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## Phosphatidylinositol 4-phosphate and Rab inputs collaborate in myosin-V-dependent transport of secretory compartments in yeast

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### Summary

Cell polarity involves transport of specific membranes and macromolecules at the right time to the right place. In budding yeast, secretory vesicles are transported by the myosin-V Myo2p to sites of cell growth. We show that phosphatidylinositol 4-phosphate (PI4P) is present in late secretory compartments and is critical for their association with, and transport by, Myo2p. Further, the Trans-Golgi network (TGN) Rab Ypt31/32p and secretory vesicle Rab Sec4p each bind directly, but distinctly, to Myo2p, and these interactions are also required for secretory compartment transport. Enhancing Myo2p's interaction with PI4P bypasses the requirement for interaction with Ypt31/32p and Sec4p. Together with additional genetic data, the results indicate that Rab proteins and PI4P collaborate in the association of secretory compartments with Myo2p. Thus, we show that a coincidence detection mechanism coordinates inputs from PI4P and the appropriate Rab for secretory compartment transport.

### Introduction

Cell polarity is achieved largely through the selective transport of cargoes by molecular motors moving along microtubules and/or microfilaments (Goode et al., 2000). By selectively localizing macromolecules and organelles to specific areas of the cell, processes such as migration, secretion, growth, and division can occur, which ultimately, are essential for the development of the organism. To achieve such transport, mechanisms must exist for motors to recognize specific organelles for their transport to the correct place at the appropriate time. A potential candidate to provide organelle selective transport includes Rab GTPases that associate with specific membrane compartments (Grosshans et al., 2006; Zerial and McBride, 2001). Indeed, Rab27a is linked through melanophilin to a specific splice form of myosin-Va for melanosome capture at the cell cortex (Wu et al., 2002). Similarly, Rab11 associates with myosin-Vb to facilitate exit from the recycling endosome (Hales et al., 2002; Lapierre et al., 2001). Another family of candidate molecules is the phosphoinositides that are enriched in specific membrane-bound compartments. For example, PI3P is enriched in endosomes where it binds effectors and, together with Rab5, plays a critical role in endocytic trafficking (Wurmser and Emr, 1998; Zoncu et al., 2009);

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PI4,5P<sub>2</sub> is enriched at the plasma membrane and regulates a myriad of processes, from endocytosis to cytoskeletal organization (Audhya et al., 2004; Zoncu et al., 2007); and PI4P is enriched in the Golgi compartment where it is critical for exit of cargo from that organelle (D'Angelo et al., 2008; Szentpetery et al., 2010; Walch-Solimena and Novick, 1999). In these ways PIs can regulate and control membrane trafficking, often together with Rab GTPases (Di Paolo and De Camilli, 2006). In this study we extend this concept by demonstrating a collaborative role for both Rab GTPases and PI4P in the myosin-V based transport of secretory compartments in the budding yeast *Saccharomyces cerevisiae*.

The ability of this yeast to grow is dependent on the selective and polarized transport of secretory vesicles into the bud where cargo enzymes remodel the cell wall for bud expansion. Transport 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). The cables serve as tracks for the transport of secretory vesicles by yeast's essential myosin-V (Schott et al., 1999, 2002), whose heavy chain is encoded by *MYO2* (Johnston et al., 1991). Myo2p also contributes to organelle segregation during the cell cycle by actively transporting vacuole fragments (Hill et al., 1996), peroxisomes (Hoepfner et al., 2001), mitochondria (Altmann et al., 2008), and the TGN (Arai et al., 2008; Rossanese et al., 2001) into the bud, and by binding the ends of cytoplasmic microtubules for nuclear orientation prior to mitosis (Yin et al., 2000). Cargo-specific receptors for Myo2p on almost all of these compartments have been identified, including the vacuole receptor Vac17p (Ishikawa et al., 2003), the peroxisome receptor Inp2p (Fagarasanu et al., 2006), and Kar9p that binds to Bim1p on microtubule ends (Yin et al., 2000). However, no receptor has been described for secretory vesicles, the only essential cargo of Myo2p.

Earlier work has shown that PI4P performs an essential role in the secretory pathway regulating exit of cargo from the TGN (Hama et al., 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. As Myo2p transports secretory vesicles from the Golgi complex we set out to explore whether PI4P might also participate in the recognition mechanism by which Myo2p associates with secretory vesicles. We have described seven conditional mutations in the cargo-binding tail domain of Myo2p that very rapidly uncouple the motor from secretory vesicles at the restrictive temperature (Schott et al., 1999). These mutants are not defective in secretion *per se*, as the cells grow at the restrictive temperature in an isotropic manner, giving rise to large round cells that cannot divide. In this study we show that PI4P plays an additional specific role in the association of Myo2p with both the TGN and secretory vesicles. Additionally, we confirm that Myo2p binds Ypt31/32p, the yeast homologues of Rab11, and show that it also binds directly to Sec4p, the yeast homologue of Rab8, on secretory vesicles. We report that enhanced association of Myo2p with PI4P can bypass the need for an association with Ypt31/32p or Sec4p, suggesting a model where PI4P and a compartment-specific Rab protein contribute to Myo2p binding and polarized transport of membrane-bound compartments of the secretory pathway.

## Results

### Increasing Golgi-localized PI4P specifically rescues defects in Myo2p-dependent polarized secretion

Yeast contains three PI 4-kinases, two of which are essential, the plasma membrane localized Stt4p (Audhya and Emr, 2002; Cutler et al., 1997) and the Golgi localized Pik1p (Flanagan et al., 1993), and the non-essential endosomal/plasma membrane Lsb6p (Han et al., 2002). If PI4P is important for the association of Myo2p with secretory vesicles, we reasoned that some of our mutants might be sensitive to alterations in PI4P levels. We

therefore examined if over-expression of any of the PI 4-kinases might elevate PI4P and suppress the growth defects of any of our conditional Myo2p-tail mutants. Galactose regulated expression of *PIK1*, but not of *STT4* or *LSB6*, partially suppressed the conditional growth defect of one allele, *myo2-12*, without affecting the growth of wild-type cells (Figure 1A and Table 1). Under these conditions, the total level of cellular PI4P rises about 1.5-fold as measured by thin layer chromatographic separation of radioactive PIs (Figure S1A). This suppression requires the kinase activity of *PIK1* as kinase-dead mutants of *PIK1* cannot suppress *myo2-12*, although they are all expressed at similar levels (Figure S1B, S1C). The suppression of *myo2-12* is specific for its defect in secretion, as *myo2-12* is also defective in spindle orientation (Yin et al., 2000) and this is not corrected by *PIK1* over-expression (Figure 1B).

The polarized transport of secretory vesicles can be visualized by the localization of GFP-Sec4p (Schott et al., 2002), the Rab protein associated with secretory vesicles (Goud et al., 1988). In wild-type cells, GFP-Sec4p localizes to sites of cell growth, namely the bud tip in budding cells, and the bud neck in cells just before and after cytokinesis. In *myo2-12* cells shifted to the restrictive temperature for just 5 minutes, the normal polarized distribution of GFP-Sec4p is lost (Figure 1C). However, in *myo2-12* cells over-expressing *PIK1*, GFP-Sec4p remains at least partially polarized after shifting the cells to elevated temperatures, thereby accounting for the ability of these cells to grow (Figures 1C, 1D).

