Clathrin-mediated endocytosis is a highly complex process in which the eukaryotic cell deforms its plasma membrane and pinches off a small vesicle ([Kaksonen and Roux, 2018](https://elifesciences.org/articles/44215" \l "bib44); [Kirchhausen et al., 2014](https://elifesciences.org/articles/44215" \l "bib45); [McMahon and Boucrot, 2011](https://elifesciences.org/articles/44215#bib56)). Endocytosis requires the dynamic recruitment and disassembly of dozens of different proteins in a highly stereotypical sequence to coordinate cargo recruitment, membrane reshaping, scission and vesicle uncoating ([Kaksonen et al., 2005](https://elifesciences.org/articles/44215" \l "bib43)). In budding yeast (*Saccharomyces cerevisiae*) and fission yeast (*Schizosaccharomyces pombe*), endocytic membrane bending is strictly dependent on the assembly of a dynamic, branched actin network ([Gachet and Hyams, 2005](https://elifesciences.org/articles/44215" \l "bib25); [Kaksonen et al., 2003](https://elifesciences.org/articles/44215" \l "bib42)), which is thought to provide the force to bend the membrane against the high turgor pressure of yeasts. In metazoan cells, actin polymerization is required for clathrin-mediated endocytosis under certain conditions where the membrane resists bending, for example in case of high membrane tension or strong substrate adhesion ([Batchelder and Yarar, 2010](https://elifesciences.org/articles/44215#bib8); [Boulant et al., 2011](https://elifesciences.org/articles/44215" \l "bib12)).

The endocytic actin structure in yeast consists of thousands of individual protein molecules, which transiently co-assemble to form a highly dynamic, branched actin network ([Goode et al., 2015](https://elifesciences.org/articles/44215#bib31)). Actin filaments are nucleated through activation of the Arp2/3-complex, which induces the formation of new daughter filaments branching at a 70° angle from a mother filament ([Amann and Pollard, 2001a](https://elifesciences.org/articles/44215#bib2); [Amann and Pollard, 2001b](https://elifesciences.org/articles/44215#bib3)). The main Arp2/3-activating nucleation promoting factors (NPFs) of budding yeast, Las17 (homolog of Wiskott-Aldrich syndrome protein (WASp)) and the type-I myosins Myo3 and Myo5, are recruited to a ring-shaped area on the plasma membrane surrounding the forming invagination, resulting in an inward flowing actin network ([Idrissi et al., 2008](https://elifesciences.org/articles/44215" \l "bib38); [Mund et al., 2018](https://elifesciences.org/articles/44215" \l "bib61); [Picco et al., 2015](https://elifesciences.org/articles/44215" \l "bib64)). This actin network is connected to the tip of the invaginating membrane by the epsins and Sla2, proteins that bind both lipids and actin filaments ([Skruzny et al., 2015](https://elifesciences.org/articles/44215" \l "bib69); [Skruzny et al., 2012](https://elifesciences.org/articles/44215" \l "bib68)). Though it is generally accepted that a large fraction of the force that is required to bend the membrane is provided by actin polymerization via a Brownian ratchet-type mechanism ([Mogilner and Oster, 1996](https://elifesciences.org/articles/44215#bib58); [Peskin et al., 1993](https://elifesciences.org/articles/44215" \l "bib63)), several modeling studies suggest that this force alone is not sufficient to counter the high turgor pressure of the cell ([Carlsson and Bayly, 2014](https://elifesciences.org/articles/44215#bib15); [Dmitrieff and Nédélec, 2015](https://elifesciences.org/articles/44215" \l "bib20)). Additional factors that may contribute to membrane bending include myosin motor activity, membrane curvature generation or stabilization by proteins that bind to the membrane, or local decreases in turgor pressure ([Carlsson, 2018](https://elifesciences.org/articles/44215#bib14)).

