Spatial regulation of clathrin-mediated endocytosis through position-dependent site maturation

Ross Pedersen, Julian Hassinger, Paul Marchando, and David Drubin

Corresponding Author(s): David Drubin, UC Berkeley

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April 13, 2020

Re: JCB manuscript #202002160

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

Dear Dr. Drubin,

Thank you for submitting your manuscript entitled "Cargo licenses maturation of stalled endocytic sites through a regulatory checkpoint". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that all three reviewers found the work interesting and of high overall quality. However, each raised significant criticisms. We think all are reasonable and should be addressed, requiring revision and re-review by the same reviewers. In particular, all reviewers (particularly 1 and 3) pointed to alternative interpretations not considered, and a bit of over-reaching in the claims made. Please caveat as appropriate and provide additional data or analysis to buttress support if possible. Reviewer 3 also pointed to insufficient citation and discussion of previous work by others in mammalian cells. Loerke et al 2009 is cited but not discussed in the context of later cargo control. Puthenveedu et al (Cell 2006) is not cited although this study is on point, having reached a similar overall conclusion and demonstrating invariant kinetics of dynamin recruitment after the "checkpoint".

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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Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

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The 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. * Given the current situation with lab closures we are extending the timeframe for revisions as necessary. *

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,

Mark von Zastrow, MD, PhD
Monitoring Editor
Journal of Cell Biology

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

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

The work by Pedersen et al., performed comprehensive analysis of endocytic proteins in yeast and meticulously mapped their lifetime and variations. They found high variability of the lifetimes of early arriving proteins and poor correlation between early proteins and late ones, for which they hypothesize that a cargo checkpoint might exist. The major strategies the authors used to manipulate cargo concentration to test the role of cargo in such transition are to compare the relative dynamics of CME in bud vs. mother cells, reversal of polarity by osmotic shock and Brefeldin A (BFA) treatment. Although these perturbations are rather complex and not entirely specific for plasma membrane cargo concentration, the common denominator is the cargo concentrations, so the results are consistent with the presence of a cargo-dependent checkpoint for CME to proceed from early assembly to late stages. They next used the well characterized temporal progression of various endocytic proteins to figure out exactly the point after which endocytosis do not proceed. The transition point was identified to be around the time when intermediate coat module Pan1 and Sla1 were recruited. Lastly, they show in strains that are defective in adaptor function, the polarized distribution of CME late module is absent.

Overall I think it is a very comprehensive body of work that provided a lot of quantitative information. The differential degree of robustness of early vs. later module is well documented and quite compelling which is independent of the model. The major caveat I think is the lack of direct evidence on cargo for the model proposed, but I understand this is a technical challenging issue in yeast. In the absence of more acute and cleaner evidences, however, alternative mechanisms could not be excluded. For instance, the depolarization induced Sla1 appearance in the mother cell is potentially consistent with the requirement of actin module in a high turgor pressure state (after water) but not before. In addition, bud and mother cells are likely different in terms of their Cdc42 activities, phosphatidylserine levels (Fairn et al., 2011). How could that affect the interpretation of the results? Cdc42 was mentioned in discussion but was dismissed without any real discussions. I do not think these needs to be experimentally addressed unless the authors have the data. If there are no strong arguments against these, I think the text can be toned down to emphasize more on the polarized dynamics of CME for the two independently regulated phases of endocytosis, which by itself is quite interesting with solid data presented, rather than cargo checkpoints which may be rather controversial.

With these minor reservations on a point that is rather difficult to address, I do support its publication.

Other comments:
1. "We interpreted a correlation between query protein behavior and reference protein behavior to indicate shared protein-protein interactions." Why? Everything in CME can be presumably connected as a protein-protein interaction network. On the other hand, there could be other rate-limiting steps for either the early modules or the later modules that depend on protein-lipid interactions.
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3. Most of the scatter data were fitted using linear regression and the fit was not good even for Abp1 vs. Arc15. Was binning method tried? They may be useful to handle noisy data.
4. The lifetime distributions of Las17 and Sac6 are fairly narrow in Fig S1B, which do not seem to match the visual impression of the scatter plots in Fig 2. One possibility is that the majority of the data are concentrated in the neighborhood of a very small range (i.e. the yellow blob, ~ 20 sec for Las17 and ~ 10 sec for Sac6). One wonder whether the linear regression approach makes sense as the small percentage of the outliners may contribute more to how good the fit is than the majority of the data around the average value.
5. The author thinks Ede1-GFP-marked early CME sites are not polarized in Fig. 5A. I am not sure about that. Ede1 does seem concentrated in the bud as well.
6. Is it possible to show redistribution of endocytic cargo from the plasma membrane of buds in highly polarized cells to the plasma membrane of mother cells after water shock directly? It would be useful to compare how fast is it compared to the observed changes in Sla1.

