Type I myosins anchor actin assembly to the plasma membrane during clathrin-mediated endocytosis

Ross Pedersen and David Drubin

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Review Timeline:		
	Submission Date:	2018-10-01
	Editorial Decision:	2018-10-26
	Revision Received:	2018-11-14
	Editorial Decision:	2018-12-06
	Revision Received:	2018-12-20
	Accepted:	2019-01-04

Monitoring Editor: Anthony Bretscher

Scientific Editor: Andrea Marat

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: 10.1083/jcb.201810005

October 26, 2018

Re: JCB manuscript #201810005

Dr. David Drubin
UC Berkeley
16 Barker Hall
Berkeley, California 94720-3202

Dear Dr. Drubin,

Thank you for submitting your manuscript entitled "Type I myosins anchor actin assembly to the plasma membrane during clathrin-mediated endocytosis". Your manuscript has been carefully read by three reviewers and myself. All three reviewers are very positive that new insights are provided by the new data further indicating a role for the TH1 domain of Myo5 in tethering actin assembly to the site of endocytosis. As noted in the introduction, this study grew out of earlier work (Lewellyn et al., 2015) reporting on a minimal Myo5-Las17 construct that can provide all the activities necessary for endocytic force generation.

We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

Reviewer #1 is the most critical and questions whether the data could also be interpreted by an association of the TH1 domain with the endocytic coat rather than with lipids. While this question was extensively studied in the 2015 paper, the suggestion of examining the effect of appropriate point mutations in the TH1 domain, instead of deleting it, might be informative. Alternatively, the authors might consider exploring whether a lipid-binding PH domain might be able to functionally replace the TH1 domain. This reviewer also has a number of other issues that should be considered.

Reviewer #2's only concern is over the finding of actin waves. It would be useful to know how common these events are, but it seems to me that significant quantitation of these is beyond the message of this study. S/he also makes a good point about the absence of reference to yeast: I couldn't find the word 'yeast' in either the title or abstract; I think it should be made clear to the reader that these studies were done in baker's yeast.

Reviewer #3 has a number of relatively minor comments. However, the first one says: "Some of the conclusions in this work overlap with those in a previous paper (Lewellyn et al. 2015). This fact does not degrade significance...." I fully agree - the authors should clearly define what that earlier study told them about the role of the TH1 domain, and what new insights are provided by this study. This may also help them address the main point of reviewer #1.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

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abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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Our typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Anthony Bretscher
Monitoring Editor

Andrea L. Marat
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

R. Pedersen and D. Drubin provide evidence suggesting that the myosin-I TH1 domain, which is expected to interact with negatively charged cellular membranes, is necessary to link the endocytic WASP/Myo module and the endocytic actin network to the plasma membrane. The original observation that mainly supports this conclusion is that about 20% of the cells expressing a Myo5 version lacking the TH1 domain (Myo5-TH1Δ) as the only source of myosin-I, exhibited cytosolic

actin tails, most likely seeded by the endocytic WASP/Myo module. However, the same authors show by TIRF that in the myo5-TH1Δ mutant, most actin structures formed in association with endocytic patches at the cortex, slide away in the plane of the membrane, suggesting that the connection with the endocytic coat, rather than the association with the lipid bilayer, is lost. Demonstration that the myosins-I link the endocytic actin network to the lipid bilayer rather that the endocytic coat would require either reconstitution with purified components or the use of more subtle point mutations that specifically alter the interaction of the TH1 doamin with phospholipids. Complete deletion of the TH1 domain can impact on the functionality of adjacent domains or affect not yet described protein-protein interactions.
On the other hand, the group of M. Kaksonen has described a redundant function of Ent1 and Sla2, linking actin to the endocytic coat and the lipid bilayer (Skruzny PNAS 2012). Further, the group of D. Drubin itself has previously proposed that Pan1, End3 and Sla1 links Arp2/3-dependent actin polymerization to endocytic sites (Sun, Mol Biol Cell 2015). In the presence of such many functionaly redundant links, it is difficult to explain how altering a rather unspecific interaction between the myosin-I TH1 domain and negatively charged membranes can detach actin from the endocytic sites. These multiple observations need to be discussed. Would it be possible that the TH1 domain has a regulatory role on actin polymerization and its depletion just causes ectopic or unsyncronized actin polymerization? In this context, it is worth noticing that the TH1 domain has an autoinhibitory effect on Myo5-induced actin polymerization (Grötsch, EMBO J 2010).
Other points:
In figure 1A and B, actin polymerization at endocytic sites seems to be strongly exacerbated in the myo5-TH1 mutant as compared to the wild type, but not in the myo5 deleted mutant. Is that so?
In figure 2D, the authors show that Las17, the other major activator of Arp2/3 at endocytic sites, is associated with the cytosolic actin tails in the myo5-TH1 mutant. Can the authors link those tails to endocytic sites by fusing the Myo5 TH1 domain to Las17?
In figure 3 the authors provide some evidence indicating that the actin tails might have an endocytic origin but that they are not attached to membranes. However the evidence is rather indirect and certainly not conclusive. In this context, the authors show in figure 3A that Abp1 forms tails that detach from the plasma membrane in the myo5-TH1 mutant. However, the authors show in figure 1 that Abp1 stays at the plasma membrane in the same mutant. How frequent is one phenotype as compared to the other? Is the treatment with CK-666 followed by its wash-out exacerbating the formation of Abp1 tails? It would be better to perform the CK-666 wash-out experiments with the myo5-TH1 mutant rather than the knock out.
The conclusion that the actin tails are not associated with membranes of endocytic origin is not really supported by the data neither. To sustain this view, the authors indicate that the endocytic coat is not present in the actin tails. However, the coat could prematurely disassemble before internalization in the mutant. Indeed, in the figure 3D CK-666 wash-out experiments, Sla1 seems to slightly internalize and fade away as Abp1 goes into the cytosol in the myo5 mutant. Is this always the case?. It would be more conclusive in this context to follow endocytic cargo. On the other hand, the authors state that there is only residual internalization of membrane in the mutant and as a consequence, endocytosed membranes cannot seed actin polymerization. However, this residual internalization is about 20 % as compared to the wild type and therefore, internalized membranes could accumulate in time and indeed seed persistent actin polymerization. Double labeling experiments with Abp1-GFP would be needed to properly rule out this possibility.

