His3p reporter; Figure 4.1B). The fact that I failed to see an interaction with the GTD only highlights the importance of the coiled coil region in stabilizing the interactions between Myo2p and its binding partners. To map the Myo2p interaction region on Sec2p I divided the protein in half, with the N-terminal fragment including amino acids 1 to 380 and a C-terminal fragment with amino acids 381 to 759. However, none of these constructs interacted with Myo2p and, since they also didn't interact with AD-*SEC4*(S34N), they probably don't fold correctly and/or are degraded (Figure 4.1C). I decided to clone into the two-hybrid vector previously purified constructs of Sec2p shown to be functional as they bind target proteins *in vitro* (Figure 4.1A; Ortiz *et al.*, 2002; Medkova *et al.*, 2006). Use of these constructs showed that Myo2p binds on an N-terminal region of Sec2p and that these truncations bind more strongly (Figure 4.1D), in agreement with previous reports that the C-terminal tail of Sec2p negatively regulates the N-terminal region (Medkova *et al.*, 2006). Because a two-hybrid construct containing the GEF domain only (amino acids 1- 160) autoactivates, I could not rule out the possibility that the interaction we were seeing was indirectly through Sec4p. Although Sec4p should never localize to the nucleus, I tested for this possibility using the helix 9 mutant AD-ccGTD(Y1415R) that does not bind Sec4p (see Figure 3.2A in Chapter 3) and found that both the truncations and full-length Sec2p still interact (Figure 4.1D). Because the fragment encompassing amino acids 1 to 374 has been shown to bind to Ypt31p as well as Sec15p, I wanted to further map the Myo2p interaction site. However, the GEF domain by itself autoactivates and

fragments lacking this domain were not expressed, most probably because the GEF domain with a portion of the downstream sequences are one folded unit (as judged by the robust expression of the $Sec2^{1-258}$ construct in bacteria and by the degradation pattern of bigger constructs that always accumulate a band running at the same size as the $Sec2^{1-258}$ fragment). These results clearly show that *in vivo* Sec2p and Myo2p, if not interact directly, at least associate together in a complex.

The direct *in vitro* binding of Myo2p with Sec2p is very weak

To confirm if the two-hybrid interaction was indeed direct, I cloned a GST-Sec2 construct for bacterial expression. Although the expression levels were very low, I could obtain sufficient amounts bound to the beads to carry out *in vitro* binding assays. Since I had to use GST-Sec2 (as HIS_6-Sec2 did not express), I cleaved the GST tag from the purified Myo2p tail constructs and used our Myo2p tail antibody (B74) to detect bound proteins. I carried out binding assays between these two proteins under different buffer conditions and, although variable, most of the times I could not see any binding (Figure 4.2A). At the same time, Sec4p binding to Sec2p was very strong under all conditions tested. Since the two-hybrid interaction was stronger with the N-terminal fragments of Sec2p, I decided to purify these fragments and test for binding to Myo2p (Figure 4.2B, insert). Although in some experiments I got clear binding to the Myo2p tails with these fragments, it was very variable and could not be routinely reproduced (Figure 4.2B). In fact, in the experiments

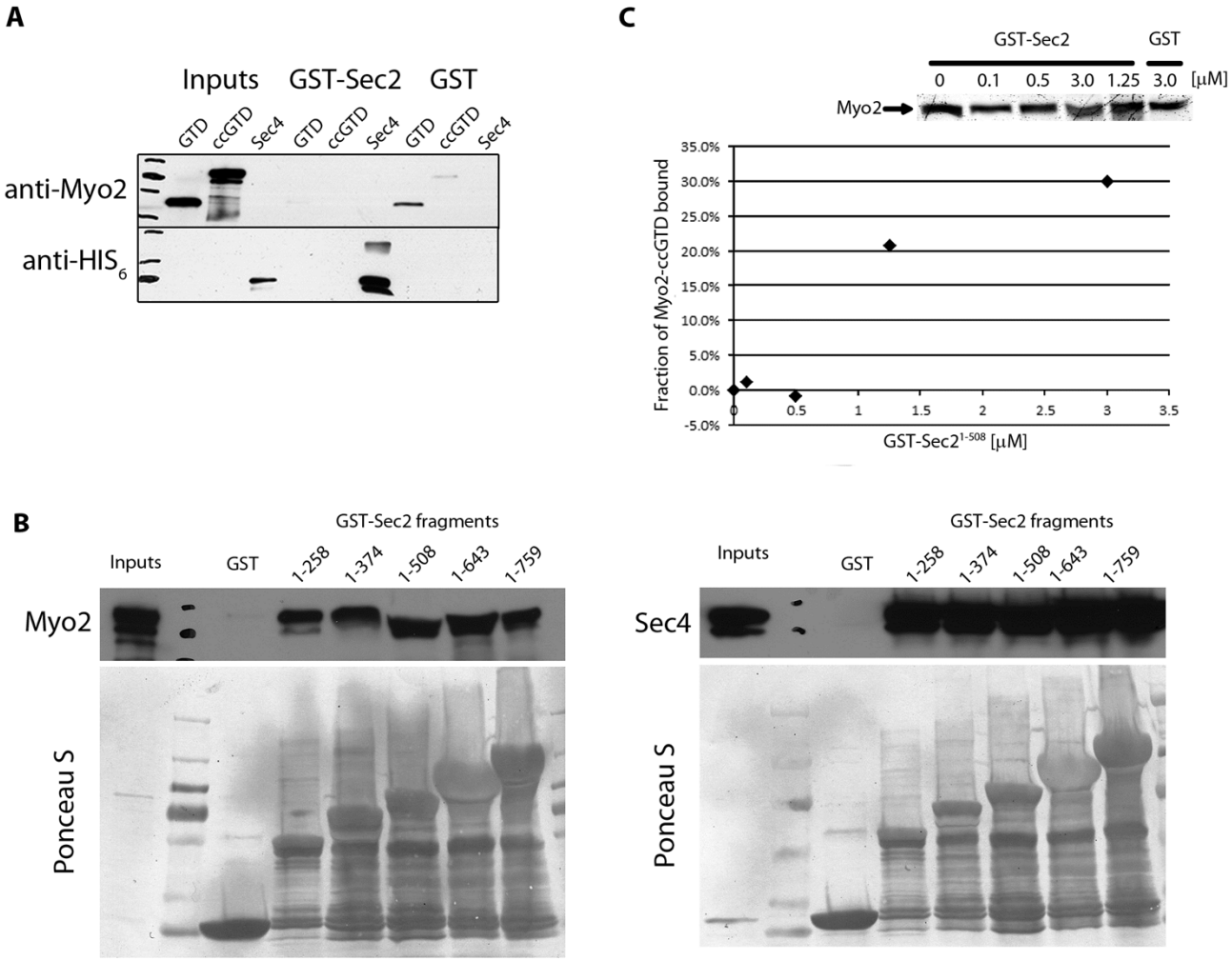


Figure 4.2 The binding of Sec2p and Myo2p is very weak *in vitro*. (A) Full-length Sec2p fused to GST was bound to glutathione resin and incubated with soluble untagged Myo2p tail constructs or HIS_6-Sec4. After washing, proteins retained in the resin were probed by western blot using anti-Myo2 or anti-HIS_6 antibodies. Different buffer conditions were tried but I could not get Myo2p binding to Sec2p while the Sec4p binding was very reproducible. (B) Same as in (A), but Sec2p fragments fused to GST were used instead. Depending on the experiment, sometimes I could get Myo2p bound, but it was not consistent (left panel is an example where all fragments bound Myo2p). Sec4p bound to all the fragments, as they all contain the GEF domain (right panel). (C) Supernatant depletion assay between Myo2p and full-length Sec2p. Soluble untagged Myo2p ccGTD was incubated with increasing concentrations of GST-Sec2 or GST resin as described in the Materials and Methods. Using this method, I could consistently get a fraction of Myo2p bound to the Sec2p resin when using concentrations above 1μM. The insert above the graph shows the staining of the membrane for this particular experiment. The bands were quantified using the Odyssey infrared system.

that showed binding, there was very little difference in the efficiency of binding between the full-length and the fragments, suggestive of mostly background binding. In my experience, the GST-Myo2p tail constructs are very sticky and unfold after just a few days kept at 4°C. Although I did not test extensively using different buffer and wash conditions, my general impression was that the two proteins do not bind directly. However, after reading an article from Tom Pollard (Pollard, 2010) about *in vitro* binding assays, I decided to try supernatant depletion assays, which are recommended for interactions with weak to modest binding constants. Another advantage of this assay is that, if large amounts of proteins can be purified, the actual binding constant can be calculated by varying the concentration of one of the reactants while leaving the other constant. However, the condition of "large amounts of proteins" was precisely the one I could never reach to be able to obtain a binding constant. Nevertheless, I was always able to deplete some amount of Sec2p (20% - 30%, depending on the experiment) from the supernatant with Myo2p tail concentrations above 1µM (Figure 4.2C). The insolubility of the Sec2p fragments and the difficulty in getting them to bind the beads (for some reason I always lost a considerable amount of the soluble Sec2p in the unbound fraction) prevented me from carrying out the assays with concentrations above 3µM. However, these results strongly suggest a binding in the tenths of µM, which is very low to be physiologically relevant. It could, however, be the case that by generating microdomains in membranes, the local concentration of these proteins gets high enough for binding to occur. This is supported in part

by the fact that these two proteins localize to the same vesicular membranes, although unpublished data from Kirk Donovan in the lab indicate that there is only about 7 to 8 Myo2p motors per vesicle (14 – 16 GTDs), too low to be in an excess over the amount of Sec2p that might be present.

Sec2p is in a complex with Myo2p *in vivo*

Since I failed to get a reproducible interaction between Myo2p and Sec2p *in vitro* and yet the two-hybrid interaction is very reproducible, I wondered if *in vivo* Sec2p is associated with Myo2p in a complex. I first tried using our anti-Myo2p tail antibody to see if immunoprecipitated Myo2p brings down Sec2p with it. A caveat of this procedure is that the antibody was generated against the GTD and could, in principle, block the interaction of Myo2p with GTD binding partners such as Sec2p. Moreover, B74 has never been used before for immunoprecipitations so I tried a standard protocol that is by no means optimized for my purposes (see Materials and Methods). I prepared cleared lysates from strains induced to express HA-tagged Ypt1p, Ypt31p, Sec4p, or Sec2p for 45 minutes, and treated the lysates with DMSO or the crosslinker agent DSP. After developing the western blot I did not find much difference between the non-crosslinked and crosslinked samples (Figure 4.3A, top gel), with Sec2p present in the immunoprecipitate under both conditions. However, some amount of Ypt1p, my negative control, also was present in both samples, although it was more prominent in the DSP-treated sample. Since DSP did not show a big difference, I decided to try one more time without DSP and also