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

Pedersen and Hassinger et al. describe a regulatory checkpoint in the CME pathway in S. cerevisiae that is released by the presence of endocytic cargos. Through a quantitative, systematic TIRF imaging based analysis of CME proteins, they characterize the behavior of many CME proteins and define two phases of CME (initiation and internalization). They identify a regulatory checkpoint between these two phases that slows the maturation of early/initiation phase endocytic sites. The authors then determine that the availability of endocytic cargo regulates endocytic site maturation through this checkpoint. They further demonstrate this finding by using perturbations such as osmotic shock and brefeldin A treatment to acutely regulate endocytic cargo availability and hence their identified endocytic checkpoint. While the concept of cargo regulating CME dynamics is not entirely new compared to studies the authors reference and more, this study provides novel insights and a significant conceptual advance through the detailed description and analysis of the many endocytic proteins involved in CME that underlie this cargo regulation.

In general, this study is of high quality and uses automated unbiased analysis to extract behaviors of many endogenously labeled proteins. The manuscript is sound and the claims made by the authors are indeed supported by the experiments shown. I only have a few comments listed below.

Specific comments:

1. In Figure 5, how are the authors determining that Sla-1 is polarized and Ede-1 is not (page 16, lines 6-8). Looking at the representative images, it looks like both Sla-1 and Ede-1 are polarized to the bud though Ede-1 less so. How is this determined/calculated? Perhaps what is most striking is the lack of Sla-1 at endocytic sites in the mother. I don't think seeing polarization of one and not the other is essential for their conclusions, but it seems overstated at present. I think the Sla1/Ede1 site ratio used in subsequent figures is more accurate and informative.

Relatedly, it seems that the strongest evidence that lack of cargo stalls endocytic sites in the mother (Figure 5B). Therefore, it's odd to me that there is a focus on the polarization of late proteins in the bud. Seems equally important that the early endocytic sites in the mother are stalled whereas in the bud, they are not.

2. If cargo proteins are acting to release a brake on CME and 7△ mutant can no longer sense cargo, what is the lifetime of endocytic events in the mother cells of the 7△ mutant? Have CME events shifted from predominantly persistent/partial as shown for WT in Figure 5B-D?

3. What is the CME brake? The 7△ data suggests one or more of these proteins is involved in sensing cargo. Do single mutants of any of these proteins recapitulate the 7△phenotype or is this likely to be an effect of many proteins? Are any of the 7△mutant proteins known targets of Hrss25? Based on previous Drubin lab work, Ede1 is a target of Hrss25? What do endocytic sites in Ede1 mutants look like? Do the authors have any further insight into the molecular brake or can they speculate on the nature of the checkpoint step(s)?

Minor points:

1. There are a lot of protein names to keep track of. When panels of proteins are shown such as in Figures 1F, 2D, 3D for example, could general description labels be added such as: early, late arriving/coat proteins, and scission/disassembly.

2. For Figure 2 and 3, it seems odd that an example graph for a scission/disassembly protein is not shown in the main figure, but in the supplement. Why not include the Ark1 or Rvs167 graph in the main figure to have an example from each phase?

3. Typo on page 18, line 23: through instead of though.

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

The paper reports analysis of clathrin pit formation in the budding yeast Saccharomyces cerevisiae, using fluorescence microscopy to investigate the role of cargo in the recruitment dynamics of endocytic coat proteins to the site of clathrin-dependent endocytosis. By tracing an impressively large number (~ 24) of fluorescently tagged endocytic proteins expressed at physiological levels from endogenous loci, Pedersen et al have obtained a systematic set of pairwise temporal interactions in relation to a subset of reference proteins. The key outcomes reported are: (1) extension to yeast cells of previous studies in mammalian cells showing that cargo stabilizes coated pit formation; (2) confirmation of the existence of three main endocytic 'modules', as previously described in yeast using ensemble-imaging approaches; (3) detection in yeast of significant fluctuations in the temporal recruitment patterns of coat components during pit formation, as previously observed in both yeast and mammalian cells.

The wealth of data generated in this study merits publication, particularly because of the importance of the topic to the community engaged in membrane traffic studies. As explained below, however, publication will be appropriate only after the authors have made major modifications to the strength of their stated conclusions and to their description of how the current work relates to earlier work. In particular, the authors must (1) substantially modulate the claims they make about the role of cargo in coated pit formation, and (2) assess more objectively the similarities and differences in the molecular organization of clathrin-mediated endocytosis in yeast cells and mammalian cells.

Editorial comments:
In the abstract, the authors incorrectly state that 'previous studies in mammalian cells suggested that cargo ensures its own internalization by regulating either CME initiation rates or frequency of abortive events'. In fact, Loerke et al and Liu et al confirmed and extended earlier work by Ehrlich et al, showing quite clearly that cargo loading stabilizes nascent clathrin coated pits and that cargo is present in maturing coated pits but absent in abortive events (Ehrlich 2004, Loerke, 2009, Liu 2010). Substantially increasd TfR expression at the plasma membrane does not change the initiation frequency or maturation rate of coated pits (Loerke, 2009), although cargo clustering might lead to more efficient clathrin coated vesicle formation (Liu 2010, Lax 1991, von Zastrow 2006, Cureton 2009).