The conditional allele *pik1-139* has reduced levels of PI4P even at the permissive temperature, but not sufficiently low to affect secretion (Sciorra et al., 2005). We examined which of the *myo2* alleles might be affected by lower PI4P levels by combining each of them with the *pik1-139* mutation. Just two of the alleles, *myo2-12* and *myo2-16*, showed synthetic growth defects when combined with *pik1-139* (Figure S1D and Table 1). Thus, 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 reductions in Pik1p's PI4P pool.

Our data, combined with earlier studies on Pik1p, suggest that PI4P plays two important roles in the secretory pathway. First, acute depletion of PI4P blocks secretion (Walch-Solimena and Novick, 1999). Second, a partial reduction of PI4P does not block secretion, but affects Myo2p-dependent membrane transport in an appropriately sensitized background.

### Modulating PI4P turnover at the Golgi affects Myo2p-dependent polarized secretion

Yeast contains three lipid phosphatases that can dephosphorylate PI4P to PI. Two of these, Sjl2p and Sjl3p, function at the plasma membrane (Stefan et al., 2005), and the third, Sac1p, functions at the ER/Golgi (Foti et al., 2001; Tahirovic et al., 2005). However, Sac1p is the main phosphatase since the level of PI4P in *sjl2Δsjl3Δ* double mutants does not increase significantly (Stefan et al., 2002) whereas in *sac1Δ* cells, the level of PI4P rises more than eightfold (Foti et al., 2001; Hughes et al., 2000; Figure S1A). We therefore explored whether this method of acutely elevating PI4P levels would also suppress some of the conditional *myo2*-tail mutants. Although *sac1Δ* slows cell growth, the higher level of PI4P was able to suppress a broader spectrum of *myo2* mutants, namely it suppressed *myo2-12* and *myo2-16* very well, and *myo2-13* and *myo2-18* to a lesser extent (Figure 2A, Table 1). Thus, altering PI4P modestly through *PIK1* manipulation, or acutely by *SAC1* deletion, reveals that the two alleles, *myo2-12* and *myo2-16*, are especially sensitive to alterations in PI4P levels.

As an additional way to examine if decreasing Golgi-localized PI4P affects polarized secretion, the effect of moderate over-expression of *SAC1* on the *myo2* mutants was

assessed. Expression of *SAC1* from a weakened *GAL1* promoter (Mumberg et al., 1994) has little effect on wild-type yeast, but it lowers the restrictive temperature of all the *myo2*-tail mutants (Figure 2C). This effect is dependent on the phosphatase activity as expression of equivalent levels of Sac1p with mutations in the catalytic domain (Figure 2B, S2B) has no effect when over-expressed (Figure 2C) or when added back to the *myo2<sup>ts</sup> sac1Δ* strains (Figure S2C).

There are at least two pools of PI4P in cells, in the Golgi apparatus and at the plasma membrane (Roy and Levine, 2004). To assess the location where the Sac1p phosphatase activity affects the *myo2* tail mutants, we made use of gene fusions that target the Sac1p phosphatase domain to different intracellular compartments to see which would compromise the ability of *sac1Δ* to suppress the *myo2* conditional mutants. The Sac1p phosphatase domain was targeted by the PH domain of PLCδ to the PI-4,5P<sub>2</sub> at the plasma membrane, or by the FYVE domain of EEA1 protein that binds PI-3P on endosomes, or by the PH domain of FAPP1 that binds specifically to the PI4P present at the Golgi (Parrish et al., 2004). Targeting the phosphatase to the Golgi reversed the ability of *sac1Δ* to rescue *myo2-12*, whereas targeting to the plasma membrane or endosome did not (Figure S2D). Again the suppression is dependent on PI4P, as expression of targeted mutant *sac1* versions did not restore the temperature sensitivity of the *myo2* alleles (Figure S2D). These data therefore support the hypothesis that PI4P at the Golgi plays an important role in Myo2p's essential function of transporting membranes for cell growth.

### PI4P is important for coupling secretory membranes to Myo2p

In wild-type cells, there is a strong colocalization between Myo2p and the secretory vesicle marker, Sec4p (Schott et al., 1999). To examine how this colocalization is affected by PI4P depletion, we localized Myo2p-GFP and GFP-Sec4p in the fast-acting *pik1-83* conditional mutant after shifting to the restrictive temperature. At the permissive temperature, Myo2p and Sec4p polarize as well as they do in wild-type cells. Upon shifting to the restrictive temperature of 35°C for just 5 minutes, Myo2p is still polarized, but Sec4p has become somewhat depolarized, and an increased percentage of cells showed this uncoupling at longer timepoints (Figure 3A). Likewise, the polarized localization of GFP-Snc1p, the v-SNARE associated with secretory vesicles, also becomes affected after inactivating Pik1p (Figure S3A, S3B), indicating that the affect on GFP-Sec4p 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 change in the amount of transport vesicle-associated Sec4p before and after shift to the restrictive temperature (P100 fraction, Figure S3C) in the same timeframe that results in a partial depolarization of GFP-Sec4p. Moreover, in the presence of a constitutive active Sec4p (Q79L), GFP-Snc1p is partially depolarized immediately after Pik1p inactivation (Figure S3A, S3B), 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. Thus, PI4P is a critical component necessary for the transport of secretory vesicles by Myo2p.

If secretory vesicles require PI4P for their transport by Myo2p, a marker for PI4P should colocalize with secretory vesicles. The PH domain from yeast Osh2p recognizes all pools of PI4P, including the significant concentrations in the Golgi and at the plasma membrane (Roy and Levine, 2004). We therefore used mCherry-PH<sup>Osh2p</sup> to explore if PI4P colocalizes with GFP-Sec4p. mCherry-PH<sup>Osh2p</sup> localizes to many punctate structures, and live cell imaging revealed many of these showed directed transport to the bud (Figure 3B, 3D and movie S1). The PI4P-rich structures overlap with GFP-Sec4p enrichments at the bud tip and bud neck, which are sites of active growth (Figure 3B, 3D). However, very few instances of colocalization in the mother cells were seen in wild-type cells, possibly because most of the

GFP-Sec4p puncta accumulate at growth sites. In *myo2-12* cells shifted to the restrictive temperature, where the secretory vesicles are not transported and accumulate in the mother cell, most of the PI4P and Sec4p puncta were distinct, but a significant number now colocalized (Figure 3C). We conclude that at least some Sec4p-containing secretory vesicles contain PI4P. This may be an underestimate as it is limited by our ability to image low levels of PI4P.