Type-I myosins play an important but enigmatic role in endocytosis. Type-I myosins are monomeric, actin-based motors that use the energy from ATP hydrolysis to power work along actin filaments. Besides an N-terminal motor domain, comprising the ATPase site and an actin-binding pocket, they contain a neck with one or more binding sites for myosin light chains, and a tail which can interact with lipids and other proteins ([Masters et al., 2017](https://elifesciences.org/articles/44215#bib53)). Generally type-I myosins are involved in linking actin filaments to membranes in diverse cellular contexts such as cell migration, cellular and organellar morphology and maintenance of cortical and plasma membrane tension ([Almeida et al., 2011](https://elifesciences.org/articles/44215#bib1); [Dai et al., 1999](https://elifesciences.org/articles/44215#bib17); [Diz-Muñoz et al., 2010](https://elifesciences.org/articles/44215" \l "bib19); [Hartman et al., 2011](https://elifesciences.org/articles/44215#bib35); [McConnell and Tyska, 2010](https://elifesciences.org/articles/44215#bib55); [Nambiar et al., 2009](https://elifesciences.org/articles/44215#bib62); [Sokac et al., 2006](https://elifesciences.org/articles/44215" \l "bib70)), but the molecular details of these interactions remain scarce. For several cell types it has been shown that myosin-I molecules are recruited to endocytic sites concomitantly with actin; examples include the two myosin-I paralogs of budding yeast named Myo3 and Myo5 ([Jonsdottir and Li, 2004](https://elifesciences.org/articles/44215" \l "bib41); [Sun et al., 2006](https://elifesciences.org/articles/44215#bib71)), the fission yeast type-I myosin Myo1 ([Arasada et al., 2018](https://elifesciences.org/articles/44215" \l "bib5); [Basu et al., 2014](https://elifesciences.org/articles/44215" \l "bib7); [Sirotkin et al., 2005](https://elifesciences.org/articles/44215" \l "bib67)), and Myo1E in metazoan cells ([Cheng et al., 2012](https://elifesciences.org/articles/44215#bib16); [Krendel et al., 2007](https://elifesciences.org/articles/44215" \l "bib47); [Taylor et al., 2011](https://elifesciences.org/articles/44215#bib73)). Deletion or depletion of these myosins blocks endocytosis ([Basu et al., 2014](https://elifesciences.org/articles/44215" \l "bib7); [Geli and Riezman, 1996](https://elifesciences.org/articles/44215" \l "bib29); [Krendel et al., 2007](https://elifesciences.org/articles/44215" \l "bib47)), but the molecular mechanism by which type-I myosins function remains elusive.