In the abstract, the authors state they have uncovered 'maturation through a checkpoint in the pathway as the cargo sensitive-step'. This mechanistic conclusion is overstated, given the experimental evidence presented, and disingenuous, since precisely the same general notion was put forth in the studies in mammalian cells cited above.

Beginning with the title and continuing throughout key sections of introduction and discussion, the authors present their results in yeast as if the molecular mechanisms were generalizable and directly applicable to the clathrin endocytic pathway in mammalian cells. But maturation and scission of clathrin pits in mammalian cells involves steps, such as dynamin recruitment, that are absent in yeast. Thus, careful distinctions need to be drawn between steps and mechanisms likely to be similar and those likely to be different, even if the resulting regulatory properties have physiological similarities. For example, in yeast (this study), cargo might reasonably release an early block in coated pit formation, but a different mechanism appears to apply in mammalian cells, as there are no 'holding' intermediates during coated pit assembly (Erlich 2003, Loerke 2009, Liu 2010). A more accurate title would therefore be: 'Cargo licenses maturation of stalled endocytic sites in yeast through a regulatory checkpoint'. It is reasonable to state that it was important to test in the yeast system, the 'prediction' that 'longer assembly times at endocytic sites would lead to greater abundances' (p12, l 5). This 'prediction' has already been ruled out in published work on mammalian cells.

Experimental comments:

(1) The first (and principal) conclusion of this study concerns the role of cargo in coated pit maturation. None of the observations from the imaging studies reported here directly link presence of cargo to the maturation properties of a nascent coated pit, however, unlike the imaging studies previously carried out in mammalian cells. The authors have instead introduced global perturbations affecting the relative localization of cargo (stages during cell division, osmotic shock, interference with exocytosis) to make mechanistic inferences about the dynamics of coated pit formation and its regulation. As a result, the data presented do not show whether differences in coat formation are direct consequences of specific molecular interactions between cargo and the early endocytic machinery or indirect effects in response to the strong physiological perturbations. The conclusions should therefore be qualified with appropriate interpretive reservations.

(2) The inference, that presence of cargo modulates or releases a checkpoint, comes from correlative data such as those shown in Fig. 3. In almost all cases, however, the R2 values are less than 0.17. This relatively modest correlation is not generally considered statistically significant. There is indeed some correlation of the R2 values with the stage of pit maturation, but it is a stretch to postulate the existence of a 'checkpoint', rather than simply to suggest such a mechanism as a potential explanation. Moreover, the term 'checkpoint' is itself merely a conceptual restatement of the observations, rather than a specific mechanism.

(3) Quantitative fluorescence microscopy should include determination of number of fluorescent molecules particularly when the imaging is done at diffraction-limited sites in organisms in which the fluorescent protein was expressed at physiological levels. This is technically feasible for imaging studies and crucial for mechanistic understanding, as emphasized by Pollard (Pollard 2005, Sirotkin 2010) and others, both for yeast and for mammalian cells. The authors should try to meet these standards, since their experiments consist almost entirely of quantitative analysis data from fluorescence microscopy imaging.

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

The work by Pedersen et al., performed comprehensive analysis of endocytic proteins in yeast and meticulously mapped their lifetime and variations. They found high variability of the lifetimes of early arriving proteins and poor correlation between early proteins and late ones, for which they hypothesize that a cargo checkpoint might exist. The major strategies the authors used to manipulate cargo concentration to test the role of cargo in such transition are to compare the relative dynamics of CME in bud vs. mother cells, reversal of polarity by osmotic shock and Brefeldin A (BFA) treatment. Although these perturbations are rather complex and not entirely specific for plasma membrane cargo concentration, the common denominator is the cargo concentrations, so the results are consistent with the presence of a cargo-dependent checkpoint for CME to proceed from early assembly to late stages.

We appreciate this reviewer's understanding that, while we did not specifically alter plasma membrane cargo concentration, we did use a battery of manipulations, the "common denominator" of which being cargo concentration. We drew our conclusions based on results from these diverse experiments. Concerns from this reviewer and others make it clear that we stated some conclusions too strongly and that we did not clearly explain our logic of using multiple perturbations that share a common theme of altering plasma membrane cargo concentration. We have altered the text to better reflect the caveats of our results and to more explicitly outline the reasoning that led to our conclusions.

We would like to clarify one point. Comparisons of CME dynamics in buds vs. mother cells were not used extensively in this study. Instead, we compared endocytic dynamics in mother cells that had either large or small buds. When mother cells have small buds, the cell polarity machinery directs secretion to the bud tip. At this stage of the cell cycle, we observed slow maturation of early endocytic sites into late endocytic sites in mothers, accounting for the observation of few late sites but plentiful early sites in mothers in this condition. When mother cells have large buds, the cell polarity machinery directs secretion to both sides of the bud neck, so endocytic cargos are delivered to both the bud and the mother plasma membrane. At this stage of the cell cycle, we observed fast maturation of early endocytic sites into late sites in mothers, accounting for the relative parity of early and late endocytic sites in these mothers.