### Secretory membranes transported by Myo2p are rich in PI4P

Since PI4P is necessary for the transport of secretory vesicles by Myo2p, we explored whether other Golgi membranes enriched in PI4P are also transported by Myo2p. The PH domain from human FAPP1 binds only to a PI4P rich Golgi compartment as it also requires the presence of Arf1p (Levine and Munro, 2002). We therefore used GFP-PH<sup>FAPP1</sup> to image the movements of this compartment *in vivo*. Directed movements towards the bud at 2.54 $\mu$ m/sec ( $\pm$ 0.23) were observed in wild-type cells (Figure 3E, movie S2), a rate close to that found for secretory vesicles (Schott et al., 2002). To determine whether this movement is dependent on Myo2p, we observed it both in a *myo2* mutant that transports organelles at a slower velocity due to a shortened lever-arm (Schott et al., 2002), and in the *myo2* tail mutants at their restrictive temperature. The movement of the GFP-PH<sup>FAPP1</sup> was slower in a cell with the shortened Myo2p lever arm, and transport of GFP-PH<sup>FAPP1</sup> ceased in a *myo2-12* conditional mutant shifted to the restrictive temperature. These data suggest that the PI4P rich compartment is recognized and transported by Myo2p in a manner similar to that used for secretory vesicles.

The compartment marked by GFP-PH<sup>FAPP1</sup> precisely colocalizes with RFP-Sec7p (Figure 3H), a guanine nucleotide exchange factor for Arf1p, located in a late Golgi compartment (Franzoso et al., 1992; Rossanese et al., 2001). Consistent with the GFP-PH<sup>FAPP1</sup> results, the Sec7p compartment also exhibits rapid directed movements towards the bud (Figure 3F, movie S3). The related and redundant Rab proteins Ypt31/32p are enriched in the TGN (Jedd et al., 1997), and overlap extensively with RFP-Sec7p (Figure 3J). Imaging GFP-Ypt31p or Ypt32p in wild-type and *myo2* lever-arm mutants showed that this compartment too is transported by Myo2p (Figure 3G, movie S4), consistent with the recent results of others (Casavola et al., 2008; Lipatova et al., 2008). Moreover, co-imaging of GFP-Ypt31p with the PI4P reporter shows that most of these compartments are also rich in PI4P (Figure 3I), as expected. No directed movement of these compartments was observed in *pik1-83* cells at their restrictive temperature. 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.

### Linking Myo2p directly to PI4P can rescue specific *myo2* alleles

Our data suggest that PI4P is important for the association of secretory compartments with the Myo2p tail, and that some of our *myo2* alleles are especially sensitive to changes in PI4P levels. To test if Myo2p's compromised association with secretory vesicles in these alleles is in part due to defective PI4P binding, we explored the possibility that the Myo2p tail itself binds PI4P. However, bacterially expressed tail constructs failed to show any PI specificity by lipid-protein blot overlays (not shown) or showed any preference for PI4P-containing vesicles by liposome floatation assays (Figure S4B). Another possibility is that Myo2p interacts with PI4P 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. We made a construct in which the C-terminal domain (CTD) of Myo2p was replaced by either the PH<sup>FAPP1</sup> domain (Myo2- $\Delta$ CTD-PH<sup>WT</sup>) or an equivalent construct with mutations (Q16E, R18E) that compromise the ability of the PH domain to bind PI4P (Myo2- $\Delta$ CTD-PH<sup>EE</sup>; Figure 4A, S4A). 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 CTD, and one with the mutated CTD. The Myo2- $\Delta$ CTD-PH<sup>WT</sup> completely suppressed the temperature sensitivity of *myo2-12* and *myo2-16*, the two alleles that are especially sensitive to reduced levels of PI4P (Figure 4B and Table 1). Importantly, it also partially suppressed just those mutants that could also be suppressed by *sac1 $\Delta$* , but not those, like *myo2-14*, unaffected by elevation of PI4P (Figure 4B and Table 1), indicating that it is not a general non-specific mechanism to suppress conditional *myo2* mutants. Thus, the ability of Myo2- $\Delta$ CTD-PH<sup>WT</sup> to rescue a specific class of *myo2* mutants indicates that it cannot supply all the essential functions of the tail. Consistent with this, Myo2- $\Delta$ CTD-PH<sup>WT</sup> was not able to replace the function of the chromosomal *MYO2* gene (Figure 4C). The Myo2- $\Delta$ CTD-PH<sup>EE</sup> construct defective in lipid binding was unable to rescue any alleles albeit being expressed at similar levels as the PH<sup>WT</sup> fusion (Figure 4B, S4C), indicating that the effect is due to an interaction with PI4P. Moreover, expression of this mutated version had a dominant-negative effect causing a decrease in the restrictive temperature of many *myo2* alleles (Figure S4D and Table 1). Consistent with this dominant negative effect, expression of either Myo2- $\Delta$ CTD-PH<sup>WT</sup> or Myo2- $\Delta$ CTD-PH<sup>EE</sup> caused a partial depolarization of GFP-Sec4p in *myo2-12* cells at the permissive temperature (Figure 4E). Nevertheless, at the restrictive temperature, Myo2- $\Delta$ CTD-PH<sup>WT</sup> allowed sufficient polarization of GFP-Sec4p for growth, whereas Myo2- $\Delta$ CTD-PH<sup>EE</sup> did not (Figure 4D and 4E). Thus, while the *myo2-12* allele disrupts association with secretory vesicles at the restrictive temperature, enhancing the connection of Myo2-12p with PI4P now allows for an association with membranes that contain GFP-Sec4p.

The complementation of the essential function of *myo2-12* by the introduction of Myo2- $\Delta$ CTD-PH<sup>WT</sup> did not extend to other functions of Myo2p; indeed it acted as a dominant negative for both spindle orientation and vacuole inheritance (Figure S4E, S4F). Thus, it specifically rescues the transport of compartments of the secretory pathway.

### The Rab proteins Ypt31/32p and Sec4p provide an additional linkage between Myo2p tail and secretory membranes

Two-hybrid studies have shown that Ypt31/32p binds the tail of Myo2p (Casavola et al., 2008; Lipatova et al., 2008), as does another Rab protein, Ypt11p (Itoh et al., 2002), and Smy1p (Benigno et al., 2000). Our two-hybrid studies confirm these interactions and additionally show that Sec4p also binds the Myo2p tail (Figure 5A). Interestingly, the interactions of the Rabs with the tail are not identical, as we get a comparable 2-hybrid signal with Sec4p only when the upstream coiled-coil region of Myo2p is present (Figure 5A). Additionally, by two-hybrid analysis, the interaction of Sec4p and Ypt31p with the Myo2p tail is GTP-dependent (Figure S5A). In vitro, Myo2p exhibited Rab specificity by binding directly to both 6HIS-Ypt31p and 6HIS-Sec4p, but not to 6HIS-Ypt11p, although we were unable to recapitulate the GTP-dependence seen by two-hybrid analysis (Figures 5B, S5B). Residues required for Ypt31/32p to bind the tail, including the Y1415R mutation, were recently reported (Casavola et al., 2008; Lipatova et al., 2008). This mutation also abolishes the interaction with Sec4p and Ypt11p, but not Smy1p (Figure 5A). When introduced into the chromosomal locus, this mutation renders Myo2p non-functional and is therefore lethal (Casavola et al., 2008; Lipatova et al., 2008; Figure 5C). Remarkably, when the Myo2- $\Delta$ CTD-PH<sup>WT</sup> construct, with the PI4P-binding module, is introduced into the *myo2-Y1415R* strain it can now grow, whereas with the lipid binding defective construct, Myo2- $\Delta$ CTD-PH<sup>EE</sup>, it cannot (Figure 5C). 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-Sec4p (Figure 5D). Thus, although Myo2-Y1415R