Reviewer #2 (Comments to the Authors (Required)):

This manuscript describes the role of Myosin I as a membrane anchor in endocytosis in budding

yeast. In MyoI null or tailess mutants, actin is nucleated by Arp2/3 at the surface, but the endocytic vesicle fails to invaginate, and the actin forms a comet tail that often moves into the cell interior with some WASP. The membrane binding portion of Myosin I but not the motor domain is responsible for this anchoring function. These studies thus nicely define a function of Myosin I in mooring assembly factors to the plasma membrane for proper organization and force production required for efficient endocytosis.

In general, this is a very well written and interesting manuscript with a significant message. The data are strong and of technically high quality. All the main points of the paper are well supported. This will likely be an defining, impactful paper in the field and therefore is suitable for publication in JCB. I only have minor comments.

Specific comments

The behavior of the actin comet tails/ waves should be described more fully at a quantitative level. In particular, it is not so clear how rare or common these events are. Although it is stated that every cell exhibits some wave or comet actin, what percentage of the patches end up forming one of these structures or some other structure, like an actin dot or no actin? Does the comet all form after a certain time period relative to Sla1 dynamics? Do the comets have a limited lifetime?

The word "yeast" is only mentioned once in the title/abstract. To distinguish from other types of yeast, term "budding yeast" or "Saccharomyces cerevisiae" should be used.

Reviewer #3 (Comments to the Authors (Required)):

Authors investigate roles of type 1 myosin, Myo5, in clathrin-mediated endocytosis in yeast. This work builds on a previous study from this lab, in which Lewellyn et al. have identified a number of critical domains within a multiprotein actin-nucleating complex that controls actin-mediated internalization of endocytic pits. These critical domains include the Myo5 motor and its membrane-targeting TH1 domain. The current work focuses primarily on the roles of Myo5-dependent membrane targeting of the actin nucleation complex. The main finding here is that Myo5 restricts actin polymerization to endocytic sites by targeting of the nucleation complex to the membrane via the TH1 domain. This targeting allows for efficient internalization. In the absence of Myo5-dependent targeting, actin nucleation machinery detaches from the membrane and travels around the cell in the form of comets. This study provides important new insights into functions of class 1 myosin in endocytosis. The work is carefully done and analyzed. Although the study is performed in yeast, the conclusions can be applicable to other cell types. I have relatively minor comments that should be addressed.

1. Some of the conclusions in this work overlap with those in the previous paper (Lewellyn et al. 2015). This fact does not degrade significance of the current follow-up study, which is more advanced. However, it would be helpful if authors made more connections and comparisons between these two studies, so that the current advance was more obvious.
2. Figure 1B: label mutants used in individual panels.
3. Figure 1C: What is the meaning of dots in the graph?
4. Figure 2B shows that treatment of cells with CK-666 inhibits cytoplasmic comets, but not cortical patches. Why? Cortical patches do disappear in supplemental figure S1E and F. The concentrations of CK-666 and DMSO for figure 2B are not shown in the figure or in the legend.

5. Data for Bbc1 and Bzz1 are shown in figure 2D and corresponding movies, but are not described in Results.
6. Figures 3D and S2B show that Sla1-GFP does not move inward in myo5Δ mutants after CK-666 washout. However, both figures also show that Sla1 intensity decreases when actin reassembles. Does it happen regularly? If yes, what does it mean?
7. p. 11: "However, unlike Sla1-GFP, which remained on the plasma membrane (Figure 3D), Vrp1-GFP puncta splintered ... (Figure 3E)". How often do they splinter? Always?
8. Figure 4G and 5A: Blue shape in 4G are annotated in the legend as "WASP/Myosin complex", but myosin is shown separately. Is it meant to be "WASP/WIP" complex? In 5A, it appears that "yellow bananas" correspond to Myo5, but blue shapes are also labeled as WASP/Myosin complex. In fact, bananas are labeled as Myo5 in the legend, but as Myo5 membrane anchor in the figure, which makes things even more confusing.
7. Mund et al. 2018 paper in the reference list has the title typed twice.
8. It would be helpful to have a discussion about how applicable these findings could be for mammalian endocytosis.