They next used the well characterized temporal progression of various endocytic proteins to figure out exactly the point after which endocytosis do not proceed. The transition point was identified to be around the time when intermediate coat module Pan1 and Sla1 were recruited. Lastly, they show in strains that are defective in adaptor function, the polarized distribution of CME late module is absent.

Overall I think it is a very comprehensive body of work that provided a lot of quantitative information. The differential degree of robustness of early vs. later

module is well documented and quite compelling which is independent of the model. The major caveat I think is the lack of direct evidence on cargo for the model proposed, but I understand this is a technical challenging issue in yeast. In the absence of more acute and cleaner evidences, however, alternative mechanisms could not be excluded. For instance, the depolarization induced Sla1 appearance in the mother cell is potentially consistent with the requirement of actin module in a high turgor pressure state (after water) but not before. In addition, bud and mother cells are likely different in terms of their Cdc42 activities, phosphatidylserine levels (Fairn et al., 2011). How could that affect the interpretation of the results? Cdc42 was mentioned in discussion but was dismissed without any real discussions. I do not think these needs to be experimentally addressed unless the authors have the data. If there are no strong arguments against these, I think the text can be toned down to emphasize more on the polarized dynamics of CME for the two independently regulated phases of endocytosis, which by itself is quite interesting with solid data presented, rather than cargo checkpoints which may be rather controversial.

We thank the reviewer for this constructive criticism. We agree that alternative interpretations were incompletely discussed due to space considerations.

It should be noted, however, that the actin module is deployed to every endocytic site in budding yeast, whether or not turgor pressure is artificially elevated (Kaksonen, Sun, and Drubin, *Cell*, 2003, among many others). Models in which late endocytic sites appear in mothers after osmotic shock due to recruitment of the actin module to sites that would normally not require actin can therefore be discarded.

We also agree that cell polarization is much more complex than locally concentrated cargo, and that polarized lipids and protein distributions, especially Cdc42, could also affect endocytic progression. We note, however, that phosphtidylserine has previously been implicated in endocytic initiation rather than endocytic progression (Sun and Drubin, *J. Cell. Sci.*, 2012). While mutants that cannot synthesize phosphatidylserine are depolarized, they also have broadly abnormal plasma membrane phospholipid compositions, complicating interpretation (Fairn *et al., Nat. Cell Biol.* 2011). Similarly, Cdc42 itself has never been directly linked to formation or maturation of endocytic sites, although many research groups including ours have looked for a connection (our own unpublished observations). It is worth noting that while yeast Cdc42 appears to regulate formins to assemble cytoplasmic actin cables, the Arp2/3 activator Las17, yeast WASP, does not depend on Cdc42 for its activity at endocytic sites.

It should also be noted that we never quantitatively compared behavior of endocytic markers in mothers with their behavior in buds in our manuscript, wary as we were of the many molecular differences between mothers and buds. Instead, quantitative comparisons were conducted on endocytic sites localized to the mother cell plasma membrane in situations where the polarized delivery of cargo away from the

mother was experimentally varied through three orthogonal methods. While alternative explanations cannot be dismissed outright, the most parsimonious interpretation of our data is that cargo controls endocytic progression.

We have addressed this comment by (1) revising the Abstract and introduction to deemphasize framing of our results as being clear evidence of a cargo checkpoint and (2) toning down the language of cargo checkpoint conclusions within the Results section, and (3) expanding the discussion of alternative interpretations of our data in the Discussion section.

With these minor reservations on a point that is rather difficult to address, I do support its publication.

Other comments:
1. "We interpreted a correlation between query protein behavior and reference protein behavior to indicate shared protein-protein interactions." Why? Everything in CME can be presumably connected as a protein-protein interaction network. On the other hand, there could be other rate-limiting steps for either the early modules or the later modules that depend on protein-lipid interactions.

The reviewer raises a good point, and we have removed this interpretation from the text.

2. Ede1 appears to be much earlier than the rest of the proteins (~20 sec earlier than syp1 for example) tested in Fig 1 E/F but it has similar timing compared to syp1 in Fig 1D. The author mentioned that it could be due to Ede1 being the brightest of the group. It will be good to add a discussion about to what extent the differences in brightness can lead to a timing difference of ~20 sec in the quantification method. In addition, statistical tests for the rest of the data would be useful (which differences are significant?), especially if the authors intend to use this new dataset as the most updated standard in terms of the temporal progression of yeast endocytosis.

The reviewer raises an interesting point. Different proteins accumulate to different levels and at different rates at endocytic sites, which can influence when our software first detects a signal, particularly for especially "dim" proteins. Thus, comparisons between very bright proteins (like Ede1) and very dim proteins (like the rest of the early arriving endocytic proteins including Syp1) are particularly difficult. We have expanded the section of the text in the Results to include these caveats to our dataset.