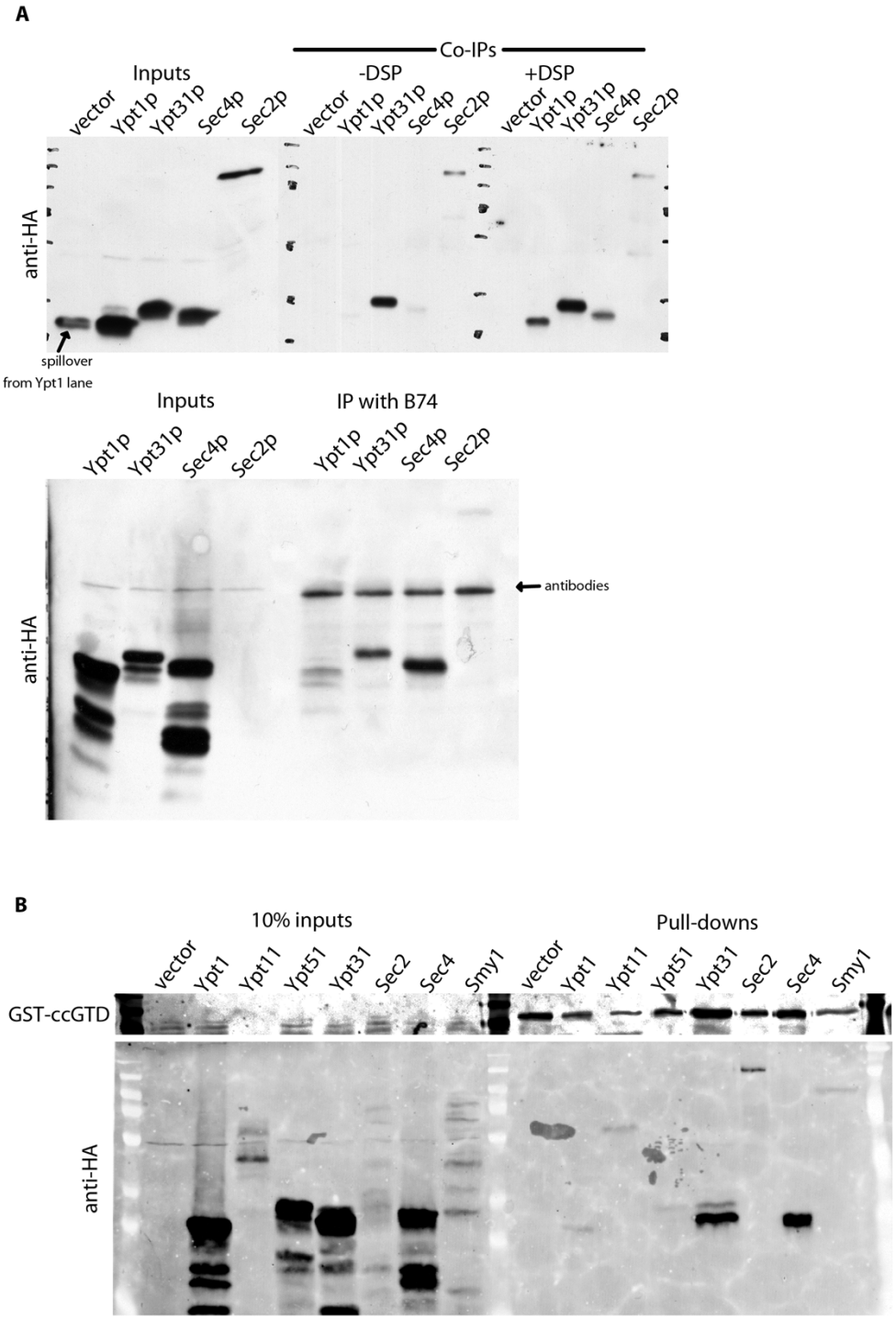


Figure 4.3 Sec2p associates with Myo2p *in vivo*. (A) Coimmunoprecipitation experiments with or without crosslinker (DSP) shows that Sec2p is associated with Myo2p. Two different examples are shown, the second one was done in the absence of crosslinker. (B) GST-pull-down experiments from yeast lysates containing HA-tagged proteins of interest. Yest strains co-overexpressing the Myo2p ccGTD and HA-tagged proteins were disrupted, and the clear lysate incubated with GSH beads for retention of the GST-ccGTD. Proteins bound to the beads were assayed by western blot. Ypt31p and Sec4p bound strongly while Sec2p and Smy1p to a lesser extent.

using normal rabbit antisera as a control for background binding. This time Ypt31p and Sec4p were clearly enriched in the B74 precipitates over background and very little Ypt1p came down under either condition. However, very little Sec2p, compared to the previous experiment, was present in the B74 precipitate, although after a long exposure it was clearly visible (Figure 4.2A, bottom gel). Because of the reasons mentioned above, there were many variables that I could change to optimize the immunoprecipitation of Myo2p by B74. However, in order to save time and antibody (as it is not commercial and there is a limited amount), I decided to try a more standard procedure to pull out Myo2p that at the same time eliminated the problem with the possible interference of the antibody by binding to the GTD.

By mildly expressing a GST-ccGTD construct in yeast, I could use GSH-beads to pull down the Myo2p tail construct without interfering with the GTD interactions. This time I also tested a broader array of HA-tagged proteins: Ypt1p and Ypt51p as negative controls; Ypt11p, Ypt31p, and Sec4p as positive controls; and Sec2p and Smy1p as my experimental samples. Under these pull-down conditions, Ypt31p and Sec4p were clearly visible as expected, but Sec2p was visible as well (Figure 4.3B). Ypt11p and Smy1p were also pulled down at lower levels, and Ypt1p and Ypt51p were hardly visible over background. I repeated this one more time obtaining the same results, and also the same problems. Since I'm inducing two constructs simultaneously, and for a very short time (30 – 45 minutes), the amount of GST-ccGTD that is

expressed varies greatly between the strains. Likewise, the Rab proteins, being very small and compact, are expressed at higher levels and are less prone to degradation than larger proteins like Sec2p or Smy1p, that are expressed at very low levels and much more degradation bands appear. These problems make the comparison and quantitation very difficult but, nevertheless, in qualitative terms it is clear that Sec2p, as well as the Rab proteins Ypt31p and Sec4p, and the kinesin-like Smy1p, all associate with Myo2p *in vivo*. I reasoned that a way to circumvent these problems was to use purified GST-ccGTD protein such that the same amount of protein was present in all lysates. Likewise, I could test a panel of different induction times for the bigger proteins to find one where the expression levels were similar to that of the Rab proteins in 30 minutes. These experiments were put on hold while writing this thesis.

Sec2p binds strongly to PI4P but does not bring Myo2p with it

I have already used a liposome floatation assay to explore whether the recombinant Myo2p-tail would bind synthetic liposomes in a PI4P dependent manner, and it does not (Chapter 2). Since the genetic, biochemical, and imaging data discussed before indicates that Myo2p responds to PI4P, but does not bind it directly, additional factors must be required to establish a stable association with PI4P-rich membranes. I reasoned that if Sec2p associates with PI4P membranes in our floatation assay, it could bring with it Myo2p if indeed the two proteins interact together. However, the assay used by the Novick lab to demonstrate PI4P binding by Sec2p was different from our assay

(Mizuno-Yamasaki *et al.*, 2010). Although they used small unilamellar liposomes as we do, they used a much greater concentration of PI4P (10% vs 4%) and precipitated the liposomes rather than floating them. A floatation assay is much more stringent than precipitation, as the proteins have to remain bound as the liposomes move upward in the gradient against drag and centrifugal forces. I wanted to test first if the Sec2p PI4P binding was strong enough to be detected in our floatation assay. As seen in Figure 4.4A, Sec2p binds very clearly to PI4P containing liposomes in a manner dependent on the region between amino acids 374 and 508, as reported by the Novick lab. This result allows me to test if more Myo2p floats with the PI4P containing liposomes in the presence of Sec2p. Since Myo2p has an inherent (albeit weak) affinity for membranes, I thought that having also Sec2p there, the combined affinity would be enough to get Myo2p floating. However, the presence of full-length Sec2p, or the truncated versions (not shown), had no effect on the amount of Myo2p binding the liposomes (Figure 4.4B, compare lanes 5,6 and 13,14). Next I wanted to test our hypothesis that Myo2p associates with secretory membranes in a PI4P and Sec4p dependent way by adding Sec4p to the reactions. At the same time, I tested if the presence of Sec2p could stabilize this complex, aiming at reconstituting the recruitment of Myo2p *in vitro*. Again, there was no difference in the amount of Myo2p in the float fraction under either of these conditions (Figure 4.4B, lanes 19 – 22 for the presence of Sec4p or 15 – 18 for the presence of Sec4p and Sec2p). However, a flaw of this experiment is that Sec4p by itself did not associate with the

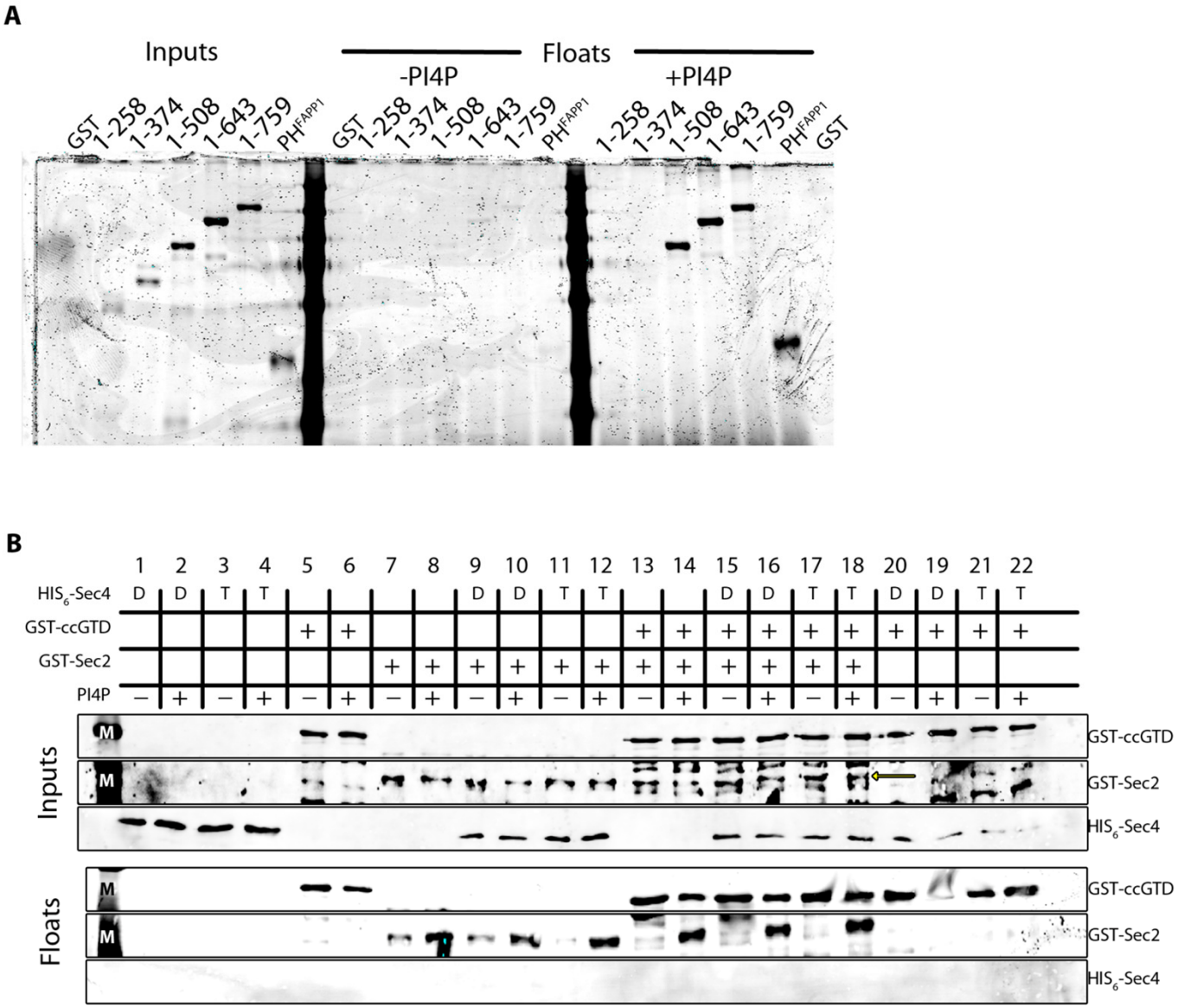


Figure 4.4 Sec2p binds to PI4P strongly but cannot bring with it Myo2p or Sec4p in floatation assays. (A) Liposome floatation assays with GST, or different Sec2p fragments fused to GST, or GST-PH^{FAPP1}. No protein floats with liposomes lacking PI4P, but the GST-PH^{FAPP1} construct and the Sec2p fragments containing the region between 374 and 508 floated with the PI4P-containing liposomes. The gel was stained with IRDye Blue Protein Stain. (B) Liposome floatation assays with Sec4p, Myo2p ccGTD, $Sec2^{1-508}$ construct, and the different combinations between them. The M to the left of the blots marks the protein ladder lane, and the yellow arrow in the GST-Sec2 Inputs panel points to the Sec2p band, which can be clearly seen in lanes 7 – 12. Sec2p floats normally with the PI4P-containing liposomes, but Sec4p does not (lanes 1 – 4), and there is no change in the amount of Myo2p that floats in the absence or presence of Sec2p (lanes 5,6 and 13,14), or of Sec4p and Sec2p (15 – 18).

liposomes (Figure 4.4B, lanes 1 – 4). Moreover, even in the presence of its GEF, Sec4p did not float with Sec2p as it would be expected to happen since *in vitro* their binding is very strong (Figure 4.4B, lanes 9 – 12). However, this was not surprising as *in vivo* Sec4p needs to be modified with lipid moieties to be able to associate with membranes and this does not occur in bacteria. I explored the possibility of carrying out this reaction in bacteria but, other than the fact that most of the Sec4p would then be insoluble, the enzyme complex responsible for the modification involves the activity of many different subunits that would make it very difficult to do in bacteria. This approach, however, is ideal to test the effects additional factors such as Mmr1p (James Young, unpublished) or Smy1p can have on Myo2p function (Chapter 3). As a consequence I am in the process of testing the effect yeast lysates or Sec4p purified from yeast might have on Myo2p incubated with liposomes with or without PI4P. The aim is to find conditions were Myo2p associates with the liposomes in a PI4P dependent manner, enabling us to then test the effect of adding or removing different Myo2p associated factors. Currently, I'm optimizing the conditions to establish this system as a way to reconstitute *in vitro* Myo2p recruitment to secretory membranes.