and Myo2- $\Delta$ CTD-PH<sup>WT</sup> cannot bind to Sec4p, Myo2- $\Delta$ CTD-PH<sup>WT</sup> 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. Even in the cells with no clear enrichment of GFP-Sec4p, the PI4P compartment was polarized which presumably provides sufficient Sec4p for bud growth (Figure 5D, lower cells). Moreover, in these cells colocalization and movement of GFP-Sec4p with PI4P in mother cells is clear (Figure 5E, S3D). This is similar to our result with *myo2-12* cells, where Sec4p is uncoupled from Myo2p at the restrictive temperature, yet can be rescued by expression of Myo2- $\Delta$ CTD-PH<sup>WT</sup> with partial restoration of GFP-Sec4p polarization (Figure 4D). The results indicate that there are two important components for the association of the Myo2p tail with compartments of the secretory pathway. The first is a Rab protein – either Ypt31/32p or Sec4p – and the second is the lipid PI4P, and enhancing the interaction with the latter can compensate for a defective interaction with the former.

### PI4P and Rab GTPases collaborate in the association of Myo2p with secretory compartments

Our results suggest a coincidence detector model for the recruitment of Myo2p to secretory membranes. A prediction of this model is that over-expression of Sec4p might be able to suppress Myo2p tail mutants that are sensitive to low levels of PI4P. Indeed, mild over-expression of Sec4p, driven by the *ADHI* promoter, is sufficient to suppress the *myo2-12* and *myo2-16* alleles (Figure 6A and Table 1), precisely those that are especially sensitive to reductions in PI4P levels. Likewise, over-expression of Ypt31p can rescue *myo2-12* specifically, albeit only partially. These results show that Myo2p requires interactions with both PI4P and Rabs, especially Sec4p, and these are affected in the *myo2-12* and *myo2-16* alleles.

Additional support for this model comes from analysis of over-expression of the Myo2p tail. Earlier studies have shown that Myo2p tail over-expression is lethal because it depolarizes secretion, presumably by binding the secretory vesicle receptor and thereby uncoupling secretory compartments from the motor (Reck-Peterson et al., 1999; Schott et al., 1999). If there are two components to the association between secretory vesicles and Myo2p, it might be possible to overcome this lethality by enhancing one of the associations. We therefore examined whether *PIK1* over-expression (to enhance the PI4P component), or Sec4p over-expression (to enhance the Rab component), can suppress the lethal phenotype conferred by over-expression of the Myo2p tail. Consistent with our model, over-expression of either *PIK1* or *SEC4* was able to partially suppress the lethal phenotype of the Myo2p tail, whereas *STT4*, *YPT31* or *SMY1* were not (Figure 6B). These growth differences were not due to decreased expression of the GST-Myo2p-tail construct as they remained equivalent in all the strains (Figure 6C). Moreover, in cells expressing the Myo2- $\Delta$ CTD-PH<sup>WT</sup> construct, the GST-Myo2p-tail is also unable to completely inhibit growth (Figure 6D). These results indicate that both PI4P and Sec4p are limiting factors for the association of Myo2p with secretory vesicles, and under conditions where one of the components can be bypassed, the GST-Myo2p-tail construct no longer completely interferes with endogenous Myo2p.

### Discussion

Most of the receptor proteins that bridge Myo2p to its organelles have been identified with the exception of the secretory compartments, which are the only essential cargo. 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 (Tang et al., 2003). This is well suited for organelles that just require a one-way trip to be delivered at a specific point in the cell cycle. By contrast, compartments of the secretory pathway are transported to specific locations throughout the cell cycle, so the receptor is

likely to persist and be recycled. Several studies have suggested Rab proteins as compartment-specific components involved in bridging to Myo2p (Casavola et al., 2008; Lipatova et al., 2008; Schott et al., 1999). In this study, we present evidence to support this idea, and importantly extend it to also include PI4P as a factor required to bridge secretory compartments to Myo2p.

In a simplistic model, organelle-specific lipids, such as phosphoinositides, could account for Myo2p's selectivity in cargo selection. We therefore focused on PI4P's role in Myo2p-dependent secretion for two reasons: first, PI4P is greatly enriched at the TGN, where secretory vesicles originate; second, genes encoding enzymes responsible for PI4P regulation show genetic interactions with *myo2-66* and the Rab proteins *sec4-8* (Walch-Solimena and Novick, 1999) and *ypt31Δ* (Sciorra et al., 2005). These observations point to a functional relationship between Myo2p, Pik1p, Ypt31p and Sec4p. In agreement, we found that two *myo2* alleles that are especially sensitive to levels of PI4P can be rescued by increases in PI4P while being highly sensitive to reductions of the lipid. However, PI4P alone cannot account for the recruitment of Myo2p to secretory membranes because we failed to see an in vitro interaction between Myo2p tail and PI4P and, most importantly, PI4P is also present in the plasma membrane. To achieve specificity, a factor must also be present only on the membranes to be transported by Myo2p. Indeed, we found that the exocytic Rabs that identify specific secretory membranes moved by Myo2p bind the Myo2p tail directly, an interaction that is essential as demonstrated by the lethality of the *myo2-Y1415R* mutation. Therefore, our results indicate that PI4P and exocytic Rabs function as a coincidence detector for the polarized transport of secretory compartments by Myo2p.

Which compartments of the secretory pathway are transported by Myo2p in a PI4P-dependent manner? By using two different PI4P reporters, we have shown that all the secretory membranes actively transported by Myo2p, the Sec7p late Golgi, the TGN Ypt31/32p compartment, and the Sec4p-labeled secretory vesicles, are enriched in PI4P. The PI4P compartment labeled by PH<sup>FAPP1</sup> coincides precisely with the Sec7p compartment. PH<sup>Osh2p</sup> illuminates additional smaller tubular and punctate structures that overlap with Ypt31p staining, and Sec4p overlaps strongly with PH<sup>Osh2p</sup> in small budded cells. Thus, all three compartments contain PI4P. A more stringent test would be the ability to co-visualize markers for both PI4P and each secretory compartment during Myo2-directed movement. This is technically challenging due to their small size and rapid rate of movement. When we look at PI4P using PH<sup>Osh2p</sup>, we see small vesicular as well as tubular structures moving in a Myo2p-dependent manner. Many of the tubular structures seem to fuse and divide while they move towards growth sites, and they are usually bigger than the GFP-Sec4p puncta. These vesiculotubular structures are reminiscent of the membranes labeled by Ypt31p. Secretory vesicles, however, with their small size may have amounts of PI4P that are generally 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, as well as in the *myo2-Y1415R* mutant covered by the Myo2-PH fusion, we can see an increased amount of colocalization of Sec4p with the PI4P reporter in the mother cell. 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. Supporting this hypothesis, the Novick lab recently suggested that the vesicle's PI4P levels have to decrease in order to recruit Sec15p to its surface (Mizuno-Yamasaki et al., 2010). It is not clear where, and by what phosphatase (or kinase), this happens, but vesicle PI4P hydrolysis (or conversion) could be one way membrane transport by Myo2p is integrated with exocytosis. Nevertheless, Sec7p, Ypt31p, and Sec4p directed movements are all lost in the *pik1-83*



mutant at the restrictive temperature, indicating that PI4P throughout the secretory pathway is needed for Myo2p-dependent movement.