Variation in the accumulation profiles of different endocytic proteins is also why we were cautious about using the kinds of statistical tests normally deployed by cell biologists (t-tests, ANOVA, etc.) on our high throughput dataset. Because we were not measuring the same response while varying the treatment condition, we felt that direct comparisons between values measured on different proteins were

inappropriate. We instead elected to provide as many descriptive statistics as possible in supplemental tables.

Finally, it is important to note that, because of caveats mentioned above, we did not mean for our dataset to become the updated standard in terms of temporal progression of CME. Minute differences in timing are likely better detected in studies dedicated to mechanistically dissecting one or two events in the CME pathway. Our intention with the first figure of the current manuscript was to demonstrate that our high throughput method yields data that agree remarkably well with previously published results. We felt that the more detailed analysis that we carried out was only appropriate after we had performed this sanity check, given the mature state of the yeast CME field.

3. Most of the scatter data were fitted using linear regression and the fit was not good even for Abp1 vs. Arc15. Was binning method tried? They may be useful to handle noisy data.

The choice to show all of our data was intentional. We were surprised by how variable/noisy the data were given the deterministic way that the process of CME has been described in the past. We wanted readers of our manuscript to appreciate this variability. We therefore elected not to average out the noise.

4. The lifetime distributions of Las17 and Sac6 are fairly narrow in Fig S1B, which do not seem to match the visual impression of the scatter plots in Fig 2. One possibility is that the majority of the data are concentrated in the neighborhood of a very small range (i.e. the yellow blob, ~ 20 sec for Las17 and ~ 10 sec for Sac6). One wonder whether the linear regression approach makes sense as the small percentage of the outliners may contribute more to how good the fit is than the majority of the data around the average value.

We apologize for this confusion. In Figure 2, the scale of the X-axis is different for each graph, so the Ede1 graph on the left is 140s wide, while the Las17 graph is only 60s wide and the Sac6 graph is narrower still, just 30s wide. Thus, although the plots in Figure 2 are scaled to display the full spread of the data, the spreads of the data for Las17 and Sac6 are, in fact, narrow relative to Ede1.

The reviewer is correct that the majority of the data are concentrated in the "yellow blob" region. The colors of the points reflect how dense the data points are in the surrounding region. We have more clearly defined the meaning of data point colorization in the methods section and have also added contour lines to the plots in the supplement to more clearly demonstrate where the majority of the data are concentrated.

While the linear trends that we report are far from perfect correlations, we are convinced that our approach is justified. The correlations that we observed varied in biologically relevant ways, because proteins from the same endocytic modules

showed similar correlative relationships, giving credence to our analysis method. We also anticipate that the linear regression approach will be more accessible to an audience of cell biologists than more complex analyses, improving the readability of our manuscript.

5. The author thinks Ede1-GFP-marked early CME sites are not polarized in Fig. 5A. I am not sure about that. Ede1 does seem concentrated in the bud as well.

The data presented in Figure 5A are maximum intensity projections which make the small, highly curved bud appear more crowded with Ede1 sites than it really is in single focal plane images. Nevertheless, there is some polarization of Ede1, just markedly less than for Sla1. We have edited the text to reflect this.

6. Is it possible to show redistribution of endocytic cargo from the plasma membrane of buds in highly polarized cells to the plasma membrane of mother cells after water shock directly? It would be useful to compare how fast is it compared to the observed changes in Sla1.

Live imaging of any endocytic cargo in budding yeast is difficult. Individual cargoes tend to be too dim to reliably detect at endocytic sites, and we are unaware of a method to simultaneously image all cargos.

While our experiments indicate that osmotic shock results in dispersal of localized secretion, prior work has also hinted that acute glucose depletion creates endocytic cargo by triggering widespread, nonspecific internalization of plasma membrane proteins (Lang *et al.*, *J. Biol. Chem.*, 2014). Since this effect does not require membrane trafficking, cargo redistribution is likely to be even faster than the redistribution of secretory traffic that we describe.

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

Pedersen and Hassinger et al. describe a regulatory checkpoint in the CME pathway in S. cerevisiae that is released by the presence of endocytic cargos. Through a quantitative, systematic TIRF imaging based analysis of CME proteins, they characterize the behavior of many CME proteins and define two phases of CME (initiation and internalization). They identify a regulatory checkpoint between these two phases that slows the maturation of early/initiation phase endocytic sites. The authors then determine that the availability of endocytic cargo regulates endocytic site maturation through this checkpoint. They further demonstrate this finding by using perturbations such as osmotic shock and brefeldin A treatment to acutely regulate endocytic cargo availability and hence their identified endocytic checkpoint. While the concept of cargo regulating CME dynamics is not entirely new compared to studies the authors reference and more, this study provides novel insights and a significant conceptual advance through the detailed description and

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Specific comments:

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We thank the reviewer for raising this point. The data presented in Figure 5A are maximum intensity projections, which make the small, highly curved bud appear more crowded with Ede1 sites than it actually is in single focal plane images. Nevertheless, there is some polarization of Ede1, just markedly less than for Sla1. We have edited the text to reflect this.