Setting up a two-hybrid screen for Sec2p mutants that no longer associate with Myo2p

Lastly, in order to prove that the association of Sec2p with Myo2p is physiologically relevant, we wanted to obtain a Sec2p mutant that selectively

abolishes the interaction with Myo2p by two-hybrid. Once obtained, this mutant would be put into a cell to determine if any defect in polarity is seen. We opted for a scheme that would allow us to simultaneously screen different Sec2p binding partners to rule out the complete loss of function mutations and focus in the ones that selectively abolish Myo2p interaction (Figure 4.5A). I generated a library of Sec2p mutants by PCR using as a template the shortest fragment that gave a positive interaction with Myo2p, $Sec2^{1-258}$. After transforming into yeast strain pJ694-α, I followed the scheme outlined in Figure 4.5A to mate with strains containing the Sec2p binding partners AD-ccGTD, AD-*SEC4*(S34N), or AD-*SEC15*. At the time of assembly of this thesis, I have screened ~300 clones and found 13 that abolished both Myo2p and Sec4p interactions (Figure 4.5B). I have not so far found one that selectively abolishes one or the other. I also found out that the Sec2p fragment used in this screen does not interact with Sec15p, as previously shown with purified proteins *in vitro* (Medkova *et al.*, 2006).

DISCUSSION

Sec2p has always been suggested to be involved in the Myo2p association with secretory vesicles as in the original *sec2* mutants, which are delocalized at all temperatures, upon shifting to the restrictive temperature vesicles accumulate randomly throughout the cell. Consistently, Sec2p is the link between Ypt31p and Sec4p signaling, coupling activation of the upstream Rab GTPase with the one downstream in the pathway. More importantly,

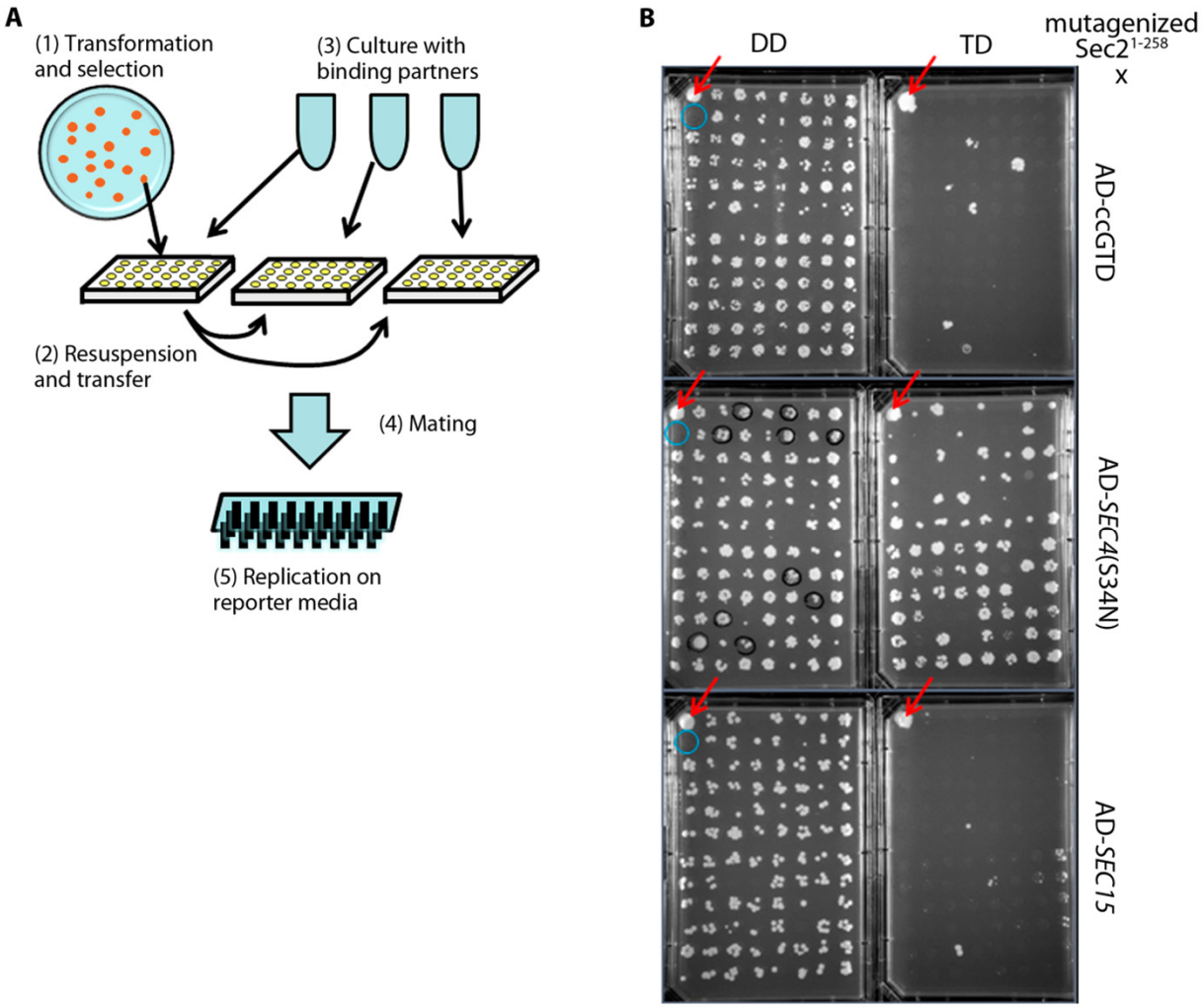


Figure 4.5 Isolation of Sec2p mutants defective in the interaction with Myo2p. (A) Scheme for the isolation of Sec2p mutants that have lost specifically the Myo2p interaction. See Materials and Methods for more details. (B) Example of a set of plates where different mutagenized AD-Sec2p clones were mated to strains carrying AD-ccGTD, AD-*SEC4*(S34N), or AD-*SEC15*. The red arrows show the positive control for mating, and the blue circle the negative control. We were aiming at clones that do not grow in the AD-ccGTD TD plate but grow normally on the other plates. I later found out that the minimal Sec2 fragment used in this screen does not interact with *AD-SEC15*.

Sec2p was recently identified as a Golgi PI4P effector, the binding of which not only regulates its localization but also its function in exocytosis, therefore it is a central player in the regulation of vesicle biogenesis, transport, and subsequent fusion. Although we hypothesized that Sec2p was the bridge between Myo2p and PI4P signaling, our data do not support or refute that model. First, we have no evidence of a strong direct interaction between Myo2p and Sec2p, as the affinity between purified proteins is too low to be physiologically relevant. Second, in some *sec2* temperature sensitive alleles, the PI4P binding site is absent, accounting for the mostly diffuse localization of those mutants, but also indicating that PI4P-binding by Sec2p is not completely essential as it would be expected for a protein linking Myo2p to PI4P. Moreover, I reasoned that if indeed Sec2p was playing a role in bridging Myo2p to vesicles, the Myo2-PH domain fusion protein used in Chapter 2 should be able to bypass this function. I transformed the Myo2-PH^{WT} fusion into *sec2-41* (a truncation at amino acid 397) and found no effect at any temperature (not shown). Similarly, fusion of Myo2p to the Sec2p tail domain (amino acids 450 to 759), which regulates Sec2p localization, also has no effect on *sec2* or *myo2* mutants (not shown). I conclude that Sec2p, based on these results, is not the bridge between Myo2p and the PI4P and Sec4p signaling. Nevertheless, our results clearly indicate that Sec2p associates with Myo2p, most probably by being part of, or closely associated with, the secretory vesicle receptor for Myo2p. This last statement is supported by the two-hybrid analyses and by the GST pull-downs from yeast lysates.

Our data is also consistent with previous reports that the C-terminal tail of Sec2p regulates its localization and function (Elkind *et al.*, 2000; Ortiz *et al.*, 2002; Medkova *et al.*, 2006). *Sec2-78* is a point mutation in the C-terminal tail, away from the Ypt31p and PI4P binding sites (Figure 4.1A), and yet causes a mislocalization of the protein. It was also found that this mutant protein binds more strongly to Sec15p, which also binds at a location in the N-terminal region. Limited proteolysis analysis showed that Sec2-78p adopts a different conformation as it resulted in a different degradation pattern than wild-type (Medkova *et al.*, 2006). Likewise, binding to PI4P decreases the amount of Sec15p that associates with Sec2p *in vitro*, and the *sec2* mutant lacking the PI4P binding sites co-immunoprecipitates much more Sec15p than wild-type (Mizuno-Yamasaki *et al.*, 2010). Thus, Sec2p is somehow conformationally regulated by its tail domain and that regulation can respond to PI4P binding. In a similar manner, the presence of the C-terminal tail negatively regulates its association with Myo2p. It would be interesting to see if association with Sec15p is mutually exclusive with Myo2p association. This would be consistent with our model that there is a gradient of PI4P in the secretory pathway, with high levels in late Golgi cisternae, and lowered levels in secretory vesicles. As the secretory vesicles arrive to their destination, Sec2p binding of Sec15p is more important for its exocytosis function than at the early steps of vesicle biogenesis and transport. Therefore, PI4P plays a fundamental function along the whole secretory pathway, coupling generation of vesicles with their transport and finally with their tethering at the destination.

I prefer the idea that rather than being a bridge, Sec2p association with the secretory vesicle receptor results in a microdomain of active Sec4p in PI4P rich membranes. Myo2p, modulated by Smy1p, would strongly bind to active Sec4p, resulting in an association with membrane domains destined to become secretory vesicles. As explained in the introduction, Myo2p binds *in vitro* directly to Sec4p independent of the nucleotide status. I found that the Rab GTPases bind Myo2p in a cooperative manner and, therefore, constraining the Rab signaling to a small membrane domain would result in a rapid conversion of TGN membranes (labeled by Ypt31p) into secretory vesicles (labeled by Sec4p) coupled to Myo2p recruitment. Isolation of a specific Myo2p-binding defective Sec2p mutant would be very informative in this matter.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Involvement of PI4P and Rab GTPases in the recruitment of Myo2p to secretory membranes

In recent years, an ever-growing number of publications report the direct involvement of lipids in the recruitment of specific proteins to their site of action (Hurley, 2006; Lemmon, 2008). These reversible recruitments are mediated by lipid-binding domains present on the protein and thus are dependent on the spatial and temporal regulation of the specific lipid ligand. To achieve the specificity needed to carry out cellular processes, these lipids have to be regulated either in time, in space, or both, and very frequently are seen associated with other signaling molecules like small GTPases. A lipid family that has the capacity to do just that is the phosphoinositides, which with the reversible phosphorylation of its inositol ring at positions 3, 4, or 5 can generate up to seven distinct species. Some of the species are present all the time (PI3P or PI4P) but restricted to specific membranes, while others are generated upon specific stimuli ($PI3,5P_2$ or $PI3,4,5P_3$). Due to this ability of being able to be generated upon demand and to restrict their production, or degradation, to specific membranes, phosphoinositides join the ranks of small GTPases as critical regulators of many essential cellular processes.

One of these fundamental processes in cellular function is membrane

trafficking, and budding yeast is an excellent model to study actin-based transport. Polarized transport along actin is essential in yeast, and is analogous to the transport of cargo to and from the actin-rich cell cortex in higher organisms. In order to grow and divide, yeast need to deliver cell-wall modifying enzymes and new membranes to the growing bud. Then, before cytokinesis, it needs to segregate all its organelles, followed by deposition of the septum to ensure cell separation. All of these processes are carried out by the myosin-V Myo2p, transporting secretory membranes to the different sites in the cell undergoing active remodeling. Remarkably, how Myo2p associates with its secretory cargo, its only essential cargo, was not understood.