Reviewer #1 (Comments to the Authors (Required)):

R. Pedersen and D. Drubin provide evidence suggesting that the myosin-I TH1 domain, which is expected to interact with negatively charged cellular membranes, is necessary to link the endocytic WASP/Myo module and the endocytic actin network to the plasma membrane. The original observation that mainly supports this conclusion is that about 20% of the cells expressing a Myo5 version lacking the TH1 domain (Myo5-TH1∆) as the only source of myosin-I, exhibited cytosolic actin tails, most likely seeded by the endocytic WASP/Myo module. However, the same authors show by TIRF that in the myo5-TH1∆ mutant, most actin structures formed in association with endocytic patches at the cortex, slide away in the plane of the membrane, suggesting that the connection with the endocytic coat, rather than the association with the lipid bilayer, is lost. Demonstration that the myosins-I link the endocytic actin network to the lipid bilayer rather that the endocytic coat would require either reconstitution with purified components or the use of more subtle point mutations that specifically alter the interaction of the TH1 doamin with phospholipids. Complete deletion of the TH1 domain can impact on the functionality of adjacent domains or affect not yet described protein-protein interactions.

We are not sure that we understand this reviewer’s model, but think that she/he is proposing that a link between the myosin and the endocytic coat is lost when the TH1 domain is absent. First of all, we favor a different interpretation of the TIRFM data. Although the waves of actin assembly move in the plane of the plasma membrane, the fact that the actin tails can also often be observed to leave the plasma membrane (see, for example, figure 3D) indicates that they are no longer attached to the membrane. It should be noted that the type 1 myosin stays at the base of the endocytic invagination (near the plasma membrane) while the endocytic coat (typically imaged with Sla1-GFP) internalizes upon actin assembly during wild-type endocytosis (Sun, Martin, and Drubin, *Cell,* 2006). Any connection between Myo5 and the endocytic coat must therefore be transient and absent during actin assembly and membrane invagination, even in the presence of full length Myo5.

The phenotype when there is a lost connection between the actin cytoskeleton and the endocytic coat is quite distinct from what is observed in the Myo5 TH1 mutants. In mutants lacking Sla2, a protein that binds to both clathrin and actin filaments, long actin tails form at endocytic sites, but they are associated stably with the cell cortex at endocytic sites and never leave the cell cortex. In contrast, in the Myo5 TH1 mutants, the actin assembles at endocytic sites but then becomes detached and travels into the cell interior in rocket-like structures that resemble intracellular pathogens like Listeria.

While complete deletion of the TH1 domain may not in principle be the ideal genetic manipulation, the resulting Myo5 deletion mutant protein is stable (Fig. S1A), suggesting that adjacent domains are properly folded.

We agree that point mutants in the TH1 domain would be attractive for studies of TH1 function. Unfortunately, point mutations in the non-specific anionic phospholipid-binding

TH1 domain of the human homologue of Myo5, Myosin 1e, which we might have used as a model for construction of analogous mutants, have little effect on phospholipid affinity *in vitro* or localization of the protein *in vivo*. We therefore believe that any effects of the analogous point mutations in Myo5 would be uninterpretable (Feeser *et al., Biochemistry,* 2010). We previously tried to replace the TH1 domain of Myo5 with the TH1 domain from mouse Myosin 1c. This TH1 domain binds specifically to phosphoinositides and its membrane binding can be ablated through introduction of point mutations (Hokanson *et al., MBoC,* 2008). Unfortunately, replacement of the Myo5 TH1 domain with the wildtype Myo1c TH1 domain failed to support robust growth (Lewellyn *et al., Dev. Cell,* 2015). Upon obtaining this result, we abandoned plans to replace the TH1 domain with a PH domain. We reasoned the PH domain would be more likely to disrupt the structure of the Myo5 tail than the more homologous Myo1c TH1 domain, though still binding specifically to phosphoinositides.

Based on previous results, it seems unlikely that elimination of the Myo5 TH1 domain alters binding of the Myo5 tail to actin assembly factors. Geli *et al.* (*EMBO J,* 2000) demonstrated that a recombinant protein consisting of only the TH2 and SH3 domains of Myo5 was able to initiate actin assembly in crude yeast extracts, indicating that these domains can fold and interact with their binding partners in the absence of the TH1 domain. Moreover, Sirotkin *et al.* (*JCB,* 2005) showed that a similar recombinant fragment of the *S. pombe* Myo5 homologue, Myo1, can activate the Arp2/3 complex in pyrene actin assembly assays.

Finally, we favor our model because previous experiments provide strong evidence that the Myo5 TH1 domain can bind phospholipids. Feeser *et al.* (2010) definitively demonstrated that the human Myo1e TH1 domain binds to liposomes *in vitro*, and we have shown that the same Myo1e TH1 domain can replace the Myo5 TH1 domain (Lewellyn *et al.* 2015). Fernandez-Golbano *et al.* (*Dev. Cell,* 2014) provide the most direct evidence that the Myo5 TH1 domain binds phospholipids through use of lipid strips.

We thus reasoned that complete deletion of the Myo5 TH1 domain would be the best way to reliably eliminate any interactions that it makes without significantly perturbing the rest of the Myo5 tail, and concluded that its loss results in loss of actin cytoskeleton association with the plasma membrane.