The reviewer is correct in noting that the most striking phenotype reported in Figures 5-6 is the stalling of endocytic sites in the mother cell. In fact, all quantitative analysis was performed by comparing mother cells where endocytosis was stalled or not stalled. We have edited the text to make the fact that analysis was carried out exclusively in mother cells more clear.

2. If cargo proteins are acting to release a brake on CME and 7△ mutant can no longer sense cargo, what is the lifetime of endocytic events in the mother cells of the 7△ mutant? Have CME events shifted from predominantly persistent/partial as shown for WT in Figure 5B-D?

The reviewer raises an interesting point. In *7Δ* cells, the lifetimes of the few pre-transition point proteins that remain are shorter, and CME sites turn over more quickly. These data are reported in Brach *et al., Curr. Biol.* 2014. We would love to determine whether CME sites shift from predominantly persistent/partial, but we are unable to conduct this experiment due to the pandemic and current lab closures.

However, because similar data are available in the literature, we have added a sentence to the text pointing out that these previous data are consistent with the notion of a checkpoint being removed.

3. What is the CME brake? The 7△ data suggests one or more of these proteins is involved in sensing cargo. Do single mutants of any of these proteins recapitulate the 7△phenotype or is this likely to be an effect of many proteins? Are any of the 7△mutant proteins known targets of Hrss25? Based on previous Drubin lab work, Ede1 is a target of Hrss25? What do endocytic sites in Ede1 mutants look like? Do the authors have any further insight into the molecular brake or can they speculate on the nature of the checkpoint step(s)?

The *7Δ* phenotype is not significantly more severe than the phenotype of cells that only have *EDE1* knocked out. In *ede1* mutants, endocytic sites are less abundant on the plasma membrane and turn over more quickly, consistent with the possibility that Ede1 is a component of the CME brake. This is an exciting avenue for future research. We have added additional speculation into the nature of the CME brake into the Discussion section.

Both Syp1 and Ede1 are confirmed substrates of Hrr25, other early arriving proteins have not been tested. Identifying the brake is a high priority for future studies.

Minor points:

1. There are a lot of protein names to keep track of. When panels of proteins are shown such as in Figures 1F, 2D, 3D for example, could general description labels be added such as: early, late arriving/coat proteins, and scission/disassembly.

We thank the reviewer for this suggestion. We have added a color code to some of the figures that list many protein names, grouping them as early arriving, coat proteins, etc. We have also added labels to panels A-C of Figures 2, 3, and 4 to give context to the protein names.

2. For Figure 2 and 3, it seems odd that an example graph for a scission/disassembly protein is not shown in the main figure, but in the supplement. Why not include the Ark1 or Rvs167 graph in the main figure to have an example from each phase?

We again thank the reviewer for this suggestion. We have replaced the Sac6 data (panels C for Figures 2, 3, and 4) with the Rvs167 data from the supplement.

3. Typo on page 18, line 23: through instead of though.

Corrected.

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

The paper reports analysis of clathrin pit formation in the budding yeast Saccharomyces cerevisiae, using fluorescence microscopy to investigate the role of cargo in the recruitment dynamics of endocytic coat proteins to the site of clathrin-dependent endocytosis. By tracing an impressively large number (~ 24) of fluorescently tagged endocytic proteins expressed at physiological levels from endogenous loci, Pedersen et al have obtained a systematic set of pairwise temporal interactions in relation to a subset of reference proteins. The key outcomes reported are: (1) extension to yeast cells of previous studies in mammalian cells showing that cargo stabilizes coated pit formation; (2) confirmation of the existence of three main endocytic 'modules', as previously described in yeast using ensemble-imaging approaches; (3) detection in yeast of significant fluctuations in the temporal recruitment patterns of coat components during pit formation, as previously observed in both yeast and mammalian cells.

The wealth of data generated in this study merits publication, particularly because of the importance of the topic to the community engaged in membrane traffic studies. As explained below, however, publication will be appropriate only after the authors have made major modifications to the strength of their stated conclusions and to their description of how the current work relates to earlier work. In particular, the authors must (1) substantially modulate the claims they make about the role of cargo in coated pit formation, and (2) assess more objectively the similarities and differences in the molecular organization of clathrin-mediated endocytosis in yeast cells and mammalian cells.

We appreciate the reviewer's close reading of our manuscript and generally positive assessment. Below we detail how we have altered the manuscript in response to the reviewer's specific concerns.