The budding yeasts type-I myosins Myo3 and Myo5 are highly similar and functionally redundant. Deletion of both Myo3 and Myo5 leads to a complete block in endocytosis and a severe growth defect, while single deletions result in no or only very minor defects in endocytosis only under heat stress conditions, and show no growth phenotype ([Anderson et al., 1998](https://elifesciences.org/articles/44215#bib4); [Geli and Riezman, 1996](https://elifesciences.org/articles/44215" \l "bib29); [Goodson et al., 1996](https://elifesciences.org/articles/44215#bib32); [Goodson and Spudich, 1995](https://elifesciences.org/articles/44215#bib33)). Myo3 and Myo5 share a domain structure consisting of a myosin motor domain, a neck containing two IQ-motifs, and a tail comprising a tail homology 1 (TH1)-domain, an Src homology 3 (SH3)-domain and a central/acidic (CA)-domain ([Goodson and Spudich, 1995](https://elifesciences.org/articles/44215#bib33)). Myo5 has been shown to have motor activity in vitro ([Sun et al., 2006](https://elifesciences.org/articles/44215#bib71)), and Myo3 and Myo5 rigor mutants - which bind to actin filaments but cannot release them - block endocytic internalization ([Lechler et al., 2000](https://elifesciences.org/articles/44215" \l "bib51); [Lewellyn et al., 2015](https://elifesciences.org/articles/44215#bib52); [Sun et al., 2006](https://elifesciences.org/articles/44215#bib71)). Myo5’s TH1-domain has been shown to bind to phospholipids in vitro ([Fernández-Golbano et al., 2014](https://elifesciences.org/articles/44215#bib23)) and is required for efficient recruitment of Myo5 to endocytic sites ([Grötsch et al., 2010](https://elifesciences.org/articles/44215" \l "bib34); [Lewellyn et al., 2015](https://elifesciences.org/articles/44215#bib52)). The SH3-domain of Myo5 (and presumably Myo3) regulates protein-protein interactions with other endocytic proteins such as Vrp1, Las17, Bbc1 and Pan1 ([Anderson et al., 1998](https://elifesciences.org/articles/44215#bib4); [Barker et al., 2007](https://elifesciences.org/articles/44215#bib6); [Evangelista et al., 2000](https://elifesciences.org/articles/44215#bib22); [Geli et al., 2000](https://elifesciences.org/articles/44215" \l "bib28); [Lechler et al., 2000](https://elifesciences.org/articles/44215" \l "bib51); [Mochida et al., 2002](https://elifesciences.org/articles/44215#bib57); [Sun et al., 2017](https://elifesciences.org/articles/44215#bib72)). Myo5 SH3-domain interaction with Vrp1 is essential for recruitment of Myo5 to endocytic sites ([Lewellyn et al., 2015](https://elifesciences.org/articles/44215#bib52); [Sun et al., 2006](https://elifesciences.org/articles/44215#bib71)). Finally, the CA-domain together with Vrp1 possess NPF-activity in vitro ([Evangelista et al., 2000](https://elifesciences.org/articles/44215#bib22); [Galletta et al., 2012](https://elifesciences.org/articles/44215" \l "bib27); [Galletta et al., 2008](https://elifesciences.org/articles/44215" \l "bib26); [Geli et al., 2000](https://elifesciences.org/articles/44215" \l "bib28); [Lechler et al., 2000](https://elifesciences.org/articles/44215" \l "bib51)).

While the biochemical properties of Myo3, and Myo5 in particular, have been well characterized in vitro, their functions within the endocytic machinery in vivo remain enigmatic. Myo3 and Myo5 were initially presumed to act mainly as NPFs ([Evangelista et al., 2000](https://elifesciences.org/articles/44215#bib22); [Giblin et al., 2011](https://elifesciences.org/articles/44215#bib30)), however both their CA-domains can be deleted without affecting endocytosis ([Galletta et al., 2008](https://elifesciences.org/articles/44215" \l "bib26)), indicating that Myo3 and Myo5 have additional functions within the endocytic machinery. Recently it was proposed that Myo3 and Myo5 might dynamically anchor actin filaments to the endocytic site; without this binding the actin filaments would then splay out over the plasma membrane surface, which would interfere with actin network force production ([Evangelista et al., 2000](https://elifesciences.org/articles/44215#bib22); [Lewellyn et al., 2015](https://elifesciences.org/articles/44215#bib52)). Furthermore, myosins could contribute to endocytic force production, for example by pushing actin filaments inwards ([Lechler et al., 2000](https://elifesciences.org/articles/44215" \l "bib51); [Sun et al., 2006](https://elifesciences.org/articles/44215#bib71)). This is in line with observations that transiently lowering the force requirements can rescue the endocytic defects of Myo1 deletion in fission yeast ([Basu et al., 2014](https://elifesciences.org/articles/44215" \l "bib7)).

By combining quantitative live-cell imaging with genetic perturbations in budding yeast, we found that the yeast type-I myosins promote actin network growth, and thereby define the speed at which the membrane reshapes. The ability of the myosins to stimulate actin network growth does not depend on their NPF-activity, nor can a deficiency of myosin motors be rescued by increasing actin nucleation, indicating that myosins stimulate actin polymerization rather than nucleation. We propose a model whereby type-I myosins stimulate actin filament polymerization, which contributes to actin network expansion and force production.

### Deletion of *MYO5* slows down invagination growth and delays scission

Previous studies have shown that deletion of both *MYO3* and *MYO5* results in a complete block in endocytosis ([Geli and Riezman, 1996](https://elifesciences.org/articles/44215" \l "bib29); [Goodson et al., 1996](https://elifesciences.org/articles/44215#bib32); [Sun et al., 2006](https://elifesciences.org/articles/44215#bib71)), making it difficult to narrow down how these proteins contribute to each subsequent phase of the endocytic process. Therefore, we decided to quantitatively assess the impact of deleting a single myosin-I gene on the endocytic machinery.