On the other hand, the group of M. Kaksonen has described a redundant function of Ent1 and Sla2, linking actin to the endocytic coat and the lipid bilayer (Skruzny PNAS 2012). Further, the group of D. Drubin itself has previously proposed that Pan1, End3 and Sla1 links Arp2/3-dependent actin polymerization to endocytic sites (Sun, Mol Biol Cell 2015). In the presence of such many functionaly redundant links, it is difficult to explain how altering a rather unspecific interaction between the myosin-I TH1 domain and negatively charged membranes can detach actin from the endocytic sites. These multiple observations need to be discussed. Would it be possible that the TH1 domain has a regulatory role on actin polymerization and its depletion just causes ectopic or unsyncronized actin polymerization? In this context, it is worth noticing that the TH1

domain has an autoinhibitory effect on Myo5-induced actin polymerization (Grötsch, EMBO J 2010).

As this reviewer points out, it is interesting to think about the various linker proteins required for robust endocytosis. Experimental evidence indicates that each of the linkages described by us and others serves a different purpose. The Pan1/End3/Sla1 complex is responsible for the initial recruitment of Arp2/3 activator Las17 to endocytic sites. The Ent1/Sla2 membrane-actin linkage is believed to be that the tip of endocytic invaginations, coupling inward flux of the actin network to invagination of the plasma membrane. The type 1 myosin linkage described here serves a distinct role, anchoring the WASP/Myosin complex to the plasma membrane at the time of actin assembly. We have added discussion of all three of these linkages to the manuscript for the sake of clarity, and we thank the reviewer for raising this interesting issue.

It should be noted that the phenotype of complete deletion of *MYO5* closely resembles the phenotype when the TH1 domain is eliminated. We therefore conclude elimination of membrane binding masks any gain-of-function effects due to loss of Myo5 autoinhibition in the TH1Δ mutant.

Other points:
In figure 1A and B, actin polymerization at endocytic sites seems to be strongly exacerbated in the myo5-TH1Δ mutant as compared to the wild type, but not in the myo5 deleted mutant. Is that so?

We thank the reviewer for pointing out this potential area of confusion. An error was made in the scaling of the Abp1-mRFP channel for display in Figure 1A. We have corrected this error. We did not directly compare levels of actin assembly in these mutants. Although care was taken to scale all images the same way, we do not believe that quantitative fluorescence comparisons should be made between cells that are not in the same field without rigorous controls.

In figure 2D, the authors show that Las17, the other major activator of Arp2/3 at endocytic sites, is associated with the cytosolic actin tails in the myo5-TH1Δ mutant. Can the authors link those tails to endocytic sites by fusing the Myo5 TH1 domain to Las17?

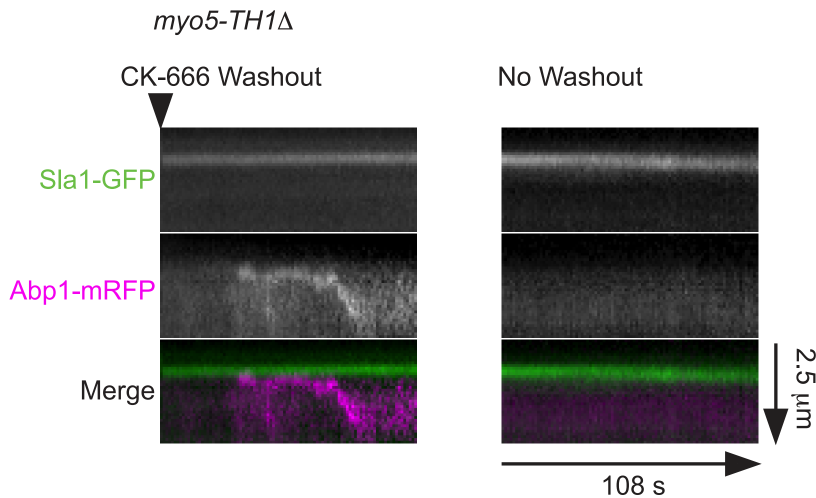
We thank the reviewer for this suggestion and for reading our manuscript with a curious eye. However, it is unclear at the current time what mechanistic information such an experiment would provide. We have therefore elected not to carry out this experiment at this time, in the context of the current study.

In figure 3 the authors provide some evidence indicating that the actin tails might have an endocytic origin but that they are not attached to membranes. However the evidence is rather indirect and certainly not conclusive. In this context, the authors show in figure 3A that Abp1 forms tails that detach from the plasma membrane in the myo5-TH1Δ mutant. However, the authors show in figure 1 that Abp1 stays at the plasma membrane