Editorial comments:
In the abstract, the authors incorrectly state that 'previous studies in mammalian cells suggested that cargo ensures its own internalization by regulating either CME initiation rates or frequency of abortive events'. In fact, Loerke et al and Liu et al confirmed and extended earlier work by Ehrlich et al, showing quite clearly that cargo loading stabilizes nascent clathrin coated pits and that cargo is present in maturing coated pits but absent in abortive events (Ehrlich 2004, Loerke, 2009, Liu 2010). Substantially increasd TfR expression at the plasma membrane does not change the initiation frequency or maturation rate of coated pits (Loerke, 2009), although cargo clustering might lead to more efficient clathrin coated vesicle formation (Liu 2010, Lax 1991, von Zastrow 2006, Cureton 2009).

We apologize for the confusion. The quoted text from the abstract was intended to summarize previous work described by the reviewer. If cargo stabilizes nascent clathrin coated pits and clathrin coated pits without cargo abort, as the reviewer says, isn't it then correct to say that cargo ensures its own internalization by

regulating the frequency of abortive events? Perhaps we are splitting hairs here. We emphasized how our data differed from published studies of mammalian cells to highlight the novelty of our work, but we agree with the reviewer that our results reveal as many similarities between budding yeast and mammalian cells as differences. We have altered the text to deemphasize listed differences between CME in mammalian cells and yeast, and instead focus more attention on the similarities. We have now dedicated a paragraph of the Discussion section to comparing and contrasting modes of CME regulation in yeast and mammalian cells.

As a point of clarification: the reviewer's quote of our text from the abstract was not quite correct. We said that "Previous studies *in yeast and mammalian cells* suggested that cargo ensures its own internalization through regulating CME initiation rates or frequency of abortive events…" The reference to regulation of initiation frequency refers to decades of work on budding yeast that assumed that the polarized distribution of CME sites was due to cargo-accelerated CME initiation in regions where the secretory machinery was active.

In the abstract, the authors state they have uncovered 'maturation through a checkpoint in the pathway as the cargo sensitive-step'. This mechanistic conclusion is overstated, given the experimental evidence presented, and disingenuous, since precisely the same general notion was put forth in the studies in mammalian cells cited above.

We felt that the mechanism that we proposed differed from the situation previously reported in mammalian cells in that we observed widespread stalling of partially assembled endocytic sites in the absence of cargo which, to our knowledge, has not been widely reported in mammalian cells. While the general notion of cargo-mediated control of CME seems similar, the specific mechanism is not precisely the same.

Beginning with the title and continuing throughout key sections of introduction and discussion, the authors present their results in yeast as if the molecular mechanisms were generalizable and directly applicable to the clathrin endocytic pathway in mammalian cells. But maturation and scission of clathrin pits in mammalian cells involves steps, such as dynamin recruitment, that are absent in yeast. Thus, careful distinctions need to be drawn between steps and mechanisms likely to be similar and those likely to be different, even if the resulting regulatory properties have physiological similarities. For example, in yeast (this study), cargo might reasonably release an early block in coated pit formation, but a different mechanism appears to apply in mammalian cells, as there are no 'holding' intermediates during coated pit assembly (Erlich 2003, Loerke 2009, Liu 2010). A more accurate title would therefore be: 'Cargo licenses maturation of stalled endocytic sites in yeast through a regulatory checkpoint'. It is reasonable to state that it was important to test in the yeast system, the 'prediction' that 'longer assembly times at endocytic sites would lead to greater abundances' (p12, l 5). This 'prediction' has already been ruled out in published work on mammalian cells.

We thank the reviewer for this constructive criticism. Taking into account comments from this reviewer and comments from reviewer one, we have changed the title of the manuscript to "Spatial regulation of clathrin-mediated endocytosis through position-dependent site maturation."

We do indeed believe that our findings in budding yeast are likely to apply in more complex eukaryotes owing to the highly conserved nature of the CME pathway. We have nevertheless taken care to point out differences in the budding yeast and mammalian CME pathways per the reviewer's request. Specifically, we added a paragraph to the end of the Discussion section comparing and contrasting the mechanism we describe in the current study with mechanisms that have been described in mammalian cells.

The reviewer specifically mentions that mammalian cells and yeast differ in the involvement of dynamin in CME. We intentionally chose not to take a position on the role of dynamin in CME in the current manuscript, as reports on the role of the dynamin homolog Vps1 in CME are conflicting (Kishimoto *et al., PNAS,* 2011, Smaczynska-de Rooij *et al., J. Cell Sci.,* 2010). Since this study comments minimally on the process of scission, we prefer to eschew comparisons of scission mechanisms.

Experimental comments:

(1) The first (and principal) conclusion of this study concerns the role of cargo in coated pit maturation. None of the observations from the imaging studies reported here directly link presence of cargo to the maturation properties of a nascent coated pit, however, unlike the imaging studies previously carried out in mammalian cells. The authors have instead introduced global perturbations affecting the relative localization of cargo (stages during cell division, osmotic shock, interference with exocytosis) to make mechanistic inferences about the dynamics of coated pit formation and its regulation. As a result, the data presented do not show whether differences in coat formation are direct consequences of specific molecular interactions between cargo and the early endocytic machinery or indirect effects in response to the strong physiological perturbations. The conclusions should therefore be qualified with appropriate interpretive reservations.