To assess how Myo5 contributes to membrane invagination, we analyzed the dynamics of eight key proteins of the coat (Sla1, Sla2), actin network (Abp1, Cap1, Arc18, Sac6 and Act1) and scission modules (Rvs167) in wild type (WT) and *myo5Δ* cells ([Figure 1A–I](https://elifesciences.org/articles/44215#fig1)). For each protein, we generated a C-terminally tagged version by integrating EGFP at the genomic locus in a WT or *myo5Δ* background (all proteins in this paper, except for Act1, were tagged at their genomic locus). We performed live-cell imaging and centroid tracking to obtain average trajectories for each protein ([Picco et al., 2015](https://elifesciences.org/articles/44215" \l "bib64); [Picco and Kaksonen, 2017](https://elifesciences.org/articles/44215" \l "bib66)). Additionally we created strains co-expressing Abp1-mCherry, which allows for the precise alignment of the trajectories within each dataset (WT or *myo5Δ*) in respect to the Abp1 trajectory ([Picco et al., 2015](https://elifesciences.org/articles/44215" \l "bib64); [Picco and Kaksonen, 2017](https://elifesciences.org/articles/44215" \l "bib66)). Finally we measured the median number of protein molecules at the endocytic site over time ([Joglekar et al., 2006](https://elifesciences.org/articles/44215" \l "bib40); [Picco et al., 2015](https://elifesciences.org/articles/44215" \l "bib64)). Trajectories were plotted so that for each dataset (WT or *myo5Δ*) the Sla2 trajectory starts at y = 0 (representing the position of the plasma membrane; [Picco et al., 2015](https://elifesciences.org/articles/44215" \l "bib64)), while the onset of Abp1 assembly is taken as t = 0.

For the coat module, we analyzed two coat proteins Sla1 and Sla2. Sla1 is an abundant component of the endocytic coat and can be used to track the tip of the growing invagination and the newly formed vesicle ([Kaksonen et al., 2003](https://elifesciences.org/articles/44215" \l "bib42); [Kukulski et al., 2012a](https://elifesciences.org/articles/44215" \l "bib49)). In addition, we chose to analyze N-terminally-tagged Sla2, since the fluorophore is located near Sla2’s membrane binding domain, meaning it reports the position of the plasma membrane ([Picco et al., 2015](https://elifesciences.org/articles/44215" \l "bib64)).

Deletion of *MYO5* resulted in a decrease in Sla1 inward movement speed ([Figure 1B](https://elifesciences.org/articles/44215#fig1)). Note that this defect was present throughout the invagination process, and not limited to any specific phase of the invagination. Sla1 movement speed was reduced from 26.2 ± 1.4 nm/s (mean ± SE) in WT cells to 17.1 ± 1.4 nm/s in *myo5Δ* (p<0.001, Welch 2-sample t-test, see also [Supplementary file 1](https://elifesciences.org/articles/44215/figures#supp1) table 1). Similar to Sla1, the coat protein Sla2 showed a reduction in inward movement speed ([Figure 1C](https://elifesciences.org/articles/44215#fig1)). While Sla2’s movement speed was reduced in *myo5Δ*, the period during which it moves was prolonged, resulting in a similar end-position for the trajectory. These data suggest that the growth rate of the membrane invagination is reduced in *myo5*∆ cells.