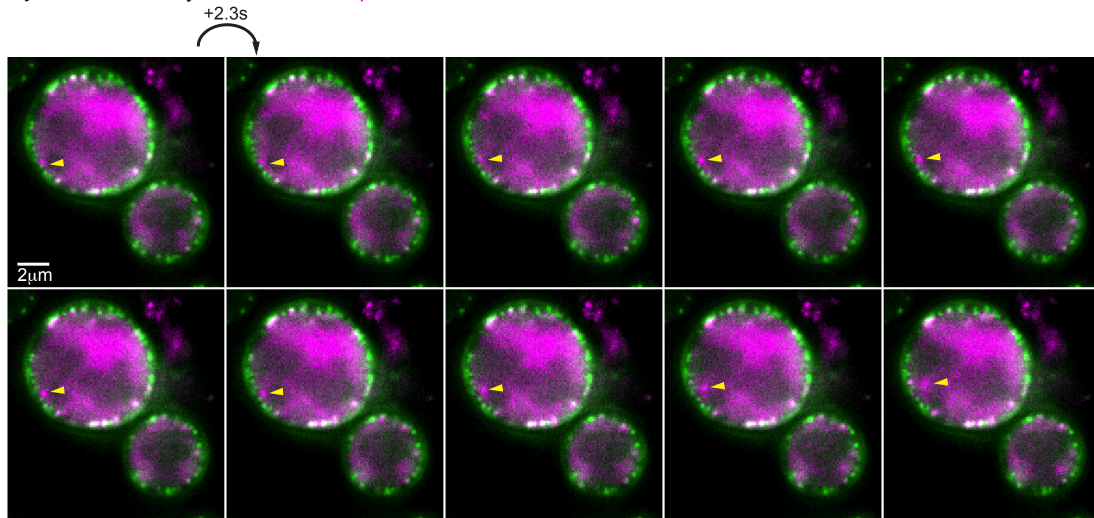
in the same mutant. How frequent is one phenotype as compared to the other? Is the treatment with CK-666 followed by its wash-out exacerbating the formation of Abp1 tails? It would be better to perform the CK-666 wash-out experiments with the myo5-TH1Δ mutant rather than the knock out.

The actin tails have been observed to detach from endocytic sites in every direction: towards the cytoplasm in epifluorescence movies and parallel to the plasma membrane in TIRFM movies. This behavior is not any more common after CK-666 treatment.

In the early figures of the paper, we demonstrate that the phenotype of *myo5Δ* cells closely resembles the phenotype of *myo5-TH1Δ* cells. We conducted CK-666 washouts in *myo5Δ* cells because we reasoned that the clean knockout would be considered by readers to be the more obvious way of eliminating membrane-bound Myo5. However, the result can also be replicated in *myo5-TH1Δ* cells. Below are kymographs from CK-666 washout experiments performed with the myo5-TH1Δ mutant, along with a montage of the movie from which the CK-666 washout kymograph was derived:



*myo5-TH1*Δ-13Myc,Sla1-GFP, Abp1-mRFP



The conclusion that the actin tails are not associated with membranes of endocytic origin is not really supported by the data neither. To sustain this view, the authors indicate that the endocytic coat is not present in the actin tails. However, the coat could prematurely disassemble before internalization in the mutant. Indeed, in the figure 3D CK-666 wash-out experiments, Sla1 seems to slightly internalize and fade away as Abp1 goes into the cytosol in the myo5Δ mutant. Is this always the case?. It would be more conclusive in this context to follow endocytic cargo. On the other hand, the authors state that there is only residual internalization of membrane in the mutant and as a consequence, endocytosed membranes cannot seed actin polymerization. However, this residual internalization is about 20 % as compared to the wild type and therefore, internalized membranes could accumulate in time and indeed seed persistent actin polymerization. Double labeling experiments with Abp1-GFP would be needed to properly rule out this possibility.

The conclusion that actin tails are not associated with membranes is supported by the data, although we admit that the conclusion is not demonstrated definitively. Proving absence of membranes through any type of microscopy is difficult. We did initially try to follow an endocytic cargo (fluorescently tagged alpha factor). However, we could not reliably follow internalization of individual cargo-filled vesicles by epifluorescence microscopy in wild-type cells. Reliably observing such events in the past has required TIRFM, disallowing observation of vesicle movement off of the plasma membrane (Toshima *et al., PNAS,* 2006). We therefore elected to follow an endocytic coat protein. Coat proteins have previously been shown to reliably report on the location of the vesicle during endocytosis (see, for example, Kaksonen, Toret, and Drubin*, Cell,* 2005) while being considerably easier to image, which is crucial for an experiment with many

“moving parts”. The residual 20% dye internalization observed in FM4-64 pulse-chase experiments is likely to result from either leakiness of the plasma membrane or internalization through non-clathrin-mediated endocytic pathways (Prosser *et al., JCB,* 2011). The *sla2Δ* mutant, considered to be essentially completely defective in clathrin-mediated endocytosis, also internalizes dye to a similar degree (Peng *et al., Dev. Cell,* 2015). Although we feel that our manuscript presents the most parsimonious interpretation of our data, we have nonetheless added a sentence to the text acknowledging that other interpretations are possible (bottom of page 9).

With regard to the Sla1-GFP signal flickering upon actin assembly in figure 3D, yes, the Sla1-GFP intensity fluctuation is a fairly regular occurrence in these movies when F-actin is present at endocytic sites for prolonged periods. We interpret this fluctuation to be due to recruitment of endocytic disassembly factors (such as the Ark1/Prk1 kinases) by F-actin, as reported in Toret *et al.* 2008 (*Traffic*). When the actin comets then move away from the plasma membrane, they presumably pull the disassembly factors with them, leading to the observed restoration of Sla1-GFP signal on the plasma membrane. We have added a sentence describing our interpretation of this phenomenon.

Reviewer #2 (Comments to the Authors (Required)):

This manuscript describes the role of Myosin I as a membrane anchor in endocytosis in budding yeast. In MyoI null or tailess mutants, actin is nucleated by Arp2/3 at the surface, but the endocytic vesicle fails to invaginate, and the actin forms a comet tail that often moves into the cell interior with some WASP. The membrane binding portion of Myosin I but not the motor domain is responsible for this anchoring function. These studies thus nicely define a function of Myosin I in mooring assembly factors to the plasma membrane for proper organization and force production required for efficient endocytosis.