Although secretory vesicles do not need to deliver PI4P to the plasma membrane, since the PI 4-kinase Stt4p synthesizes it there (Audhya and Emr, 2002), the strong accumulation of PI4P seen in small buds where secretory vesicles accumulate suggests that PI4P is present during transport of secretory vesicles to their destination. Further, under special conditions of growth, the PI 4-kinase Stt4p is not essential (Cutler et al., 1997), and, although the cells grow very slowly, arriving secretory vesicles can supply a sufficient trickle of PI4P for plasma membrane PI(4,5)P<sub>2</sub> synthesis and cell viability, again supporting the concept that secretory vesicles must contain PI4P. This last statement is clearly shown in a recent paper where they assess the role of PI4P in trafficking (Szentpetery et al., 2010). Lastly, in mutants where Myo2p cannot associate directly with GFP-Sec4p-labeled membranes, the Myo2- $\Delta$ CTD-PH<sup>FAPP1</sup> construct can still polarize these membranes, indicating that PI4P and Sec4p are present, to some extent, in the same membranes.

Our data therefore suggest the following model. PI4P is a general factor necessary for secretory compartments to associate with and be transported by Myo2p. Since a Myo2p-tail mutation that eliminates interactions with the exocytic Rab proteins is lethal, an additional requirement is for a Rab protein, potentially Ypt11p, Ypt31/32p and Sec4p. Ypt11p is known to contribute to the polarized transport of the Sec7p compartment (Arai et al., 2008), Ypt31/32p could contribute to the transport of the TGN, and Sec4p to the transport of secretory vesicles. The idea that different Rabs can use the same molecular motor is consistent with the fact that Myo2p recognition of Ypt31/32p and Sec4p is not entirely equal. Although they both need determinants in the globular tail, Sec4p binding also requires the coiled coil region of Myo2p. This is not unprecedented, as Myosin Vb shows similar structural requirements for Rab11a and Rab8a, the homologues of Ypt31p and Sec4p, respectively (Roland et al., 2007). The requirement for both a Rab protein as well as PI4P may serve as a coincidence detector to allow Myo2p to distinguish between the plasma membrane and secretory compartment pools of PI4P. At the same time, since the structural requirements for Myo2p binding of the Rabs are distinct, this might still allow for compartment-specific regulation.

How does Myo2p associate with PI4P? Since we have not found a direct interaction in vitro between the Myo2p tail and PI4P, we hypothesize that the binding to PI4P is indirect through an adaptor protein. We reasoned that if the *myo2* alleles sensitive to low PI4P were partially defective in binding the PI4P adaptor protein, we could bypass 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- $\Delta$ CTD-PH<sup>FAPP1</sup> 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. One of these necessary components, as described above, is the compartment-specific Rab protein. This requirement can also be bypassed by the Myo2- $\Delta$ CTD-PH<sup>FAPP1</sup> construct, indicating that Rabs are not the only other component of the receptor. Furthermore, this result implies a model whereby PI4P and Rabs collaborate as part of the receptor for secretory compartments. It would be very interesting to test if enhancing Rab binding could bypass the need for PI4P, the same way we show the opposite is true (Figure 4 and 5). Although this experiment would be extremely difficult since PI4P has other essential functions, we also showed that you can selectively rescue the PI4P sensitive *myo2* alleles by over-expressing the Rabs *YPT32* or *SEC4*. This result suggests that high levels of Rab proteins, by enhancing

the Myo2p-Rab interaction, provide a remedial effect to strains sensitive to low levels of PI4P. This interpretation is also supported by our finding that the lethality associated with Myo2p tail over-expression can be suppressed by over-expression of either *PIK1* or *SEC4*. However, in these experiments, the lethality of the GST-Myo2p-tail over-expression is only partially suppressed, suggesting that another component may be titrated out by the over-expressed fusion protein. Our model therefore proposes at least three components for the Myo2p secretory compartment receptor: PI4P, a compartment-specific Rab, and an unidentified third component that probably binds PI4P and/or the Rab.

How general might the requirement for phosphoinositides be in tethering cargo to a molecular motor? In plants, the delivery of secretory vesicles necessary for pollen tube and root tip growth depend on PI4P, synthesized and regulated by the *Pik1p* and *Sac1p* homologs *PI4K $\beta$ 1* and *SAC7* (Preuss et al., 2006; Thole et al., 2008). Moreover, *PI4K $\beta$ 1* is an effector of RabA, the homologue of yeast *Ypt31p*, an interaction that is conserved in mammals between *PI4K $\beta$*  and *Rab11* (de Graaf et al., 2004). Microtubule based kinesins also function with phosphoinositides. The nematode kinesin *Unc104* has a PH domain that binds *PI-4,5P<sub>2</sub>* directly to transport synaptic vesicles (Klopfenstein et al., 2002), and special vesicles rich in *PIP<sub>3</sub>* are delivered to the tips of neurites by the kinesin *GAKIN* linked through a *PIP<sub>3</sub>*-interacting protein (Horiguchi et al., 2006). As far as we are aware, Myo2p is the first myosin shown to require a phosphoinositide to bind its cargo.

We propose a model of the late secretory pathway (Figure 6E) in which PI4P is present from the donor compartment until the target membrane is reached, but the Rab protein changes along the way together with the morphology of the carrier. We speculate that PI4P may be an ancient conserved component of this pathway. As cells evolved and different compartmentalization was required, Rabs diversified to subdivide the already segregated PI4P marked membranes. Thus PI4P would be the identification marker for secretory compartments in general, whereas the Rab protein would identify the specific compartment. The whole process can then be terminated at the plasma membrane with the degradation or conversion of PI4P into PI or *PI-4,5P<sub>2</sub>*, respectively. The simple view where Myo2p tethers secretory vesicles through Rab GTPases can now be expanded to include another component, PI4P, and an additional unidentified adapter protein that may bind PI4P.

## Experimental Procedures

### Yeast Strains and molecular biology techniques

Strains used in this study are listed in Table S1. Standard media and techniques for growing and transform yeast were used (Sherman, 1991). For more details on strain and plasmid construction see Supplemental Experimental Procedures.

### 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). Movie S2 and figure 3D were taken using an Axiovert conventional fluorescence microscope. Images were further analyzed and adjusted using Slidebook 5. For panel assembly, the images were cropped and further adjusted in Photoshop (Adobe) to give the clearest presentation of the results. Quantification of GFP markers was done as described in figure legends.