To assess the invagination length at the moment of scission, we followed Rvs167, a BAR-domain-containing protein which assembles around the invagination neck, and whose abrupt disassembly coincides with scission in WT cells ([Kukulski et al., 2012a](https://elifesciences.org/articles/44215" \l "bib49); [Picco et al., 2015](https://elifesciences.org/articles/44215" \l "bib64)). In *myo5Δ* the onset of Rvs167 assembly was delayed relative to the onset of Abp1 assembly ([Figure 1D](https://elifesciences.org/articles/44215#fig1), WT: 3.1 s, *myo5Δ*: 5.5 s). However, in both *myo5Δ* and WT cells, Rvs167 was recruited once Sla2 reached an inward depth of ~45 nm ([Figures 1C](https://elifesciences.org/articles/44215#fig1) and [2A](https://elifesciences.org/articles/44215#fig2), WT: 39 ± 6 nm, *myo5Δ*: 47 ± 7 nm, p=0.381). This suggests that Rvs167 recruitment is triggered when the invagination reaches a certain length. In WT cells, the length of the accumulation period of Rvs167 molecules was 3.1 ± 0.1 s (median ± SE, n = 103) while in *myo5Δ* it was extended to 5.5 ± 0.3 s (median ± SE, n = 29), resulting in similar peak amounts of Rvs167 molecules (peak number of molecules and standard error for WT: 136 ± 16, for *myo5Δ:* 110 ± 15, p=0.234). The lengths of accumulation periods for Rvs167 molecules in the two samples were different (Mann-Whitney U-test, p value < 0.0001 ). Therefore, Rvs167 molecules accumulated at a slower pace in *myo5Δ* cells, in line with the hypothesis that the invagination growth rate is reduced in *myo5Δ*. At the moment when Rvs167 molecule numbers peaked (indicated by dashed and dotted vertical lines for WT and *myo5Δ* respectively), Sla2 had reached an inward depth of ~150 nm in both WT and *myo5Δ* ([Figures 1C](https://elifesciences.org/articles/44215#fig1) and [2A](https://elifesciences.org/articles/44215#fig2), WT: 145 ± 6 nm, *myo5Δ*: 169 ± 9 nm). Taken together these results indicate that in *myo5Δ* the invagination growth rate is reduced, but since the growth phase is prolonged, similar final invagination lengths are reached.

### Myo5 is required for efficient actin network growth

As myosins are known to interact with actin, we decided to investigate how the actin network is affected by deletion of *MYO5.* We analyzed the dynamics of the actin binding protein Abp1, the capping protein subunit Cap1, the Arp2/3-subunit Arc18, the crosslinker Sac6, and Act1, the actin monomer, in WT and *myo5Δ* cells ([Figure 1E–I](https://elifesciences.org/articles/44215#fig1)). As genomic tagging of Act1 is not tolerated ([Wu and Pollard, 2005](https://elifesciences.org/articles/44215#bib74)), GFP-Act1 was expressed from a plasmid on top of endogenous Act1 expression ([Picco et al., 2015](https://elifesciences.org/articles/44215" \l "bib64)). Furthermore, Arc18 was tagged with myEGFP, a better tolerated fluorophore by the Arp2/3 complex than EGFP ([Picco et al., 2015](https://elifesciences.org/articles/44215" \l "bib64)).

We first measured the accumulation rates of these proteins until they reached a peak. We found that the assembly rates for all these proteins were strongly reduced in *myo5Δ* cells as compared to WT cells ([Figure 2B](https://elifesciences.org/articles/44215#fig2)). However, the durations of the accumulation periods were extended by ~30%, except for Arc18 for which the period was not prolonged. This resulted in comparable peak amounts of the actin network proteins in WT and *myo5Δ* cells ([Figure 2D](https://elifesciences.org/articles/44215#fig2)), except for Sac6 which was significantly reduced (p=0.018 vs WT).

To check if we could see differences in the actin network using an independent method, we measured filamentous actin amount by quantifying phalloidin-staining intensity in fixed WT and *myo5Δ* cells. We detected a small but significant decrease in the amount of phalloidin per endocytic actin patch in *myo5*∆ cells (12% decrease, p=0.022) ([Figure 2—figure supplement 1](https://elifesciences.org/articles/44215/figures#fig2s1)).

The absence of Myo5 also resulted in a decrease in centroid movement speed for Act1, Abp1, Cap1 and Sac6, but not for Arc18 ([Figure 2C](https://elifesciences.org/articles/44215#fig2)). However, as scission was delayed in *myo5Δ*, the centroids nearly reached the same inward distances at the time of scission as in WT cells.