With all the current evidence for PI4P and Sec4p as essential factors for post-Golgi transport, we set out to explore the role this lipid and Rab GTPase could have in Myo2p-dependent transport of secretory vesicles. We found evidence for a coincidence detection mechanism on the association of Myo2p with secretory membranes although the molecular identity of all the components involved remains unclear. We identify two *myo2* alleles that are especially sensitive to Golgi levels of PI4P, and can be suppressed to different degrees by overexpressing the Golgi PI 4-kinase *PIK1* or deleting the major PI4P phosphatase, Sac1p. Under these conditions, the localization of GFP-Sec4, the Rab GTPase associated with secretory vesicles, is polarized upon shifting to the restrictive temperature, indicative of a restored association of Myo2p with secretory vesicles. Likewise, increasing the levels of Sec4p or Ypt31p can

partially rescue the growth of the same *myo2* alleles and restore localization of GFP-Sec4, suggesting that PI4P and these Rabs are working in the same pathway with Myo2p. Consistent with this, these PI4P-sensitive alleles were found to be synthetic sick with *pik1* mutations as well with *sec4-8*, and *pik1* mutations interact genetically with *MYO2* and *YPT31* (Schott *et al.*, 1999; Walch-Solimena and Novick, 1999; Sciorra *et al.*, 2005). These interactions strongly suggest a functional relationship between PI4P, the Rabs, and Myo2p. Moreover, membranes labeled by a PI4P reporter or by these Rabs are rapidly moved towards growth sites in a Myo2-dependent manner and interfering with the production of Golgi PI4P, or with the function of Sec4p, prevents this movement. Consistently, membranes labeled by Ypt31p or by Sec4p showed partial co-localization with PI4P reporters and are moved by Myo2p, while other membrane devoid of detectable PI4P and labeled by related Rab proteins, were not. Moreover, rapid inactivation of Golgi PI 4-kinase uncouples secretory membranes from Myo2p, which still walks down the cables but without its cargo.

The fact that there is a clear correlation between membranes having PI4P and being moved by Myo2p, prompted us to test if Myo2p would be recruited to these membranes by a dual recognition of PI4P and exocytic Rab GTPases. Although the exocytic Rabs do provide a direct linkage between Myo2p and secretory membranes, a direct specific binding between Myo2p-tail domain and PI4P was not found in two different *in vitro* assays. In a protein-lipid dot blot

assay (PIP strips) Myo2p-tail showed no specificity for any of the PIs, binding most of them at very high Myo2p-tail concentrations while none of them at lower concentrations. In a more physiological assay, liposomes with lipid compositions mimicking the TGN, with or without PI4P, were incubated with Myo2p-tail and a sucrose step-gradient layered on top. Because the liposomes are filled with buffer, they will float to the top of the gradient upon ultracentrifugation, bringing with it proteins that stably bind lipids. We found no change in the amount of Myo2p-tail that floated with or without PI4P. An unexpected result was that, although Myo2p-tail does not stably bind to PI4P, it does have a weak affinity for curved membranes as judge from the fact that a small portion floats with small liposomes regardless of the composition, but not with giant unilamellar vesicles (>1μm) of the same lipid composition. Despite not finding a specific interaction between PI4P and the Myo2p-tail domain, we found additional data that strongly support the coincidence detection hypothesis. Linking Myo2p directly to the normal levels of Golgi PI4P could rescue several of the *myo2* alleles, and moreover, could bypass the otherwise essential interaction with the Rab GTPases. Surprisingly, although the Myo2-PH fusion could rescue the PI4P-sensitive *myo2* alleles and support viability of the Rab GTPase binding-deficient *myo2* mutant, it could not complement a *myo2*Δ. This means that either there is another essential function for the Myo2p-tail in secretory compartment transport, or there is another component important for the association of Myo2p with membranes distinct from PI4P and Rab GTPases. This work also presents evidence supporting the idea that

secretory vesicles contain some amount of PI4P, a claim also supported in a recent paper looking at the contributions Golgi PI4P may have on the maintenance of plasma membrane PI4,5P_2 levels (Szentpetery *et al.*, 2010). Although at first this finding would seem to be at odds with the work from the Novick lab (Mizuno-Yamasaki *et al.*, 2010), we do believe that the levels of PI4P diminish as the secretory membrane matures along the exocytic pathway. We hypothesize that controlling the levels of PI4P, along with the cycling nature of the Rab GTPases between on and off states, could be a mechanism to couple transport with the later steps of tethering and fusion of the carrier (see below). Notably, it was reported recently that PI4P may have an analogous role in regulating fusion of COPII vesicles with cis-Golgi membranes, suggesting that although most of the PI4P at the Golgi is on the trans-side, a discontinuous gradient may exist, with minimal but essential amounts at the cis-side (Lorente-Rodriguez and Barlowe, 2011). Similarly, smaller amounts should be present in vesicles to allow for continuous transport by Myo2p, but perhaps are too small to be easily seen given the small and fast moving nature of secretory vesicles.

How Myo2p respond to the Golgi PI4P and active Sec4p signaling?

A big gap in our story, however, is the fact that Myo2p does not seems to recognize PI4P directly. Analysis of the surface charge of the Myo2p tail reveals a pocket lined by positive charge side chains, however it is on the vacuole binding region, opposite to the vesicle binding region. Also, the fact that

Myo2p binds Sec4p in both the GDP and GTP states is surprising. All other examples of motor proteins associated with Rab GTPases exhibited nucleotide preference (especially myosin-V motors, (Lapierre *et al.*, 2001; Roland *et al.*, 2007). We do find that Smy1p can modulate the specificity of Myo2p for active Sec4p, however Smy1p is not an essential protein, suggesting that in its absence Myo2p can still recognize active Sec4p *in vivo*, assuming that recognition of active Sec4p is necessary for Myo2p function. It is, on the other hand, accepted that inactive Rabs are rapidly extracted from membranes and kept in the cytosol in a complex with Rab GDI. Therefore, in principle, the only Sec4p on membranes that Myo2p could associate with is active Sec4p. This, however, cannot explain the specificity of Myo2p for secretory vesicles, as there is another pool of PI4P, and of mostly inactive Sec4p (by subcellular fractionation, most of Sec4p is at the plasma membrane, although this is hard to see by imaging GFP-Sec4), at the plasma membrane. All of these observations point towards the existence of additional factors that would link Myo2p to PI4P and ensure that it will associate with active Sec4p. I propose one genetic screen in Appendix II to identify genes whose products may be involved in the transmission of the PI4P and active Sec4p signal to Myo2p.

The role of Sec2p in coupling Myo2p to microdomains rich in PI4P and active Sec4p

As explained above, the dual signal required by Myo2p to associate with secretory membranes involves PI4P and the Rab GTPases Sec4p and, to a

lesser extent, Ypt31p. However, Myo2p does not bind directly to PI4P or discriminate between active or inactive Sec4p. Recently Sec2p, the GEF for Sec4p, was identified as a Golgi specific PI4P effector (Mizuno-Yamasaki *et al.*, 2010), positioning it as an ideal candidate to link Myo2p to the coincidence signal. Indeed, the mammalian homolog of Sec2p, Rabin8, was recently shown to be an effector of Rab11, the homolog of Ypt31p, in an interaction necessary for ciliogenesis (Knodler *et al.*, 2010). Moreover, MyoVb, one of the three myosin-V isoforms in mammals, binds both Rab8 and Rab11, which are associated with non-overlapping membrane tubules destined to the plasma membrane (Roland *et al.*, 2007). Although speculation at this point, Rabin8 could be playing an analogous role to Sec2p in coupling the maturation of Rab11 membranes into Rab8 vesicles to the actin-dependent transport to specific areas of the plasma membrane. In fact, very recently a sequential series of Rab11/Rab8 re-localizations in ciliary assembly was reported (Westlake *et al.*, 2011). When cilia formation is induced, Rabin8 re-localization to Rab11-positive membranes causes these membranes to be transported to the centrosome and subsequent accumulation of Rab8 is seen at the base of the cilia. After ciliogenesis, Rab8 accumulation at the cilia is strongly reduced, and this reduction correlates with a loss of Rabin8 from the centrosomal location. These observations link Rab11, Rabin8, and Rab8 in a concerted cascade that results in the delivery of ciliary proteins to the plasma membrane were the cilium is going to be formed. The fact that Sec2p requires both active Ypt31p and PI4P ensures its localization to membranes destined to become

secretory vesicles. Once in these PI4P-rich membranes, Sec2p can presumably recruit and activate Sec4p, resulting in membranes now rich in PI4P and active Sec4p. Sec2p mutants missing the PI4P binding region are mostly mislocalized, but by overexpressing the other ligand, enough Sec2p can now localize to provide the essential function. This is one of the hallmarks of a coincidence detector, and we see the same for the PI4P and Sec4p sensitive Myo2p mutants, overexpression of either signal can partially compensate for the defective one. There are several examples of complexes containing a GEF and an effector of the same Rab, all involved in the maturation or conversion of membranes in membrane trafficking pathways (the Rab5/Rabex5/Rabaptin5 complex or the Rab7/HOPS complex). The fact that in the truncated *sec2* alleles overexpressing *YPT31*, growth is restored with only partial secretion as compared to wild-type (Ortiz *et al.*, 2002), indicates that the other essential role of Sec2p, in addition to activation of Sec4p, is not in exocytosis but in an upstream process, one after vesicle generation (since *sec2* mutants accumulate vesicles), most probably in vesicle transport. This is consistent with our finding that the association of Sec2p with Myo2p is dependent on the N-terminal half, which is present in these truncated *sec2* alleles. Identification of a Sec2p mutant deficient in the two-hybrid interaction with Myo2p would be invaluable to dissect Sec2p function in secretory vesicle transport.

In vitro reconstitution of the Myo2p secretory vesicle receptor

Since we have identified several components that are associated with

Myo2p and that modulate its function, a biochemical reconstitution of Myo2p recruitment to secretory vesicles would be very informative. It would allow us to investigate in detail how Myo2's binding to secretory cargo occurs and is regulated. Once we set up this system, we could easily test the coincidence detection model by having both signals, Sec4p and PI4P, on liposomes mimicking secretory vesicles. We could then add additional components, such as Ypt31p, Sec2p, Smy1p, or Mmr1p (a Myo2p tail binding protein; Itoh *et al.*, 2002), singly or in combination to see if we can reconstitute PI4P-dependent binding. This assay would shed light on why Myo2p does not discriminate nucleotide status *in vitro* and could also be used to determine which protein links Myo2p to PI4P. We already have optimized protocols for the purification of the Myo2p tail, and candidate proteins like Sec4p, Ypt31p, and Sec2p. I have also tried to carry out these assays in the presence of yeast high speed clear lysates. Although I ran into problems with degradation and proteolysis of the GST-tag, in preliminary experiments I could see a clear increase of Myo2p in the floating fraction over the sample without lysate. I never tried a lysate from a protease-deficient strain or, even better, from a protease-deficient strain with a mutated version of Myo2p, to lower the competition between the recombinant GST-wild-type tail and the endogenous full-length Myo2p present in the lysate. If successful, this will allow us to identify novel factors required in the process by fractionation of lysates and looking for the minimal fraction that still gives a PI4P-dependent floatation of Myo2p with liposomes.

Coupling Myo2p transport to release of cargo at the destination

We have also noticed that it is very difficult to co-image PI4P and Sec4p in moving membranes. Moreover, very little co-localization of the two is seen in the mother cell, and in some cells with GFP-Sec4 caps but no enrichments of PI4P, vesicles and tubules labeled with the PI4P reporter are seen moving towards the cap and then disappear as they get there. We interpret this as a conversion of PI4P either into PI by a phosphatase or $PI4,5P_2$ by a kinase. There is evidence in the literature and from my own work supporting both options. The Novick lab has suggested that PI4P is either excluded from vesicles or is degraded on route to the plasma membrane (Mizuno-Yamasaki *et al.*, 2010). Their interpretation is based on the observation that GST-Sec2p does not bind Sec15p efficiently in the presence of liposomes containing PI4P, whereas PI4P had no effect on the binding to Ypt31p. They had previously reported that the region of Sec2p that interacts with Ypt31p also interacts with Sec15p, a component of the exocyst involved in tethering secretory vesicles at their site of exocytosis (Medkova *et al.*, 2006). Because presumably the interaction with Sec15p is essential to tether vesicles at the plasma membrane, and since Sec15p is supposed to ride on vesicles on its way to the plasma membrane (Boyd *et al.*, 2004), secretory vesicles cannot contain PI4P. However, they did not consider that Sec15p localization does not depend on Sec2p binding, and that there is at least another way that Sec15p can localize to sites of growth independent of vesicular traffic (France *et al.*, 2006). A way

to reconcile our findings with their interpretation is that Ypt31/32p and PI4P recruit Sec2p to the TGN and after the vesicle forms, the levels of PI4P drops allowing some Sec2p to switch from a Ypt31p-binding conformation to a Sec15p favorable one, but other Sec2p remains bound to PI4P until reaching the sites of growth. At that point, the remaining PI4P is converted, Sec4p hydrolyses GTP by the action of its GAPs, Myo2p dissociates from the membranes, and the vesicle undergoes fusion. Preliminary data from Kirk Donovan in the lab supports the hypothesis that Sec4p inactivation has to occur prior to Myo2p dissociation. He also has found that Myo2p dissociation occurs after vesicle tethering but prior to vesicle fusion. I have also seen that PI4,5P_2 is enriched at sites of growth, an observation also reported by others (Baird *et al.*, 2008; Garrenton *et al.*, 2010). The kinase responsible for production of this lipid is, however, distributed evenly along the whole plasma membrane (Audhya and Emr, 2003). This discrepancy on the localization of the enzyme and its product could be explained by a selective activation of the enzyme at sites of growth or by the fact that there is more substrate being delivered to these sites. Under this model, PI4P on the secretory vesicles would be converted to PI4,5P_2 that, coupled to Sec4p GTP hydrolysis, would promote fusion and at the same time prime those membranes for endocytosis (as at least in mammals PI4,5P_2 is essential for endocytosis to occur). Although it is not clear where, and by what phosphatase (or kinase), vesicle PI4P hydrolysis (or conversion) happens, this process could be one way membrane transport by Myo2p is integrated with exocytosis. Consistently, genetic interactions between

pik1 mutants and *sjl2*Δ have been reported (Nguyen *et al.*, 2005). Presumably, in cells with already low levels of PI4P, there is a reduced delivery of vesicles to sites of growth. In the absence of Sjl2p, the synaptojanin associated with actin patches, which are in close proximity to sites of growth, the defect in protein delivery is exacerbated by an additional defect in vesicle fusion, as in the *pik1*^{ts}*sjl2*Δ cells, compared with *pik1*^{ts} cells, there is a block in invertase secretion. Taken together, all these observations suggest an essential balance between the starting PI4P levels at the TGN and those at the very last steps.