## Analysis of PI4P-Myo2p tail interaction

Binding of recombinant Myo2-tail to PI4P was tested by phosphoinositide-protein dot blots (PIP strips) and by liposome floatation assays. See Supplemental Experimental Procedures for details.

## In vitro binding assays

Purification of GST-Myo2p-tail and 6xHis-Rab GTPases recombinant proteins was done as described (Legesse-Miller et al., 2006; Du and Novick, 2001). GST-PH<sup>FAPP</sup> and GST-Sec2p were purified following the standard pGEX fusion system instructions, except that for GST-Sec2p the induction was done at 20°C with 0.4mM IPTG overnight. The Rab GTPases were preloaded with GDP or GTP- $\gamma$ S by resuspending 125nM of the protein in binding buffer (25mM Tris, pH 7.4, 80mM NaCl, 1mM MgCl<sub>2</sub>, 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<sub>2</sub> to 5mM final. The GST-Myo2p-tail bound to the beads was added (~1mM, usually 10-20 $\mu$ L of slurry) and the total volume adjusted to 600 $\mu$ L. Reactions were incubated for 1hr at 4°C with rotation. Beads were washed four times with wash buffer (binding buffer except 5mM MgCl<sub>2</sub>, 10% glycerol, 0.1% triton X-100) and then resuspended in 2 $\times$  protein sample buffer. Bound Rabs were separated by SDS-PAGE and analyzed by western blotting with anti-6xHIS antibodies (Sigma).

## Yeast two-hybrid analyses

Yeast strain AH109 was co-transformed with both activation and DNA binding domain fusion proteins and the presence of both plasmids selected in media lacking leucine and tryptophan (DD) while an interaction was tested by growing in media additionally lacking histidine (TD). The Myo2p-tail constructs were done in pGADT7, the Rab proteins in pBridge, and the Smy1p construct in pAS2-1.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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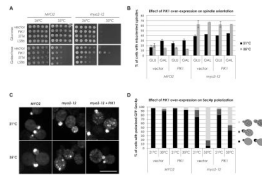
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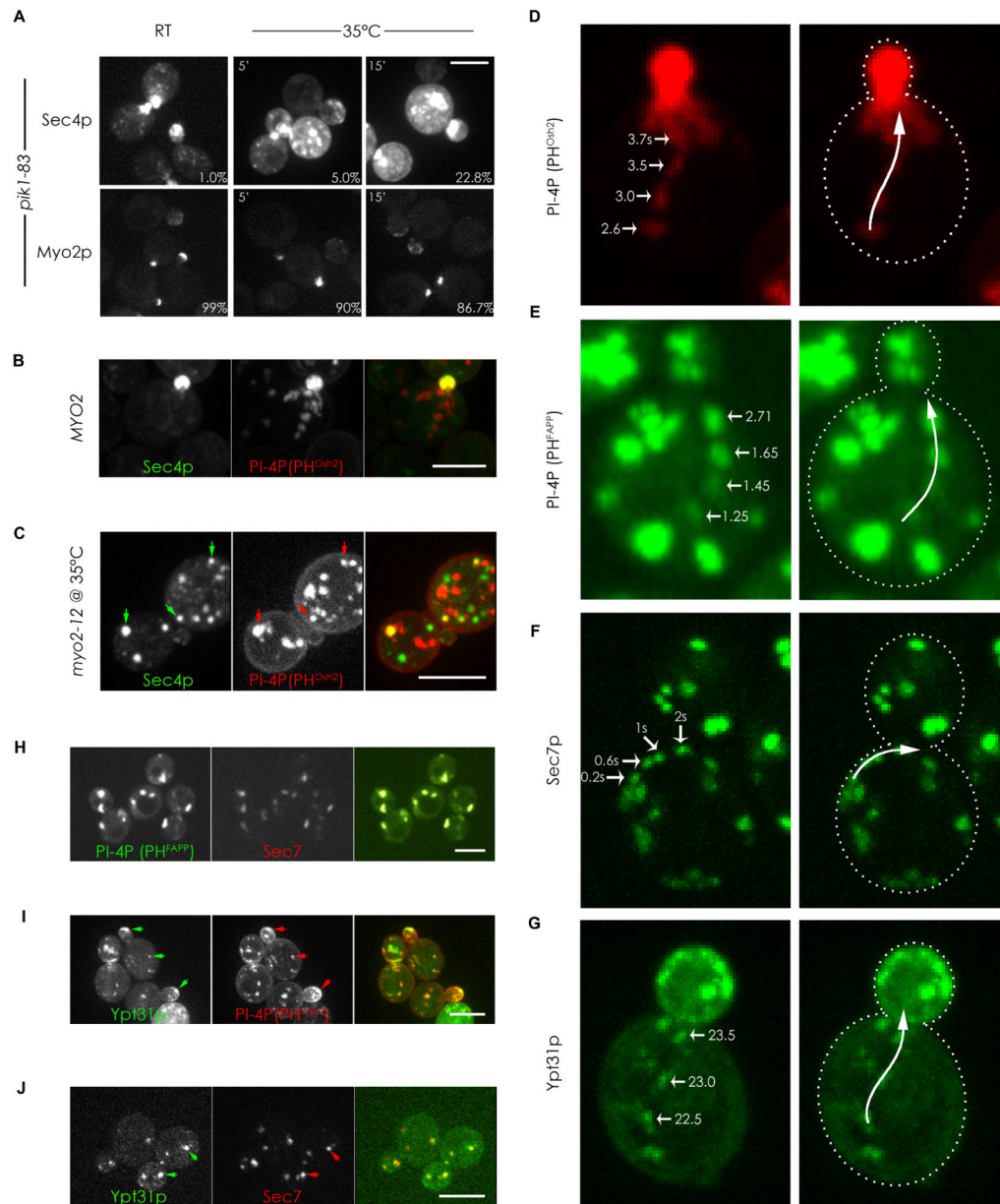


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**Figure 1. Modestly increasing Golgi PI4P can rescue a specific *myo2* conditional tail mutant** (A) Wild-type *MYO2* and the *myo2-12* cells expressing the indicated genes from the *GAL1* promoter after growth on glucose (promoter off) or galactose (promoter on) at the indicated temperatures. See also Figure S1. (B) The percentage of cells with misoriented spindles (scored as in Yin et al., 2000) was determined before and after a 10-min shift to 35°C under conditions that repress (GLU) or over-express (GAL) *PIK1*. Error bars represent standard deviation of the sample. (C) GFP-Sec4p localization in the indicated strains at either room temperature or after 30-min incubation at 35°C. (D) Quantitation of GFP-Sec4p localization of cells in (C). >100 small/medium budded cells were examined as wild-type cells polarize GFP-Sec4p to the bud tip. Since there is punctate GFP-Sec4p staining in the mothers of *myo2-12* cells even at the permissive temperature, a separate category was used to score these (dark gray). Bar is 5µm.