In general, this is a very well written and interesting manuscript with a significant message. The data are strong and of technically high quality. All the main points of the paper are well supported. This will likely be an defining, impactful paper in the field and therefore is suitable for publication in JCB. I only have minor comments.

We thank the reviewer for their positive assessment of our manuscript.

Specific comments

The behavior of the actin comet tails/ waves should be described more fully at a quantitative level. In particular, it is not so clear how rare or common these events are. Although it is stated that every cell exhibits some wave or comet actin, what percentage of the patches end up forming one of these structures or some other structure, like an actin dot or no actin? Does the comet all form after a certain time period relative to Sla1 dynamics? Do the comets have a limited lifetime?

Previous reports indicate that essentially all Sla1 puncta eventually assemble F-actin in wild-type cells (Kaksonen, Sun, and Drubin*, Cell,* 2003). This also appears to be true for the mutants described in this manuscript. We have now made this observation more clear in the text. We have also quantified the delay between Sla1-GFP recruitment to endocytic sites and actin assembly, and we now report these numbers in the Results.

The word "yeast" is only mentioned once in the title/abstract. To distinguish from other types of yeast, term "budding yeast" or "Saccharomyces cerevisiae" should be used.

We apologize for this omission. We have now made it more clear in the Abstract that this study was conducted in *Saccharomyces cerevisiae*. We have also added the designation “budding” in front of all instances of the word “yeast.”

Reviewer #3 (Comments to the Authors (Required)):

Authors investigate roles of type 1 myosin, Myo5, in clathrin-mediated endocytosis in yeast. This work builds on a previous study from this lab, in which Lewellyn et al. have identified a number of critical domains within a multiprotein actin-nucleating complex that controls actin-mediated internalization of endocytic pits. These critical domains include the Myo5 motor and its membrane-targeting TH1 domain. The current work focuses primarily on the roles of Myo5-dependent membrane targeting of the actin nucleation complex. The main finding here is that Myo5 restricts actin polymerization to endocytic sites by targeting of the nucleation complex to the membrane via the TH1 domain. This targeting allows for efficient internalization. In the absence of Myo5-dependent targeting, actin nucleation machinery detaches from the membrane and travels around the cell in the form of comets. This study provides important new insights into functions of class 1 myosin in endocytosis. The work is carefully done and analyzed. Although the study is performed in yeast, the conclusions can be applicable to other cell types. I have relatively minor comments that should be addressed.

We thank the reviewer for closely reading our manuscript.

1. Some of the conclusions in this work overlap with those in the previous paper (Lewellyn et al. 2015). This fact does not degrade significance of the current follow-up study, which is more advanced. However, it would be helpful if authors made more connections and comparisons between these two studies, so that the current advance was more obvious.

We thank the reviewer for recognizing that this study represents a significant conceptual advance over our 2015 study. We have added additional citations to the Lewellyn *et al.* (2015) paper and have also added a line in the fourth-to-last paragraph of the Results and Discussion section pointing out how the current study extends what was learned in the previous one.

2. Figure 1B: label mutants used in individual panels.

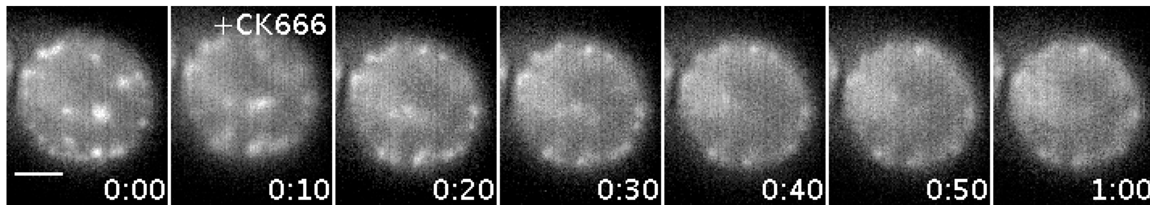
We have added the requested labels

3. Figure 1C: What is the meaning of dots in the graph?

Each dot represents the percentage of Sla1-GFP puncta internalized in a single cell. We have added this information to the figure legend.

4. Figure 2B shows that treatment of cells with CK-666 inhibits cytoplasmic comets, but not cortical patches. Why? Cortical patches do disappear in supplemental figure S1E and F. The concentrations of CK-666 and DMSO for figure 2B are not shown in the figure or in the legend.

This is an interesting observation. For an unknown reason, the cytoplasmic actin comets seem to be more sensitive to CK-666 than the cortical patches. Nevertheless, cortical patches do disappear after slightly longer amounts of time, as can be seen more clearly in this extended version of the montage. Since this figure in the paper is meant to show that the cytoplasmic comets disappear upon CK-666 treatment, we elected to show only the first 5 frames of this montage to showcase the effect of the drug on cytoplasmic comets. We have added the requested concentration information to the figure legend.



5. Data for Bbc1 and Bzz1 are shown in figure 2D and corresponding movies, but are not described in Results.

These panels were previously referred to collectively with the wording "while all members of the WASP/Myosin complex localized…" We thank the reviewer for pointing out that this wording was confusing. The proteins that compose the WASP/Myosin complex are now listed individually at that point in the text.