APPENDIX I

THE MYO2P TAIL MAY HAVE A ROLE IN VESICLE TETHERING

Rationale and Purpose

Overexpression of the Myo2p tail domain (GTD) is known to be lethal due to displacement of endogenous Myo2p from the secretory vesicle receptor. During the experiments reported in Chapter 2 and 3 involving expression of the GST-GTD, I noticed that the cells stopped growing and arrested as large budded or unbudded cells. This phenotype suggested a block in secretion rather than a depolarization of vesicle traffic. Moreover, when I looked at GFP-Sec4 in these strains, it accumulated in a central region of the cell in big patches that I call 'blobs'. I wanted to characterize these patches and identify the components present there to try and understand how overexpression of the Myo2p GTD causes this phenotype.

Results and Discussion

The observation that overexpression of the Myo2p GTD causes an arrest in surface growth was striking to me as it was reported that it depolarizes secretion, causing the cells to become big and round (Schott *et al.*, 1999). This discrepancy is apparently a function of the level of GTD expression, with very low levels resulting in the exogenous GTD piggy-backing on vesicles and becoming polarized, moderate levels depolarizing secretion, and high levels

blocking secretion. Careful literature searches confirmed my findings as two reports have showed complete inhibition of growth upon Myo2p GTD overexpression (Reck-Peterson *et al.*, 1999; Karpova *et al.*, 2000). Although one of these reports showed a depolarized Sec4p localization upon GTD induction, none tested for a secretory block.

Separation of the internal and external fractions of the beta-glucanase Bgl2p is a rapid and clear way to test for a defect in secretion. Bgl2p is a very abundant protein of the cell wall and represent a well-characterized cargo for TGN to plasma membrane post-Golgi vesicles (Harsay and Bretscher, 1995). In wild-type cells there is no detectable internal Bgl2p as judged by western blot (Figure A1.1A, rightmost lane). In contrast, in cells overexpressing GST-GTD for 4hrs, Bgl2p accumulates internally to the same extent as a *pik1* mutant incubated for 45min at 37°C, conditions known to block secretion (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999). Surprisingly, in *myo2-12* there is a slight defect at the semipermissive temperature of 30°C that is exacerbated after 45min at the restrictive temperature. However, this defect must be transient as *myo2-12* cells become big and round after 4hrs at high temperatures as a result of depolarized secretion (Schott *et al.*, 1999; not shown). This experiment clearly shows that upon high level expression of GST-GTD a secretory block is induced in the cell.

Next, I wanted to determine if the vesicles accumulated in a polarized manner, like in most of the late *sec* mutants, indicative of a defect in tethering

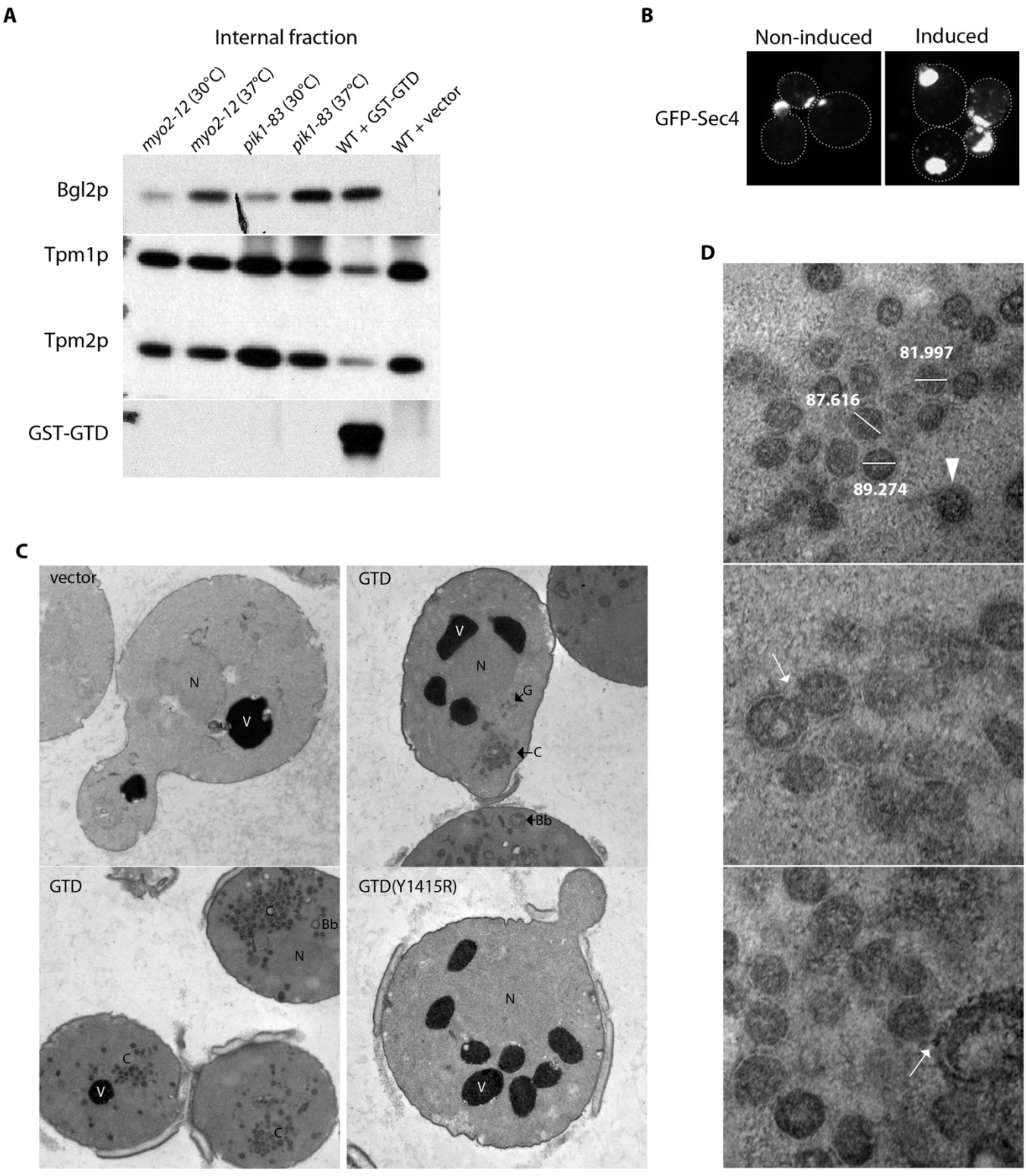

A
Internal fraction
myo2-12 (30°C)
myo2-12 (37°C)
pik1-83 (30°C)
pik1-83 (37°C)
WT + GST-GTD
WT + vector
Bgl2p
Tpm1p
Tpm2p
GST-GTD
B
Non-induced
Induced
GFP-Sec4
C
vector
N
V
GTD
V
N
G
C
Bb
GTD
C
Bb
N
C
V
C
GTD(Y1415R)
N
V
D
81.997
87.616
89.274

Figure A1.1 Overexpression of the Myo2p GTD induces clustering of post-Golgi vesicles causing a secretory block. (A) Separation of the internal and external fractions of Bgl2p was done by addition of 10X killing buffer (100mM NaF/100mM NaN_3 in 100mM Tris, pH 7.4) and incubating for 10min on ice. After washing twice in the same buffer, the cells were spheroplasted by resuspending in 100µL of buffer B (1.2M sorbitol, 25mM KPi, 25mM βME, pH 7.4) with 25µg/mL zymolyase and incubating at 37˚C for 1hour (although 20 – 30 min suffices for smaller pellets). The cells were spun down at 500xg for 5 min to separate internal (pellet) from external (supernatant) fractions, and the pellet resuspended in the same volume as the supernatant with MOPS buffer, pH 7.0 (or 10mM Tris, pH 7.4), to lyse the spheroplasts. After resolving by SDS-PAGE, the proteins were transferred to a membrane and blotted for Bgl2p, Tpm1/2p (an internal marker), and Myo2p. Only the internal fraction is shown. The amount of Bgl2p accumulated internally in cells overexpressing the GTD is an underestimation, as these cells apparently are prone to lysis since some amount of Tpm1/2p was detected in the external fraction (not shown). (B) GFP-Sec4 localization is grossly affected in cells overexpressing the GTD. It accumulates in internal patches that I termed "blobs". (C) By EM, these GFP-Sec4 blobs are clusters of vesicles. N, nucleus; V, vacuole; G, Golgi; C, clusters, Bb, Berkeley bodies. Notice how in the Rab-binding deficient mutant Y1415R, the vacuole is fragmented but no clusters are present. (D) These clusters are made up of post-Golgi secretory vesicles of the characteristic size between 80 – 100nm (top). These clusters very frequently contained chains of vesicles (arrow in middle planel) and vesicles associated with abnormal Golgi membranes (arrow in bottom panel). In a few instances, isolated vesicles were seen surrounded by a dark material, possibly a coat (arrowhead in top panel).

or fusion, or if they accumulated randomly throughout the cell, indicative of a problem with transport. For this I used GFP-Sec4 as a marker for secretory vesicles, which normally localizes to sites of active growth, namely the bud tip or the bud neck (Figure A1.1B). Surprisingly, upon induction of the Myo2p tail, GFP-Sec4 accumulated in internal patches that I called "blobs". These patches are so bright that is impossible to see the outline of the cell that normally you see because of the cytoplasmic staining of GFP-Sec4 (see color Figures 2.1, 2.5, or 2.7; on Figure A1.1B, because is black and white, is not apparent). I did a time-course experiment, adding galactose to cells growing at 30°C, and found that the blobs started to appear by 2hrs, and by 4hrs almost all the cells had blobs (in the CUY30 background, which is a strong galactose inducer). By time-lapse imaging, these blobs are fairly immobile as a whole although sometimes you can see tubules or vesicles detaching from the main blob and remaining in close proximity. Although most cells exhibited one big blob, very frequently I would see cells with multiple blobs of different sizes, and they tended to be bigger at later time points (>8hrs). These behaviors suggested that is wasn't simply an aggregate of GFP-Sec4, so we wanted to look at them with higher resolution, to determine if the blobs represented long, distended membranes, or if they were an accumulation of secretory vesicles.

I collaborated with Bret Judson, director of Imaging in the Weill Institute, to prepare and process cells overexpressing the Myo2p tail for electron microscopy (EM). In wild-type cells you very rarely see vesicles, as their

lifetime is very short (Figure A1.1C, vector panel). The nucleus and the electron dense vacuole are clearly visible, as well as other big membranes such as the mitochondria. However, in cells induced for 2 – 3hrs at 26°C, clusters of vesicles were visible in almost all cells (Figure A1.1C, GTD panels). These clusters vary in size, but were always away from sites of growth and very frequently associated with long cylindrical structures that most probably are aberrant Golgis (Berkeley bodies; Figure A1.1D). On the contrary, overexpression of the Rab-binding deficient GTD(Y1415R), did not cause this clustering of vesicles, although it still caused fragmentation of the vacuole (Figure A1.1C, Y1415R panel). These results tell us that the clustering is dependent on Rab binding (or at least on the vesicle binding region of the GTD) and that it is a direct consequence of interfering with the secretory function, as the vacuole (Figure A1.1C) or spindle orientation (not shown) functions of Myo2p are still disrupted by overexpression of the GTD(Y1415R) mutant. Consistently, other membranes transported by Myo2p such as peroxisomes are not seen clustered in the EM pictures.