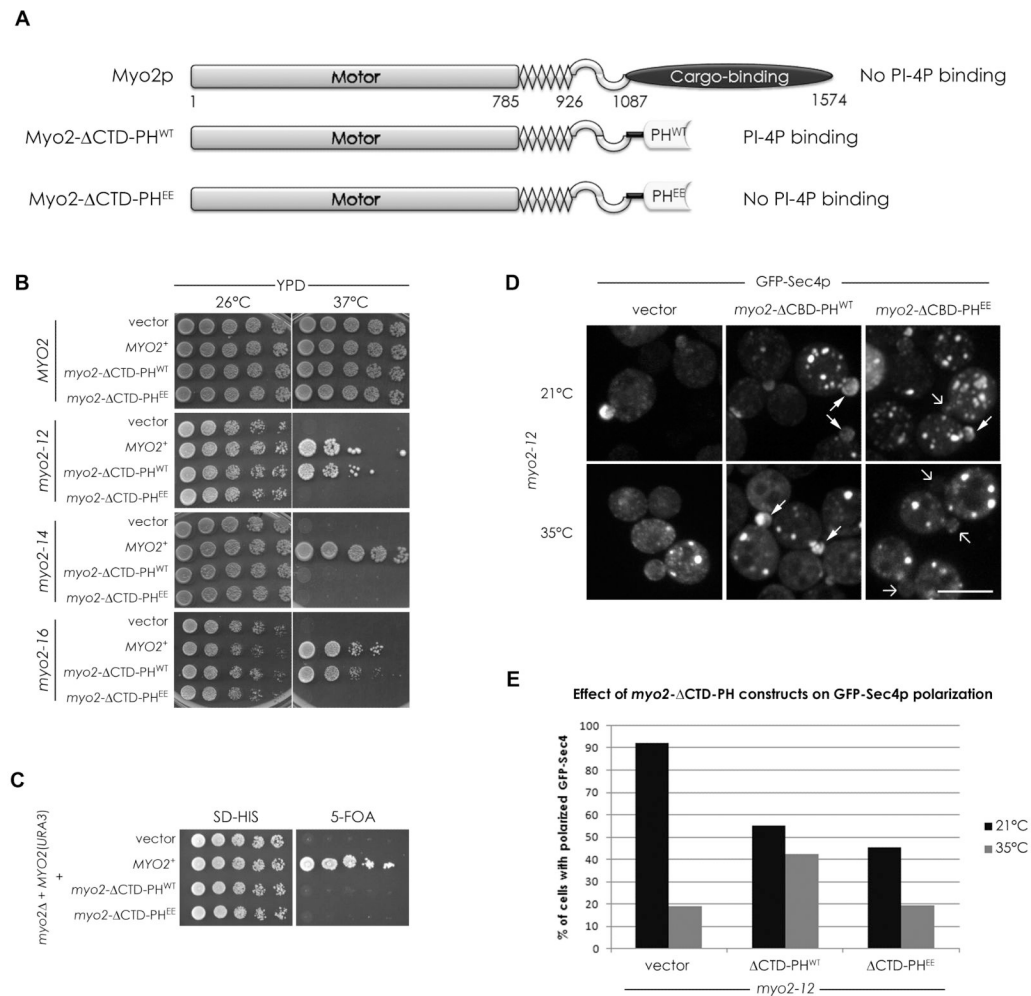


**Figure 3. PI4P is present in compartments of the late secretory pathway and is required for their transport**

(A) *ptk1-83* cells expressing either GFP-Sec4p or Myo2p-GFP were fixed and examined at different times after shifting to 35°C and the percentage of small/medium budded cells with delocalized GFP-Sec4p and polarized Myo2p-3XGFP was determined (percentages in lower right corners). (B) Small budded wild type cell expressing GFP-Sec4p and mCherry- $\text{PH}^{\text{Osh2p}}$ . (C) *myo2-12* cell expressing GFP-Sec4p and mCherry- $\text{PH}^{\text{Osh2p}}$  after 15-min shift to the restrictive temperature. Arrows indicate examples of colocalization. (D and E) Frames from Supplementary movie 1 (D) and 2 (E) showing vesicular-like structures labeled by the indicated PI4P reporters moving rapidly towards the bud. Times in the movies are indicated. (F) Frames from Supplementary movie 3 showing the directed movement of GFP-Sec7p towards the bud. (G) Frames from Supplementary movie 4 showing the directed movement of the GFP-Ypt31p towards the bud. (H, I, and J) Cells co-expressing a PI4P reporter and

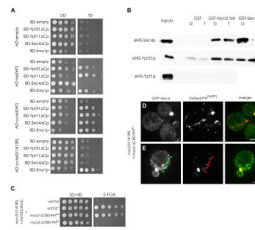
different secretory markers showing colocalization (arrows) of PI4P with Sec7p (H), with Ypt31p (I), or between Sec7p and Ypt31p (J). See also Figure S3. Bars represent 5 $\mu$ m.





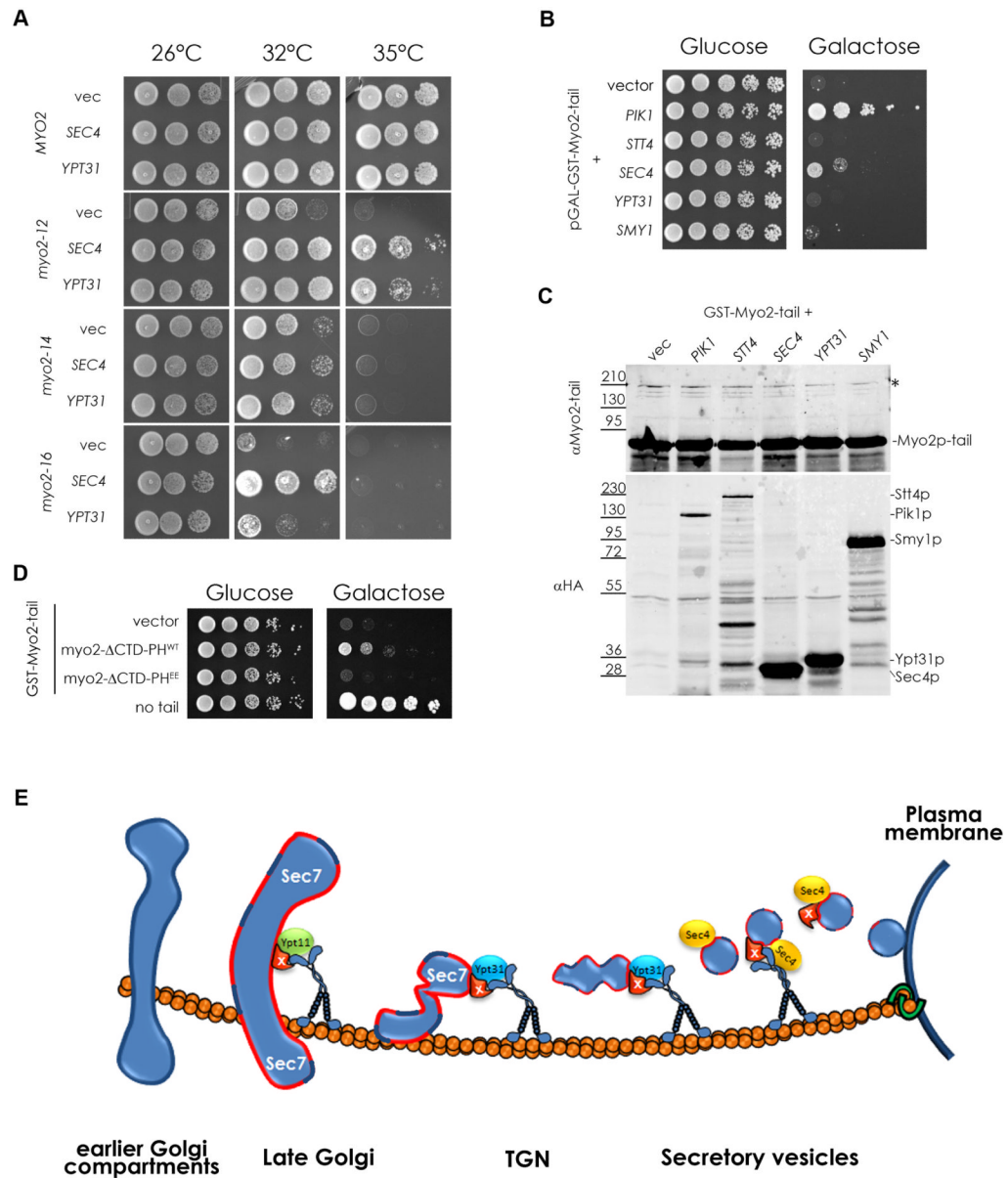
**Figure 4. Linking Myo2p directly to PI4P can contribute to its essential function**