Taken together these results show that Myo5 stimulates the addition of components to the growing actin network, leading to its expansion.

### Myo3 and Myo5 assemble at the same place and time, but in different amounts

While it is known that Myo5 is recruited to the base of the endocytic invagination at the start of the actin assembly phase ([Idrissi et al., 2008](https://elifesciences.org/articles/44215" \l "bib38); [Picco et al., 2015](https://elifesciences.org/articles/44215" \l "bib64); [Sun et al., 2006](https://elifesciences.org/articles/44215#bib71)), Myo3’s dynamics have not been quantified. In order to compare Myo3 and Myo5, we performed live-cell imaging and centroid tracking on Myo3-EGFP or Myo5-EGFP together with Abp1-mCherry ([Picco et al., 2015](https://elifesciences.org/articles/44215" \l "bib64); [Picco and Kaksonen, 2017](https://elifesciences.org/articles/44215" \l "bib66)). C-terminal tagging did not detectably affect myosin function as Abp1-mCherry lifetimes were not significantly affected ([Figure 3—figure supplement 1](https://elifesciences.org/articles/44215/figures#fig3s1)). Myo3 and Myo5 started to assemble at the endocytic site at the same place and time ([Figure 3A,B](https://elifesciences.org/articles/44215#fig3)). However, the accumulation rate was higher for Myo5, peaking at about twice as many Myo5 molecules than Myo3 (median number of molecules over the lifetime of the patch and standard error for Myo5: 77 ± 8, Myo3: 38 ± 4, p<0.001 in 1-sided z-test). The overall similarity in Myo3 and Myo5 recruitment timing and positioning is in line with their reported functional redundancy.

### Myo3 and Myo5 contribute to invagination in a dose-dependent way

Next we assessed whether the deletion of *MYO3* results in the same endocytic defects as the deletion of *MYO5*. We imaged Sla1-EGFP in WT, *myo3Δ* and *myo5Δ* cells, and found that in *myo3Δ* Sla1 dynamics were similar to WT ([Figure 3C](https://elifesciences.org/articles/44215#fig3)). Because Myo3 and Myo5 are highly similar and are recruited with similar dynamics to the endocytic site, we were surprised to find that deletion of *MYO5*, but not deletion of *MYO3*, had a strong effect on Sla1 inward movement. We wondered if the lack of phenotype in *myo3Δ* was a result of a compensatory increase in Myo5 recruitment, which could mask the effects of the absence of Myo3. We quantified Myo5-EGFP recruitment in *myo3Δ* cells and found it was unaffected by the absence of Myo3 ([Figure 3D](https://elifesciences.org/articles/44215#fig3)), nor was Myo3 recruitment increased when *MYO5* was deleted. This general lack of feedback may indicate that Myo3 and Myo5 have different binding sites within the endocytic machinery, or that their amounts are not saturating all the binding sites available. The cytoplasmic concentrations of Myo3 and Myo5 have been reported as 152 nM and 172 nM respectively ([Boeke et al., 2014](https://elifesciences.org/articles/44215" \l "bib11)). We used these values to calculate that on average 34% of the total pool of Myo3 molecules in the cell is located at endocytic sites, compared to 59% of Myo5 molecules (see Materials and methods for calculations). Therefore, Myo5 has a higher affinity than Myo3 for endocytic sites.

In order to see if the Sla1 inward movement rate is determined by the number of myosin molecules at the endocytic site, we created a series of haploid cells carrying combinations of deletions or duplications of the *MYO3* and *MYO5* alleles, and measured both myosin recruitment and Sla1 inward movement in these genetic backgrounds. We also created diploid yeasts carrying deletions of 0, 1, 2, or 3 of the four total myosin-I alleles, in order to obtain endocytic sites containing fewer myosins.