Closer examination of these clusters revealed that they are between 80 – 100nm, the characteristic size of post-Golgi secretory vesicles (Figure A1.1D). We could also see vesicles surrounded by a dense haze, most probably a coat (Figure A1.1D, arrowhead). In some clusters, vesicle chains were clearly visible, in what appeared to be incomplete fission (Figure A1.1D, arrow in middle panel), and very frequently the clusters would be associated with

Berkeley bodies (Figure A1.1D, arrow in bottom panel). All of these results indicated that the defect is a post-Golgi block, in what appears to be incomplete fission or excessive/abnormal tethering between membranes.

To characterize these blobs further, I decided to look for the presence or absence of components of the secretory apparatus, especially Rab GTPases since it is known that they regulate tethering processes (Chapter 1) and a subset of them also bind Myo2p GTD (see Chapter 3). Ypt1p is the Rab associated with the early secretory pathway, and its role in ER to Golgi transport has been well characterized (Lian *et al.*, 1994; Cao *et al.*, 1998; Kamena *et al.*, 2008). In wild-type cells, GFP-Ypt1 labels scattered membranes that represent Golgi cisterna, mostly cis membranes, but also medial and trans as Ypt1p also has a role in intra-Golgi transport (Jedd *et al.*, 1995; Sclafani *et al.*, 2010). Interestingly, upon Myo2p tail induction, GFP-Ypt1 formed blobs indistinguishable from the Sec4p blobs seen before (Figure A1.2A). Notably, GFP-Ypt6, another Rab involved in intra-Golgi transport and traffic into the TGN (Li and Warner, 1996; Tsukada and Gallwitz, 1996; Li and Warner, 1998), was not present in these blobs (Figure A1.2B), indicating that there is a selective recruitment of proteins. Surprisingly, GFP-Ypt11p, the Rab GTPase that binds the strongest to the Myo2p tail (see Chapter 3), is also not present in the blobs and its localization to the ER is undisturbed (Figure A1.2C). This further suggests that the blobs are not an indirect effect of the overexpressed GTD, as Ypt11p, a Myo2p tail binding protein that would be expected to be

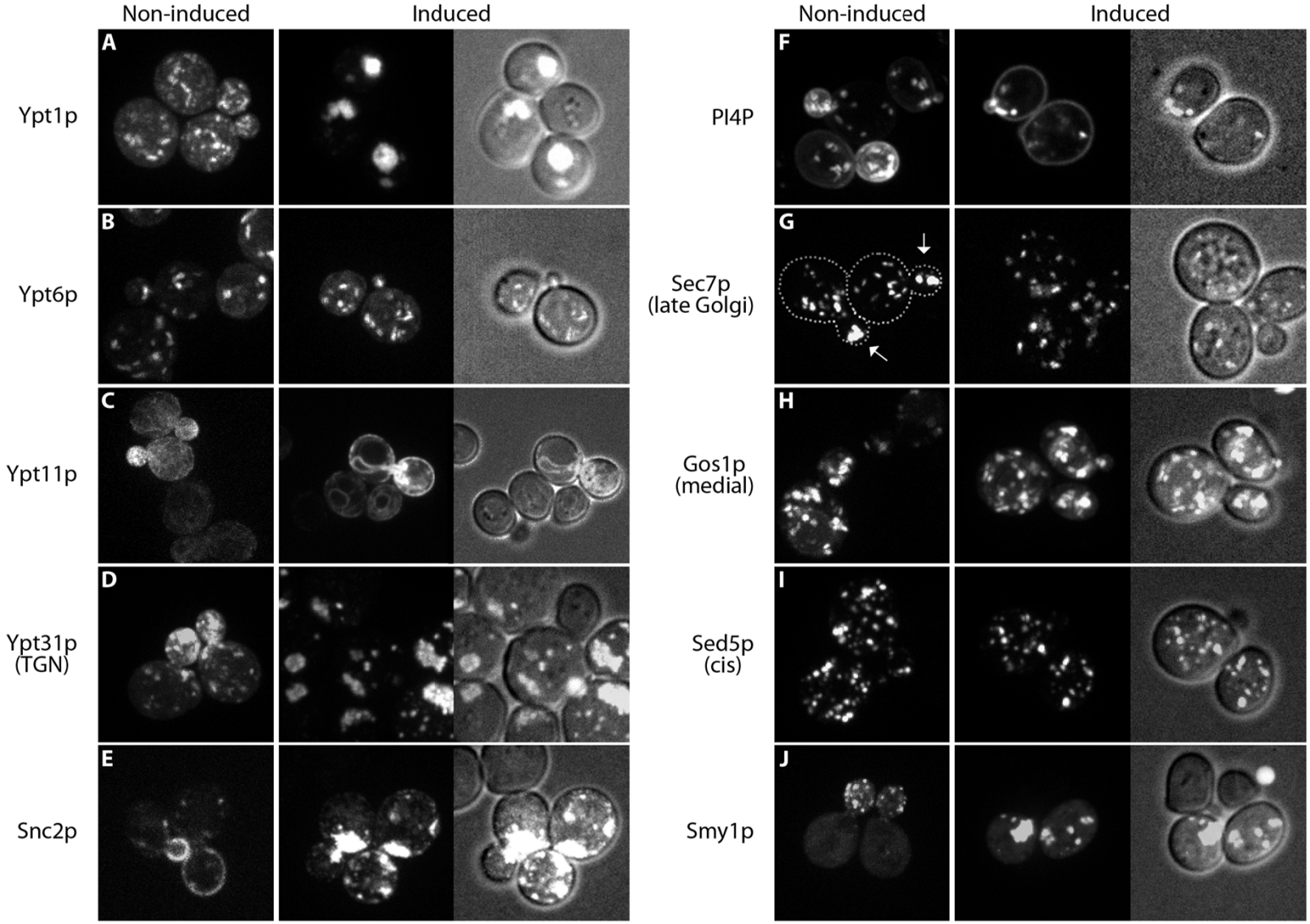

Non-induced
Induced
A
Ypt1p
B
Ypt6p
C
Ypt11p
D
Ypt31p
(TGN)
E
Snc2p
Non-induced
Induced
F
PI4P
G
Sec7p
(late Golgi)
H
Gos1p
(medial)
I
Sed5p
(cis)
J
Smy1p

Figure A1.2 Specific proteins are recruited to the blobs. Cells overexpressing the GTD and a fluorescent secretory marker were induced for 2 – 3hrs and processed for imaging. The markers used are GFP-Ypt1 (A), GFP-Ypt6 (B), GFP-Ypt11 (C), GFP-Ypt31 (D), RFP-Snc2 (E), mCherry-PH^{Osh2p} (F), Sec7-3XGFP (G), GFP-Gos1 (H), GFP-Sed5 (I), and Smy1-3XGFP (J). The fluorescent image by itself is shown under the Uninduced column as the cytoplasmic haze allows for visualization of the cell boundaries. Under the Induced column, both the fluorescent image alone and the fluorescent image overlayed over the DIC image are shown for better visualization of the cell outline.

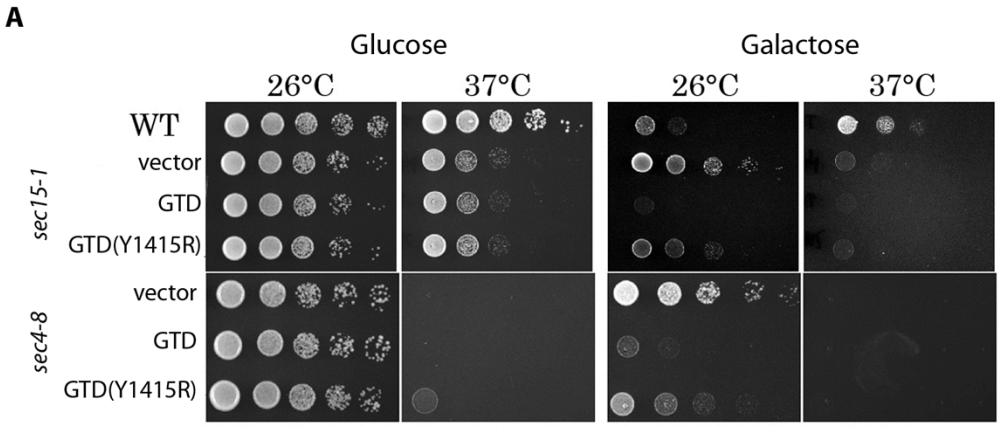
there, was not present, indicating that the clustering is caused by specific proteins that are responding to the excess Myo2p GTD. Just like Ypt11p, markers of the earlier Golgi cisternae, the cis-Golgi t-SNARE Sed5p and the intra-Golgi v-SNARE Gos1p, were unaffected by GST-GTD expression (Figure A1.2H,I). On the other hand, although the late Golgi marker Sec7p was clearly not in blobs, its localization was affected (Figure A1.2G). Some amount of Sec7-3XGFP is always present in buds (arrows in Figure A1.2G), but in cells overexpressing GST-GTD small and medium buds were devoid of Sec7p staining. It also looked as if the Sec7p positive membranes were more numerous, with several smaller profiles (that looked like a haze) surrounding bigger ones. We next looked at GFP-Ypt32, which normally resides in the PI4P-rich TGN and exhibit a polarized localization, and surprisingly it was present in the blobs but PI4P was not (Figure A1.2D,F). This suggests a problem in the maturation from Ypt31p-labeled membranes into Sec4p-secretory vesicles where Ypt31p is not leaving. Moreover, while in wild-type cells the Ypt31p membranes are rich in PI4P, upon induction of the tail, the Ypt31p clusters are mostly devoid of PI4P. This, however, could be a secondary effect of the block in secretion. The Sac1p phosphatase is known, for example, to shuttle between the ER and the Golgi (Faulhammer *et al.*, 2007; Demmel *et al.*, 2008a), and the 4-phosphatase Sjl3p is also known to play a role in TGN to endosome transport (Ha *et al.*, 2003). By accumulating membranes at this interface, these 4-phosphatases can readily gain access to PI4P and hydrolyze it. It would be informative to check the localization of these enzymes under these

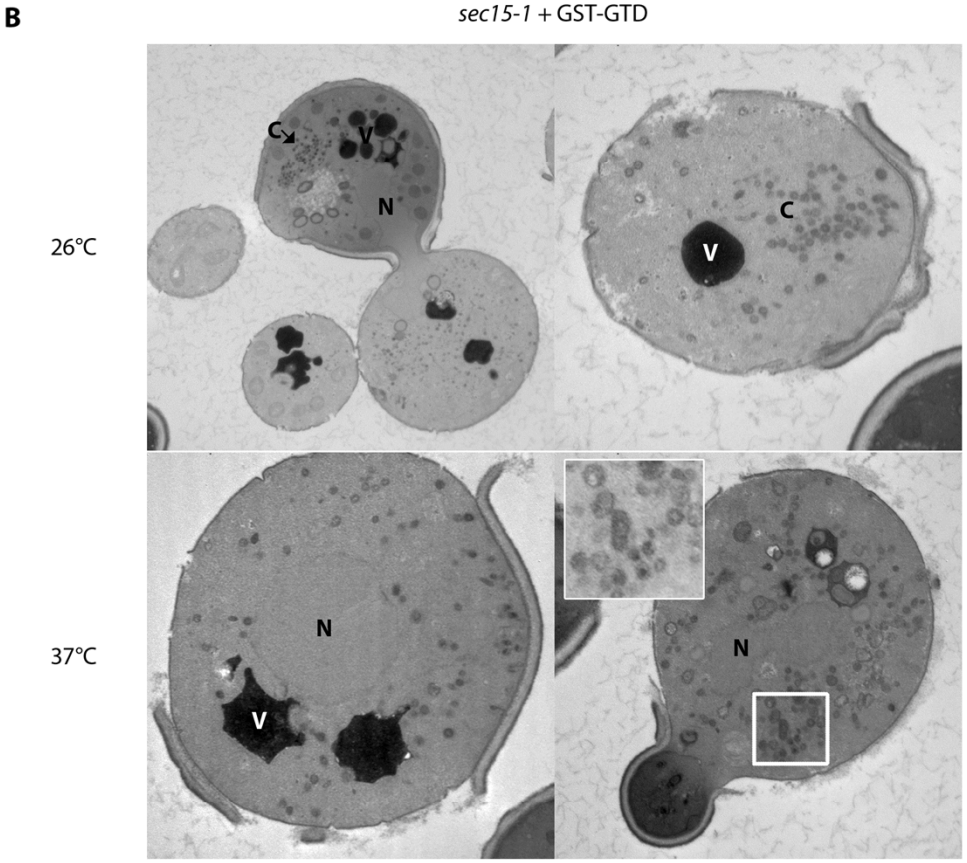
conditions. Finally, I wanted to verify if the post-Golgi v-SNARE Snc2p was present in the clusters as it would confirm that these are really correctly generated post-Golgi vesicles. Indeed DsRed-Snc2 was present in the blobs (Figure A1.2E), confirming that the vesicles that accumulate are post-Golgi and also indicating that retrograde traffic into the Golgi, for generation of secretory vesicles containing Snc2p, is not blocked upon GTD overexpression.