6. Figures 3D and S2B show that Sla1-GFP does not move inward in myo5Δ mutants after CK-666 washout. However, both figures also show that Sla1 intensity decreases when actin reassembles. Does it happen regularly? If yes, what does it mean?

Yes, the Sla1-GFP intensity fluctuation is a fairly regular occurrence in these movies when F-actin is present at endocytic sites for prolonged periods. We interpret this fluctuation to be due to recruitment of endocytic disassembly factors (such as the Ark1/Prk1 kinases) by F-actin, as reported in Toret *et al.* 2008 (*Traffic*). When the actin comets then move away from the plasma membrane, they presumably pull the disassembly factors with them, leading to the observed restoration of Sla1-GFP signal

on the plasma membrane. Reviewer #1 made a similar comment. We have added a sentence describing our interpretation of this phenomenon.

7. p. 11: "However, unlike Sla1-GFP, which remained on the plasma membrane (Figure 3D), Vrp1-GFP puncta splintered ... (Figure 3E)". How often do they splinter? Always?

Not always. Observing such “splintering” events requires that Vrp1-GFP puncta split apart far enough to be resolved by conventional fluorescence microscopy, which may not always be the case. Since our imaging experiments were conducted in a medial focal plane using epifluorescence microscopy, it also requires that the Vrp1-GFP spot leaves the endocytic site in the plane we are imaging, which also may not always be the case. Nevertheless, we observed this behavior many times across several distinct experiments. We have modified the language reporting the behavior in the manuscript so as not to mislead readers into thinking Vrp1-GFP puncta split at every endocytic site observed.

8. Figure 4G and 5A: Blue shape in 4G are annotated in the legend as "WASP/Myosin complex", but myosin is shown separately. Is it meant to be "WASP/WIP" complex? In 5A, it appears that "yellow bananas" correspond to Myo5, but blue shapes are also labeled as WASP/Myosin complex. In fact, bananas are labeled as Myo5 in the legend, but as Myo5 membrane anchor in the figure, which makes things even more confusing.

We thank the reviewer for pointing out the confusing labeling in these schematics. We have corrected the situation by re-labeling the blue shapes as “WASP complex” in both figure 4G and 5A and in the legend of figure 5.

7. Mund et al. 2018 paper in the reference list has the title typed twice.

We apologize for the error. This citation has been corrected.

8. It would be helpful to have a discussion about how applicable these findings could be for mammalian endocytosis.

We thank the reviewer for this suggestion. We have added a paragraph (third from the end of the “Results and Discussion” section) that discusses similarities between molecular functions of role of budding yeast Myo5 and its human homologue, Myosin 1e, in clathrin-mediated endocytosis.

December 6, 2018

RE: JCB Manuscript #201810005R

Dr. David Drubin
UC Berkeley
16 Barker Hall
Berkeley, California 94720-3202

Dear Dr. Drubin:

Thank you for submitting your revised manuscript entitled "Type I myosins anchor actin assembly to the plasma membrane during clathrin-mediated endocytosis". While I appreciate the concerns of Rev #1, however as Myo5 binding phospholipids is not a major point of your study, but rather that the TH1 domain binds the plasma membrane, and considering the support of the other two reviewers, I find your study suitable for publication in JCB. You are however encouraged to consider the final reviewer comments and make any text revisions you feel appropriate.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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2) Figures limits: Reports may have up to 5 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications (Fig 2 inset, S1 inset). Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be

normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition method for immunoblotting/western blots.

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a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
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g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

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12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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Sincerely,

Anthony Bretscher
Monitoring Editor

Andrea L. Marat
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Even though the authors have improved their manuscript by adding some quantifications, they have not added any data that addresses the main concerns raised in the previous review. Namely, the manuscript demonstrates that the Myo5 TH1 domain is necessary to attach the actin module to the endocytic sites (not really to the membrane, as it is concluded), but they do not demonstrate that this domain is sufficient to do so. Even though, as discussed by the authors, it is unlikely that the TH1 stably binds the Myo/WASP module to the endocytic coat, because Myo5 stays at the plasma membrane as the coat moves into the cytosol in the wild type, the TH1 domain might still be necessary for the Myo/WASP module to properly bind to other endocytic proteins that also stay at the plasma membrane (Bbc1, Bzz1, Vrp1, Las17), not necessarily to the lipids. To properly demonstrate that the function of the TH1 is direct binding to the lipid bilayer, the authors would need to either use point mutations that specifically disrupt the interaction to the lipids, rather than the complete deletion of the domain or the absence of the myosins-I, or show that another lipid anchor domain can substitute for the TH1 domain. Further, to demonstrate that the TH1 domain is sufficient (in addition to necessary) for binding of the Myo/WASP to the endocytic site or the lipid bilayer, the authors would need to demonstrate that attaching the TH1 domain to members of the Myo/WASP module other than Myo5, can rescue at least part of the phenotypes observed in the myo3D myo5-TH1D strain. The authors state that these experiments are either not possible or did not work and therefore, the experiments are still not conclusive. The editorial board might decide that the results as such are interesting for publication in J Cell Biol as a report, but the conclusions need to be rephrased.