First, we found that the numbers of protein molecules at endocytic sites in WT haploid and WT diploid cells were essentially identical, not only for myosins but also for two other endocytic proteins ([Figure 3D](https://elifesciences.org/articles/44215#fig3), [Figure 3—figure supplement 2](https://elifesciences.org/articles/44215/figures#fig3s2)). In general, Myo3 and Myo5 recruitment were largely related to their gene dosage, and unaffected by the presence or absence of the other myosin paralog. Exceptions were the *MYO5* duplication strains, where Myo5 recruitment was further increased when *MYO3* was deleted. Also, deletion of a single *MYO5* allele in diploid cells did not significantly reduce Myo5 recruitment, while deletion of a single *MYO3* allele did affect Myo3 recruitment. Taken together, we created a series of strains that have different amounts of myosins at endocytic sites ([Supplementary file 1](https://elifesciences.org/articles/44215/figures#supp1) table 2).

Intriguingly, we found that Sla1 inward movement speed increased with higher total amounts of Myo3 and Myo5 in a dose-dependent way ([Figure 3E–G](https://elifesciences.org/articles/44215#fig3); [Supplementary file 1](https://elifesciences.org/articles/44215/figures#supp1) table 1). Duplication of the *MYO5* ORF in haploid cells resulted in a slight but significant increase in Sla1 inward movement speed ([Figure 3E](https://elifesciences.org/articles/44215#fig3), 30.6 ± 1.1 nm/s, p=0.011 vs WT). Sla1 inward movement was similar between WT haploid and WT diploid cells ([Figure 3F](https://elifesciences.org/articles/44215#fig3)). Furthermore, deletion of all *MYO5* alleles in diploids strongly reduced Sla1 inward movement speeds, phenocopying the *MYO5* deletion in haploid cells, while deletion of one of the two *MYO5* alleles had no effect on Sla1 movement.

To summarize, we found a strong dose-dependent correlation between the number of myosin proteins at the endocytic site, and the Sla1 inward movement rate ([Figure 3G](https://elifesciences.org/articles/44215#fig3)). This suggests that myosins control the speed of membrane invagination, potentially via controlling actin network growth.

### The motor activity of Myo5 is necessary for membrane invagination

To test the role of the motor activity in the endocytic invagination process we introduced a glycine 132 to arginine point mutation into the *MYO5* locus. This mutation (Myo5-G132R) is a rigor mutant, which has been reported to inhibit endocytosis and has been suggested to prevent membrane invagination ([Sun et al., 2006](https://elifesciences.org/articles/44215#bib71); [Lewellyn et al., 2015](https://elifesciences.org/articles/44215#bib52)). Consistently, we observed that in Myo5-G132R cells, the Sla1-EGFP invagination movement was completely absent ([Figure 4A](https://elifesciences.org/articles/44215#fig4)). An average of ~40 Myo5-G132R molecules was recruited to endocytic sites ([Figure 4B](https://elifesciences.org/articles/44215#fig4)), which is significantly less than the number of Myo5 molecules. However, the reduction of the number of Myo5 molecules by about 50% is not enough to explain the observed strong phenotype. These data suggest that the motor activity is critical for the function of myosins in endocytic membrane invagination.

### Myo5’s effect on invagination speed is independent of the NPF activity

Deletion of *MYO5* results in a decrease in invagination speed and negatively affects actin network buildup and movement. Myo5 has a C-terminal CA-domain, which has been shown to possess NPF-activity in vitro ([Geli et al., 2000](https://elifesciences.org/articles/44215" \l "bib28); [Idrissi et al., 2002](https://elifesciences.org/articles/44215" \l "bib37); [Sun et al., 2006](https://elifesciences.org/articles/44215#bib71)). To test whether the defects in *myo5Δ* can be explained by a decrease in NPF-activity, we created a truncated Myo5 lacking the CA-domain (*myo5-CAΔ*) ([Galletta et al., 2008](https://elifesciences.org/articles/44215" \l "bib26); [Sun et al., 2006](https://elifesciences.org/articles/44215#bib71)) and measured actin network buildup and invagination rates. Intriguingly we found a slight decrease in the amount of Arc18 at endocytic sites ([Figure 5A](https://elifesciences.org/articles/44215#fig5), p=0.043 vs WT), similar to *myo5Δ*. However, while in *myo5Δ* the amount of actin (Act1) was strongly reduced, in *myo5-CAΔ* it was unchanged ([Figure 5B](https://elifesciences.org/articles/44215#fig5), p=0.647 vs WT, p=0.001 vs *myo5Δ*). Furthermore, Sla1 inward movement was essentially identical to WT, in contrast to the full *MYO5* deletion ([Figure 5C](https://elifesciences.org/articles/44215#fig5)). This indicates that the defects in actin network growth and invagination speed in the *myo5Δ* strains cannot be attributed to a decrease in NPF-activity.