The EM and florescence analyses demonstrate that these blobs are not dilated, continuous membranes but clustered 80 – 100nm vesicles. Since the overexpressed GTD also localized to other membranes, as judge by the fact that the vacuole is fragmented, and yet it did not cluster those membranes, indicates that the GTD is interfering with a normal post-Golgi vesicle specific function. In fact, this affected process could be conserved in evolution as expression of the tail domain of chicken Myo5A (which includes the coiled coil region) also caused a clustering of secretory granules in neuronal PC12 cells and of dense core vesicles in MIN6 pancreatic cells (Rudolf *et al.*, 2003; Varadi *et al.*, 2005). In these cells, induction of the Myo5A tail caused these secretory organelles to lose their cortical localization and inhibited their stimulated fusion with the plasma membrane.

One post-Golgi vesicle specific protein with tethering activity is Sec15p, one of the components of the exocyst tethering complex (TerBush *et al.*, 1996). Likewise, Sec4p is the Rab GTPase associated with post-Golgi vesicles, therefore both Sec15p and Sec4p are candidates to mediate the clustering.

Moreover, the fact that the Y1415R mutation, deficient in Sec4p binding, does not causes clustering of the vesicle strongly implicates Sec4p in the process. I obtained from the Collins lab galactose-responsive strains harboring conditional mutations in both Sec4p and Sec15p. Because originally the strain used to generate those mutants (Novick *et al.*, 1980) was galactose insensitive, I wanted to confirm that the obtained strains indeed are galactose responsive. Both strains can grow on galactose plates at 26°C as well as wild-type, indicating that they can respond to galactose (Figure A1.3A). Moreover, overexpression of the Myo2p GTD, but not the Y1415R mutant, inhibited their growth, further confirming that they can be induced by galactose (Figure A1.3A). Ultrastructural analysis of *sec15-1* strains induced at room temperature showed clustering of vesicles as wild-type cells (Figure A1.3B, top panels) with the additional presence of multiple membranes of unknown origin. Fragmentation of the vacuole could also be seen (Figure A1.3B). However, simultaneous induction of the tail with incubation at 37°C for 3hrs resulted in a less compact accumulation of vesicles. Many cells displayed scattered vesicles throughout the cytoplasm (Figure A1.3B, lower left panel) while others had clusters distributed in a larger area than it would be in wild-type (Figure A1.3B, lower right panel – compare number of vesicles in box with that in A1.3C). There was clearly an effect of Sec15p inactivation, but due to the small number of samples analyzed by EM I could not quantify. I planned to quantify by fluorescence but at the time the only known marker for the blobs was GFP-Sec4, which suppresses the *sec15-1* defect. It is clear that the overexpressed


A
Glucose
Galactose
26°C
37°C
26°C
37°C
WT
vector
GTD
GTD(Y1415R)
sec15-1
vector
GTD
GTD(Y1415R)
sec4-8


B
sec15-1 + GST-GTD
26°C
37°C
C
V
N
C
V
N
V
N

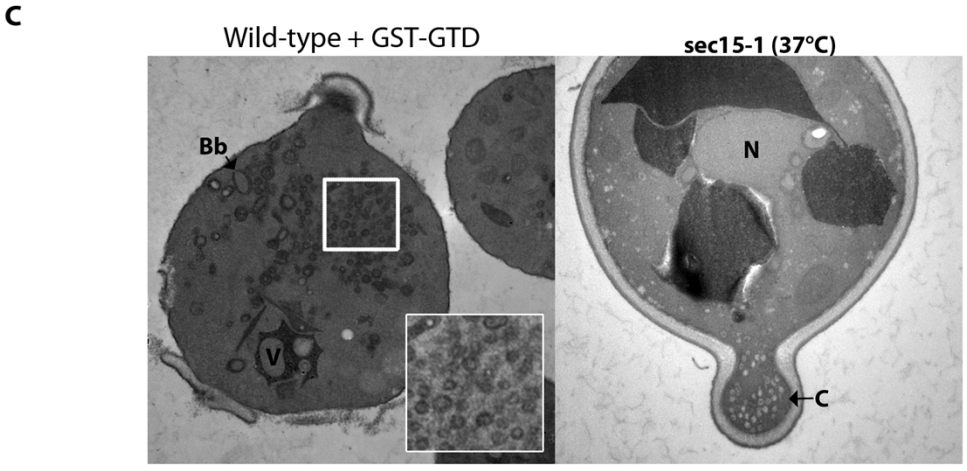

C
Wild-type + GST-GTD
sec15-1 (37°C)
Bb
V
N
C

Figure A1.3 The Sec15p tethering protein plays a role in blob formation. (A) To test if the *sec15-1* and *sec4-8* strains obtained were *GAL* responsive, they were transformed with wild-type or mutant GTD and spotted in glucose (repressive) or galactose (inducive). Both are *GAL* responsive. (B) *Sec15-1* cells were induced for 3 – 4 hours at either room temperature or 37°C and processed for EM. Even at room temperature, the clusters seem more loosely pack and some cells exhibited a random accumulation of vesicles (top panels). At 37°C, most cells had vesicles distributed throughout the cell, although they were some that still had vesicles accumulated together (bottom panels). (C) The phenotype of *sec15-1* cells overexpressing the GTD at 37°C was clearly distinct from that of inactivation of *sec15*, which results in an accumulation of polarized vesicles (right panel), while overexpression of the GTD causes the blobs to accumulate away from sites of growth (left panel).

GTD is acting upstream of the accepted Sec15p function as inactivation of Sec15p results in an accumulation of polarized vesicles (Figure A1.3C, right panel). However, *sec15-1* cells at the restrictive temperature expressing GST-GTD do not exhibit this polarized accumulation, suggesting that if indeed Sec15p is mediating the effect it is doing so by being mislocalized, recruited too early, or both, by Myo2p. These findings confirm that Myo2p transports secretory vesicles but also suggests that it may be involved in the tethering step of vesicles with the plasma membrane. This may be analogous to the capture of secretory granules or synaptic vesicles by myosin-Va in animals at the actin-rich cortex of pancreatic cells or in neurons, respectively (Rudolf *et al.*, 2003; Varadi *et al.*, 2005; Desnos *et al.*, 2007). In these cell types, myosin-Va not only transports these specialized vesicles to the cell cortex but also is involved in docking and mobilization for fusion upon stimuli. Coupling transport with tethering could be another way the cell can control vesicle transport with delivery and fusion.

APPENDIX II

MULTICOPY SUPPRESOR SCREENS FOR MYO2P TAIL ASSOCIATED FACTORS

Rationale and Purpose

I undertook two genetic screens using different types of overexpression libraries aiming at finding candidates that could work as the Myo2p secretory vesicle receptor. Presumably the reason the tail domain of Myo2p is lethal when overexpressed is because it titrates out the receptor. Increasing the levels of the receptor might overcome the lethality. Likewise, if the *myo2* GTD mutants do not associate with secretory membranes due to an impaired binding to one of the components of the receptor, increasing the concentration of that component might overcome the defective binding. I used two types of overexpression libraries to cover a wider range of expression levels as well as genome representation. We have described a cDNA library where the inserts are under the control of the strong *GAL1* promoter (Liu *et al.*, 1992). Under this approach, every clone would express an in-frame protein to high levels, with just over 20,000 clones needed to cover the whole yeast genome. On the other hand, I obtained a multicopy (2-micron) genomic library from the Eric Alani lab. In this library, however, not every insert will encode a protein but, unlike the cDNA library that only express full-length proteins, the genomic library can express fragments or isolated domains. This would require a

greater number of transformants to ensure representation of the whole genome (~100,000) but also increase the probability of obtaining a genetic interaction.

Results and Discussion

In order to identify possible Myo2p interacting proteins, I transformed the cDNA overexpression library or the 2-micron genomic library into BY4741 already containing a plasmid to overexpress GST-GTD. For the cDNA library, I grew 10mL of cells in SRaff media to de-repress the *GAL1* promoter prior to transformation and spread them directly in galactose plates. For the genomic library, the cells used for transformation were grown in SD, allowing for expression and buildup of the library proteins before the GST-GTD was induced. In combination, I screened ~109,000 transformants, of which 44 grew on galactose. After subsequent steps to ensure that the effects were due to the plasmid and not mutations elsewhere in the genome, four different genes were recovered that could efficiently restore viability to cells overexpresing the tail (Figure A2.1A,C). These genes included *LEU2* (6 times, 5 from the genomic library and 1 from cDNA), *REG1* (3 times, from the cDNA library), *ICY1* (2 times, from the cDNA library), and *RTR1* (once, from the genomic library). The multiple hits we got on *LEU2* region were not surprising, as the plasmid overexpressing the GST-GTD is in a *LEU2*-marked plasmid. Expression of Leu2p by the library plasmid would allow the cell to lose the GST-GTD expressing plasmid and still grow in media lacking leucine. All six plasmid contained only *LEU2* as a complete ORF. The second most abundant hit was

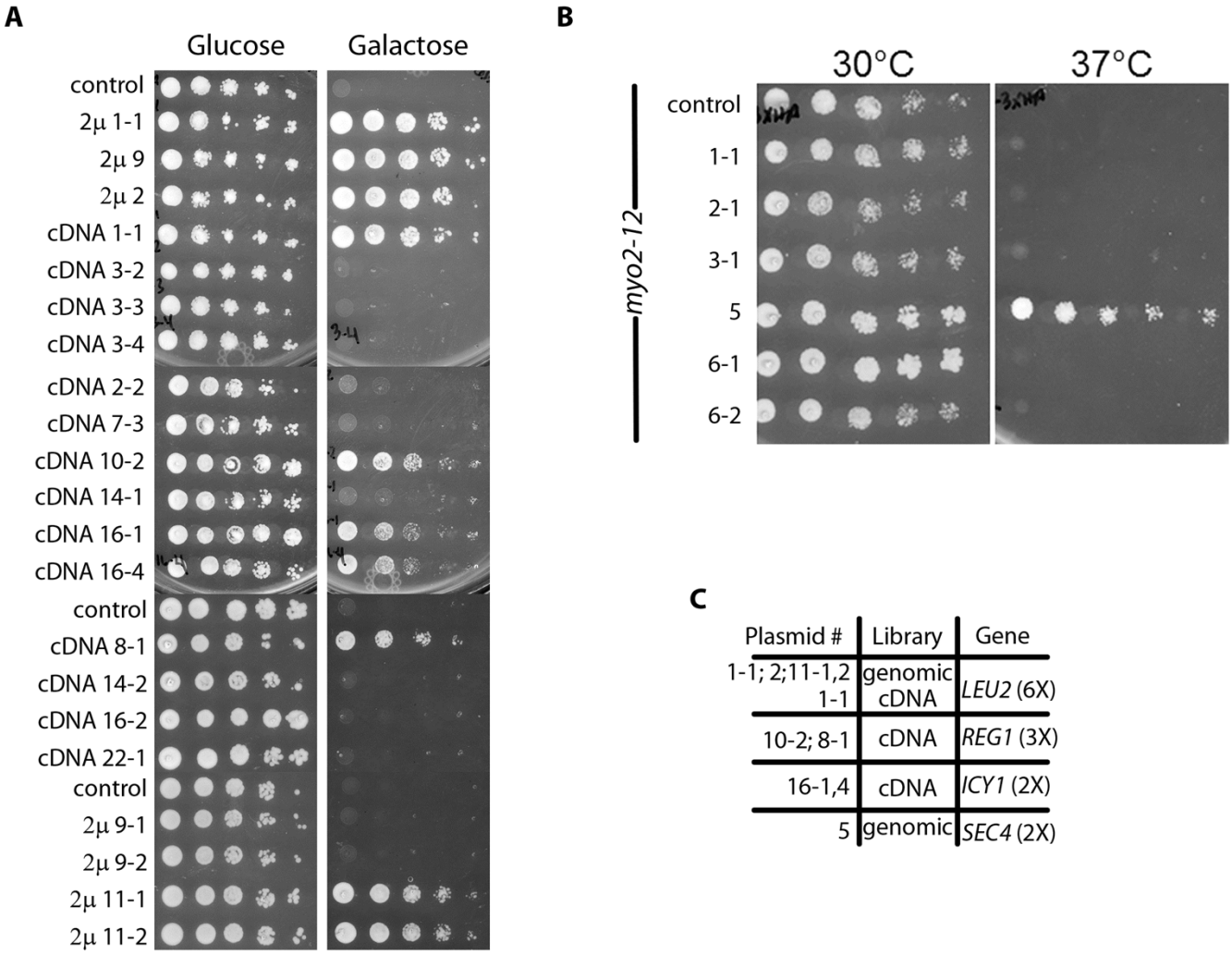


Figure A2.1 Overexpression suppression screens. (A) Candidates found to suppress the lethality of the Myo2p GTD when overexpressed. Numbers starting with 2μ were plasmids recovered from the genomic library, while numbers starting with cDNA were recovered from the cDNA *GAL1* library. (B) Candidates found to rescue the temperature sensitivity of *myo2-12*. Plasmid #5 also rescue *myo2-16*. (C) Identity of the plasmids shown in the previous panels. The number in parenthesis after the gene name shows the number of times the gene was recovered. The first three rows in the table are plasmids that suppress the lethality of the overexpress GTD, the last row containing only *SEC4*, rescues *myo2-12* and *myo2-16* growth at high temperatures.