(A) Schematic representation of the Myo2p constructs used. The C-terminal globular tail domain was replaced with either the PH domain from FAPP1 (PH<sup>WT</sup>) or a mutant version (PH<sup>EE</sup>) that does not recognize PI4P. (B) Growth of *myo2* mutants as indicated expressing either an empty vector, wild-type Myo2p, Myo2-ΔCTD-PH<sup>WT</sup>, or Myo2-ΔCTD-PH<sup>EE</sup>. (C) A strain deleted for genomic *MYO2*, covered by a *URA3*-based plasmid, and transformed with the constructs in (A), was spotted on media with or without 5-FOA, to select against the *URA3* plasmid. (D) Localization of GFP-Sec4p in *myo2-12* cells expressing the Myo2-PH fusion proteins. Arrows point to small buds with (filled arrowheads) or without (arrows) GFP-Sec4p enrichments. Quantitation of GFP-Sec4p polarization in small/medium budded cells in these strains is shown in (E). See also Figure S4. Bar represent 5μm.



**Figure 5. The essential interaction of Rab GTPases with the Myo2p tail can be by-passed by enhancing PI4P binding**

(A) Yeast 2-hybrid interactions with the Myo2p-tail (tail), or coiled-coil region and tail (cctail), or cctail with the Y1415R mutation, fused to the activating domain (AD) and the Rab GTPases and Smy1p fused to the DNA binding domain (BD). All Rab constructs were mutated in their C-terminal CXC motif (eg. Ypt31 $\Delta$ Cp) to eliminate prenylation. Growth on the control (DD) and test (TD) plates is shown. (B) GST, GST fused to Myo2p-cctail, or GST-Sec2p, was used to retain 6xHis-Rab GTPases in the presence of GDP (D) or GTP- $\gamma$ S (T). Binding assays were carried out as described in Experimental Procedures and visualized with antibodies to 6xHis. (C) A strain with the genomic *myo2* Y1415R mutation, covered by a *URA3*-based plasmid, was transformed with the constructs as indicated and spotted on media with or without 5-FOA, to select against the *URA3* plasmid. (D and E) Images of GFP-Sec4p and DsRed-PH<sup>FAPP1</sup> localization in the Rab-binding deficient *myo2*-Y1415R mutant expressing the Myo2- $\Delta$ CTD-PH<sup>WT</sup> construct. Arrows indicates examples of colocalization in the mother cell. See also Figure S5. Bar represent 5 $\mu$ m.



**Figure 6. Both PI4P and Rab GTPases are limiting factors in the association of Myo2p with secretory vesicles**

(A) Effect of *SEC4* and *YPT31* over-expression (from the strong *ADHI* promoter) on the growth of *myo2* alleles, either those sensitive to PI4P levels (*myo2-12* and *myo2-16*), or not sensitive (*myo2-14*). (B) Wild-type cells co-transformed to over-express on galactose media the GST-Myo2p-tail and different Rab proteins (*Sec4p* or *Ypt31p*), PI 4-kinases (*Pik1p* and *Stt4p*) or *Smy1p*. (C) Western blots of strains in (B) after induction with galactose for 3 hours. Same number of OD<sub>600</sub> units were loaded in a 10% protein gel, resolved and blotted with anti-Myo2p-tail antibodies and anti-HA antibodies. Asterisk marks endogenous Myo2p. (D) Wild-type cells expressing the Myo2-PH fusions and transformed to over-express the GST-Myo2p-tail. The no tail control has two empty plasmids. (E) Working model for secretory compartment association with and transport by Myo2p along polarized actin cables. The membrane lipid PI4P (red) is required for association of all transported compartments with Myo2p through an unknown factor (X). A specific Rab protein, either

Ypt11p (late Golgi), Ypt31/32 (TGN) or secretory vesicles (Sec4p), that associates with morphologically distinct compartments is also required. The association of Ypt11p with Myo2p at the late Golgi is based on the work of others (Arai et al., 2008). See text for discussion.

Table 1

Summary of genetic interactions with *myo2* tail mutants

Allele	Restrictive temperature <sup>1</sup>	Over-expression of <i>PIK1</i> <sup>2</sup>	Combined with <i>pik1-139</i>	Combined with <i>sac1Δ</i>	Expression of <i>myo2ΔCTD-PH<sup>WT3</sup></i>	Expression of <i>myo2ΔCTD-PH<sup>EE3</sup></i>	Over-expression of <i>SEC4</i> <sup>4</sup>	Over-expression of <i>YPT31</i> <sup>4</sup>
<i>MYO2</i> <sup>+</sup>	>37°C	NE	36°C	NE	NE	NE	NE	NE
<i>myo2-12</i>	33°C	35°C	30°C	37°C	>37°C	30°C	36°C	35°C
<i>myo2-13</i>	35°C	NE	NE	>37°C	37°C	33°C	NE	NE
<i>myo2-14</i>	35°C	NE	NE	NE	NE	33°C	NE	NE
<i>myo2-16</i>	32°C	NE	30°C	36°C	>37°C	30°C	35°C	NE
<i>myo2-17</i>	37°C	NE	NE	NE	NE	NE	NE	NE
<i>myo2-18</i>	36°C	NE	NE	37°C	37°C	NE	NE	NE
<i>myo2-20</i>	37°C	NE	NE	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

<sup>1</sup> Restrictive temperature on synthetic complete medium.

<sup>2</sup> *PIK1* was expressed behind the *GALI* promoter.

<sup>3</sup> The fusion constructs were expressed from the *MYO2* promoter.

<sup>4</sup> *SEC4* and *YPT31* were expressed on a low copy plasmid from the *ADHI* promoter.