Reviewer #3 (Comments to the Authors (Required)):

I am satisfied with the revision except for a few minor issues that could be easily fixed.
1. p. 5, 2nd paragraph: F-BAR should be capitalized (it is currently written as F-bar).
2. p. 7, top paragraph: While presenting quantification of the actin assembly delays in WT and mutant strains, authors use the word "however", as if these numbers are statistically different, which seems unlikely (16+/- 4 sec versus 20+/-7 sec).
3. Some reviewers' comments are addressed only in the letter, but not in the manuscript.

2nd Revision - authors' response: December 20, 2018

Reviewer #1 (Comments to the Authors (Required)):

Even though the authors have improved their manuscript by adding some quantifications, they have not added any data that addresses the main concerns raised in the previous review. Namely, the manuscript demonstrates that the Myo5 TH1 domain is necessary to attach the actin module to the endocytic sites (not really to the membrane, as it is concluded), but they do not demonstrate that this domain is sufficient to do so.

The current manuscript aims to address why a membrane binding myosin is crucial for clathrin-mediated endocytosis. Our previous publication, Lewellyn *et al.* 2015, set out to determine which activities of the WASP/Myosin complex are sufficient for endocytosis. By demonstrating that the entire WASP/Myosin complex can be replaced by a fusion protein containing only a single phospholipid binding domain (the Myo5 TH1 domain), this previous study showed that the Myo5 TH1 domain is sufficient to anchor actin assembly factors to endocytic sites.

Even though, as discussed by the authors, it is unlikely that the TH1 stably binds the Myo/WASP module to the endocytic coat, because Myo5 stays at the plasma membrane as the coat moves into the cytosol in the wild type, the TH1 domain might still be necessary for the Myo/WASP module to properly bind to other endocytic proteins that also stay at the plasma membrane (Bbc1, Bzz1, Vrp1, Las17), not necessarily to the lipids.

Our data exclude the possibility that Myo5 anchors actin through binding to Bbc1, Bzz1, Vrp1, or Las17 by demonstrating that are each of these proteins is also uncoupled from endocytic sites in *myo5-TH1Δ* cells (Figure 2D).

To properly demonstrate that the function of the TH1 is direct binding to the lipid bilayer, the authors would need to either use point mutations that specifically disrupt the interaction to the lipids, rather than the complete deletion of the domain or the absence of the myosins-I, or show that another lipid anchor domain can substitute for the TH1 domain. Further, to demonstrate that the TH1 domain is sufficient (in addition to necessary) for binding of the Myo/WASP to the endocytic site or the lipid bilayer, the authors would need to demonstrate that attaching the TH1 domain to members of the Myo/WASP module other than Myo5, can rescue at least part of the phenotypes observed in the myo3DΔ myo5-TH1DΔ strain. The authors state that these experiments are either not possible or did not work and therefore, the experiments are still not conclusive. The editorial board might decide that the results as such are interesting for publication in J Cell Biol as a report, but the conclusions need to be rephrased.

As discussed in our previous revision letter and in the comment above, these experiments are either infeasible or are included in our previous publication. Nevertheless, we have rephrased a part of the language in the discussion section as the reviewer has requested (p.13):

"Our results indicate that Myo5 is anchors Arp2/3 complex activators to CME sites, most likely through direct membrane-binding, and that this linkage is crucial for maintaining their ordered localization."

Reviewer #3 (Comments to the Authors (Required)):

I am satisfied with the revision except for a few minor issues that could be easily fixed.
1. p. 5, 2nd paragraph: F-BAR should be capitalized (it is currently written as F-bar).

We have corrected this error.

2. p. 7, top paragraph: While presenting quantification of the actin assembly delays in WT and mutant strains, authors use the word "however", as if these numbers are statistically different, which seems unlikely (16+/- 4 sec versus 20+/-7 sec).

These delays between Sla1-GFP arrival and Abp1-mRFP arrival are indeed statistically significant. We have now detailed the statistical tests used in the text.

3. Some reviewers' comments are addressed only in the letter, but not in the manuscript.

The constructive criticisms of our reviewers have been invaluable and have helped us to improve our manuscript immensely. Because our manuscript was already over the character limit for Reports, we were selective about which reviewer comments to address in the text. Responses to reviewer comments that did not change the interpretation of experiments and data that we felt were redundant with data already included were presented in our response to reviewers only. If any of these changes are deemed crucial to the manuscript, we will gladly include them, although doing so will be difficult given character limits.

January 4, 2019

RE: JCB Manuscript #201810005RR

Dr. David Drubin
UC Berkeley
16 Barker Hall
Berkeley, California 94720-3202

Dear Dr. Drubin,

Thank you for contributing your Report entitled "Type I myosins anchor actin assembly to the plasma membrane during clathrin-mediated endocytosis". It is a pleasure to let you know that your manuscript is now accepted for publication in Journal of Cell Biology. Congratulations on this interesting work.

Your manuscript will now progress through image editing, copyediting, and proofing. It is journal policy that authors provide original data upon request. You may contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu), with any questions throughout the process.

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Again, congratulations on a very nice paper. I hope you found the review process to be constructive and are pleased with how the manuscript was handled editorially. We look forward to future exciting submissions from your lab.

Sincerely,

Andrea L. Marat, PhD
Scientific Editor
Journal of Cell Biology