### Extra actin cannot compensate for a lack of myosin motors

We wondered if the reduction in Sla1 speed in *myo5Δ* could be rescued by artificially increasing actin polymerization at the endocytic site. Bbc1 is an inhibitor of NPF-activity of Las17 in vitro ([Sun et al., 2006](https://elifesciences.org/articles/44215#bib71)), and deletion of *BBC1* results in increased Las17 recruitment to the endocytic site and excessive actin polymerization ([Kaksonen et al., 2005](https://elifesciences.org/articles/44215" \l "bib43); [Picco et al., 2018](https://elifesciences.org/articles/44215" \l "bib65)). In *bbc1Δ*, Sla1 centroid movement is initially unperturbed, but accelerates around the time of scission ([Picco et al., 2018](https://elifesciences.org/articles/44215" \l "bib65)).

Combining the deletions of *MYO5* and *BBC1* balanced out their opposing effects on actin nucleation, resulting in Act1 molecule numbers that were close to WT levels ([Figure 6A](https://elifesciences.org/articles/44215#fig6)). Intriguingly, Sla1 inward movement showed the combined phenotype of both single deletions: Sla1 centroid movement was initially slowed down, resembling *myo5Δ*, but accelerated at later timepoints, like in the *bbc1Δ* strain ([Figure 6B](https://elifesciences.org/articles/44215#fig6)). So although the additional deletion of *BBC1* could compensate for the actin defect of *myo5Δ*, it was not sufficient to rescue invagination growth, suggesting that Myo5 has additional roles within the endocytic machinery besides the stimulation of actin filament nucleation.

### Ultrastructural analysis reveals subtle changes in endocytic site morphology in *myo5Δ*

As deletion of *MYO5* affects actin network buildup and coat inward movement, we wondered how the overall ultrastructure of both the actin network and the plasma membrane were impacted. We used superresolution microscopy as described in [Mund et al. (2018)](https://elifesciences.org/articles/44215" \l "bib61) to measure the dimensions of the actin network in WT and *myo5Δ* cells. For this, we determined the radial density profiles of Abp1, Cap1 and Arc18, and found that the overall protein distributions are very similar ([Figure 7](https://elifesciences.org/articles/44215#fig7)). However, deletion of *MYO5* resulted in a slight but significant reduction in the outer radii of these proteins, indicating that the actin network on average was narrower.

We were interested in how the defects in actin network assembly and coat motility seen in *myo5Δ* impacted the membrane reshaping during endocytosis. We used correlated light and electron microscopy (CLEM) to investigate membrane shapes in *myo5Δ* cells expressing Sla1-EGFP and Abp1-mCherry, using the protocol described in [Kukulski et al. (2012a)](https://elifesciences.org/articles/44215" \l "bib49); [Kukulski et al. (2012b)](https://elifesciences.org/articles/44215" \l "bib50). The *myo5Δ* dataset contained both invaginations and vesicles, indicating that productive scission had taken place ([Figure 8](https://elifesciences.org/articles/44215#fig8)). Intriguingly, the invaginations had larger tip diameters than were reported for WT cells (p=0.003, [Figure 8C](https://elifesciences.org/articles/44215#fig8); [Kukulski et al., 2012a](https://elifesciences.org/articles/44215" \l "bib49)). This increase did not correlate with invagination length ([Figure 8—figure supplement 1](https://elifesciences.org/articles/44215/figures#fig8s1)), indicating that the expansion of the invagination tip is present throughout the whole invagination process. Overall, these results indicate that myosin activity impacts the actin network architecture and endocytic membrane morphology.