REG1, which encodes a regulatory subunit for the type 1 protein phosphatase complex (PP1). The catalytic subunit of PP1, Glc7p, associates with a variety of regulatory proteins that both target the protein to its substrate or alter its phosphatase activity. Reg1p targets Glc7p to Snf1p, a serine/threonine kinase that controls transcription of genes involved in sugar catabolism. Dephosphorylation of Snf1p by the Reg1p/Glc7p complex results in its inactivation, causing an upregulation of glucose-related genes and a repression of other sugar genes. High levels of Reg1p would phenocopy a *snf1* null mutant that is unable to grow on sucrose, galactose, maltose, melibiose or other non-fermentable carbon sources, explaining why *REG1* co-overexpression suppressed GST-GTD lethality. One plasmid contained *RTR1* and two more ORFs encoding putative, uncharacterized proteins. Rtr1p is a RNA polymerase CTD phosphatase, which could also regulate the expression of the GST-GTD to levels that are not lethal. The genetic interactions of the other putative genes make them very unlikely to be involved in the suppression. The only interesting hit was *ICY1*, which encodes a protein of unknown function that seems to be involved in polarity. *ICY1*, whose name means "interactor of the cytoskeleton", is nonessential but null mutants have problems undergoing invasive growth and cells have an abnormal elongated morphology (Suzuki *et al.*, 2003). However, the reason it could be nonessential is because it has a homolog, *ICY2*, which is 45% identical. Both proteins lack any recognizable domains and have very few genetic interactions. I have not worked anymore on this hit, but it is a worthwhile candidate to keep in mind for the future,

especially since both homologs have no function described, were identified as targets of yeast's CDK (Ubersax *et al.*, 2003), and seem to be involved in polarized growth (Suzuki *et al.*, 2003). However, the fact that I did not get *PIK1* or *SEC4*, which I subsequently showed can suppress the lethality of GST-GTD (Chapter 2 and 3), suggests that either the library is not complex enough or I did not reach saturation. Additionally, when the cDNA library was made, the cDNA used was separated and purified by size, and although multiple libraries were made based on the different size fractions, the one characterized and that I used contained cDNAs that clustered around 1 – 3 kilobases. *PIK1* and *SEC4* ORFs are at the two extremes in the size range, with *PIK1* over 3kb while *SEC4* is below 1kb. Therefore is conceivable that clones of these genes were absent or largely underrepresented in the library.

The other genetic screen I carried out was using a multicopy genomic library to search for suppressors of the temperature sensitive phenotype of *myo2-12*. Previously, a different library was used and yielded *SMY1* as the only multicopy suppressor of several of the *myo2* GTD mutants (Schott *et al.*, 1999). Here I screened ~40,000 *myo2-12* transformants, obtaining 10 clones that could grow up to 36°C. Digestion analyses of the plasmids indicated 6 different constructs, and after re-transformation into *myo2-12* and *myo2-16*, 2 could rescue both alleles (Figure A2.1B). Sequencing of these plasmids revealed the same region containing the *SEC4* ORF (Figure A2.1C). Like the previous screen, I doubt the library was very complex or that I covered the genome to

saturation, as *SMY1* was previously isolated in a similar screen and I failed to find it here.

There were other screens I planned or even set up but never carried out. As a variation of the first screen described above, I reasoned that overexpression of the mutated GTD from the *myo2* alleles was going to be lethal only at room temperature, as they are non-functional at higher temperatures. This presented an advantage as I could transform the cDNA library into a strain containing, for example, the *myo2-12* GTD under the *GAL1* promoter, and select for transformants at 37°C, where the wild-type GST-GTD construct is lethal but not the one from *myo2-12*. By growing initially at 37°C, the library gene could be expressed and reach steady state levels prior to expression of a functional GST-GTD. Afterwards I replica plate and incubate at room temperature, where the GST-GTD construct should be functional and therefore lethal. However, I tested five constructs made by Daniel Schott containing the GTD (amino acids 1131 to 1574) of *myo2-13*, *myo2-14*, *myo2-16*, *myo2-18*, and *myo2-20*, and none of them was lethal when overexpressed at 30°C or 37°C (not shown). I then cloned a slightly bigger construct containing the whole GTD (amino acids 1087 to 1574) from *myo2-17*, one of the healthier alleles with a restrictive temperature of 36°C (in CUY30) or 37°C (in BY4741/2), and found that upon overexpression, it only partially inhibited growth at 26°C while it had no effect above this temperature. I never cloned longer constructs of the other alleles or tried overexpression at lower temperatures.

Lastly, the finding that the Myo2-PH fusion protein could readily rescue *myo2-12* and *myo2-16* (Chapter 2), or the Rab-binding deficient *myo2* mutants (Chapter 3), presented us with an opportunity to carry out an unbiased genome wide screen for genes involved specifically in the PI4P-dependent Myo2p function. A classic genetic screen that has been successful in the lab is the red/white colony sectoring assay (Lawrence, 2002). Strains defective in the *ade2* gene accumulate a red pigment, turning the colony red. Additional inactivation of the *ade3* gene causes the colony to be white as it blocks the pathway upstream of the Ade2p step and no pigment accumulates. Taking advantage of this genetic trick, *ade2 ade3* cells (white colonies) transformed with an unstable plasmid-borne *ADE3* will form red colonies with white sectors due to growth of cells that have lost the plasmid. Cells that are unable to lose the plasmid do not form white sectors and the colonies are red. I modified plasmid p22, which contains the full-length *ADE3* gene as well as *MYO2* and the defective origin of replication *ars1-22*, to replace the GTD with the wild-type PH^{FAPP} domain (Figure A2.2A). I then transformed this construct (called p23) into strain ABY1719 or the sensitized strain ABY1721 that has *SMY1* deleted. Under permissive conditions, both strains rapidly lose p23 and form mostly completely white rather than sectoring colonies (Figure A2.2B). These results highlights the dominant negative effects this plasmid have since cells that do not receive a copy rapidly overtake the colony, and demonstrate the feasibility of the screen, as it makes identification of red colonies easier. Under conditions were p23 becomes essential (mutations in a gene that renders

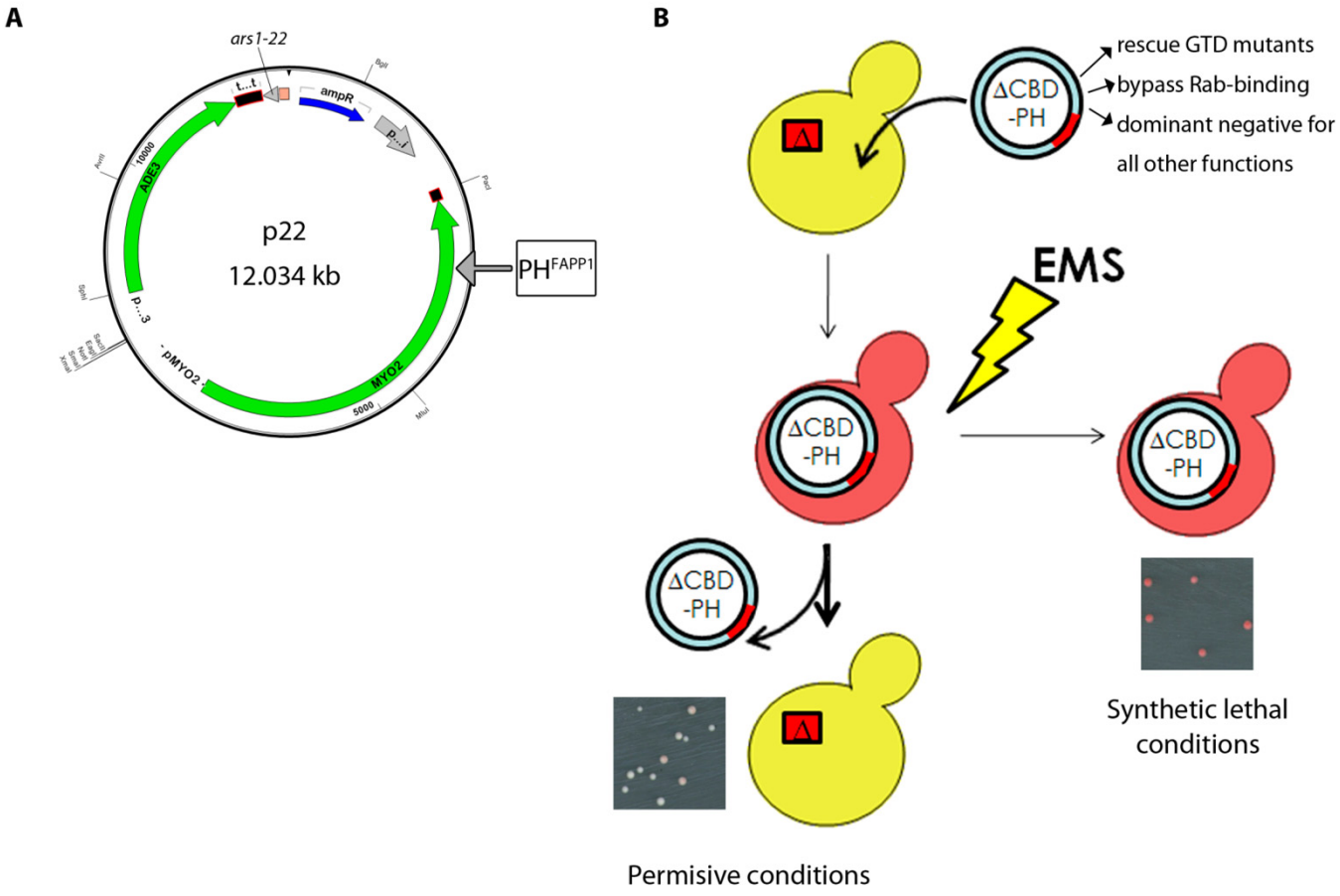


Figure A2.2 Scheme for colony sectoring assay using the Myo2-PH domain fusion. (A) Map of plasmid p22, previously used for a colony sectoring assay, indicating the area replaced by the wild-type PH domain from FAPP1. Because this plasmid is defective in replication (ars1-22), an elevated percentage of cells will fail to inherit it during division. (B) Proof of principle of the screen. Strains deleted for the "red gene" in the chromosome form white colonies. After transformation with p23 (modified p22), the cells form red colonies under conditions where the plasmid is essential (such as synthetic lethal conditions). However, upon plating on non-selective media, the cells rapidly lose the plasmid and form white colonies.

endogenous Myo2p nonfunctional), the cells cannot lose p23 and grow into solid red colonies (Figure A2.2B). I tried this scheme using ABY1719 transformed with p23 and mutagenized with EMS following the conditions used by James Young in the past. However, after the cells were washed and spread on the appropriate plates, I got no colonies growing. I later found out that the EMS was very old and it has become more toxic than mutagenic. Although I never tried it again, I realize that the outcome of the screen can be very dependent on the starting allele of *myo2* present, and using a *MYO2*$^{+}$ strain could be counterproductive as mutations in the *MYO2* gene would make p23 essential, and many of the red colonies would contain *myo2* mutations (that although informative, are not what we are aiming to obtain). Since *SMY1* is essential in all the *myo2* mutants tested, using ABY1721 or a strain already with a conditional mutation in *myo2* would ensure that the recovered mutations are outside *MYO2*. Because p23 cannot complement *myo2*Δ but rescues the PI4P or Rab sensitive *myo2* alleles, the mutants isolated in the proposed screen should be involved in the PI4P/Rab GTPase-dependent Myo2p function, such as the putative bridge connecting Myo2p to PI4P.

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- Nah! Just checking how long this is…