The reviewer is right in noting that our perturbations were global and not specific to cargo, although we note that cargo was the common denominator between these various manipulations. Nevertheless, taking into account comments from this reviewer and reviewer one, we have edited the text to weaken claims about cargo regulation of endocytic site maturation, instead elevating conclusions about spatial impact of CME site position within the polarized cells on dynamics.

(2) The inference, that presence of cargo modulates or releases a checkpoint, comes from correlative data such as those shown in Fig. 3. In almost all cases, however, the

R2 values are less than 0.17. This relatively modest correlation is not generally considered statistically significant. There is indeed some correlation of the R2 values with the stage of pit maturation, but it is a stretch to postulate the existence of a 'checkpoint', rather than simply to suggest such a mechanism as a potential explanation. Moreover, the term 'checkpoint' is itself merely a conceptual restatement of the observations, rather than a specific mechanism.

We apologize that our language came off too strong in this section of the paper. We have taken the reviewer's suggestion and simply suggested that a checkpoint is one possible explanation for the data shown in Figures 2 and 3.

(3) Quantitative fluorescence microscopy should include determination of number of fluorescent molecules particularly when the imaging is done at diffraction-limited sites in organisms in which the fluorescent protein was expressed at physiological levels. This is technically feasible for imaging studies and crucial for mechanistic understanding, as emphasized by Pollard (Pollard 2005, Sirotkin 2010) and others, both for yeast and for mammalian cells. The authors should try to meet these standards, since their experiments consist almost entirely of quantitative analysis data from fluorescence microscopy imaging.

The analyses that we carried out would not be affected by converting intensities to numbers of molecules. Molecule counting is sometimes a useful tool for mechanistic studies. For example, knowing how many molecules of Dynamin are present during scission of endocytic vesicles in mammalian cells immediately suggested rich mechanistic conclusions about the structure of dynamin helices involved in scission (Grassart *et al. J. Cell Biol.,* 2014, Coccci *et al., Mol. Biol. Cell,* 2014). On the other hand, measuring relative fluorescence levels is sufficient to make other kinds of mechanistic conclusions, like the ones presented here. Furthermore, specific numbers of various endocytic proteins recruited to CME sites in yeast are already known thanks to studies from our own lab and from others (Sun *et al.*, *eLife,* 2019, Picco *et al., eLife,* 2015). Because molecule counts are not necessary for the analyses we carried out and because these numbers are already known, we chose not to invest time and energy determining exact numbers.

August 28, 2020

RE: JCB Manuscript #202002160R

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

Dear Dr. Drubin:

Thank you for submitting your revised manuscript entitled "Spatial regulation of clathrin-mediated endocytosis through position-dependent site maturation". I am pleased to report that all of the reviewers found your revision substantially improved, and delighted to now recommend acceptance. I agree with the reviewers that your study is elegant and provides a large amount of important information. Two of the reviewers found your revised manuscript to fully address their criticisms. The third reviewer also found it significantly improved but raised two lingering concerns: (1) technical limitations of the imaging methods, particularly regarding quantification of molecule number; (2) appropriate citation of previous work. As for #1, I think your revised manuscript already includes appropriate caveats, and that the conclusions claimed are still strong in light of them. As for #2, I would ask you to consider the reviewer's concerns and exercise your good judgment in preparing a final revision.

Therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. 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.

* While we typically prefer titles that do not included a reference to the model system, if this is an important distinction for the field (as per Rev 3 comment 4) please consider editing your title accordingly.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

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.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
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. * Please note we do not allow supplemental references.

10) * Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental display items (figures and tables). We will be able to give you a bit more space, however we will still need for you to reduce the count a bit. Ensure that in reformatting you correct the callouts in the text. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the

findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

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.

B. FINAL FILES:

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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-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- 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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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of

Cell Biology.

Sincerely,

Mark von Zastrow, MD, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

Thank the authors for clarifications. It makes much more sense now that the main alternative to rule out was cell cycle effect, rather than polarity effects. And I am in full agreement with the authors and appreciate the objective, precise and understated (compared to the overstated literature) writing style in the revised version.

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

The authors have addressed my concerns through their text modifications. This manuscript contains a wealth of information regarding characterization of CME dynamics and identifies novel aspects of CME regulation related to cargo localization. It also raises interesting mechanistic questions that are now more fully addressed in the discussion section. Overall, I am in favor of publication of this work.

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

I still think that because of the wealth of data, this study merits publication. Pedersen and colleagues have addressed a number of questions asked by the other reviewers and myself.

Nevertheless, I now wish to state this clearly, the authors are often disingenuous in how they treat their data and / or published studies, particularly in mammalian systems. I insist it is a disservice and not friendly to colleagues to maintain this attitude.

Let me provide you with some general examples:

1. Their studies significantly centers on connecting cargo recruitment with fate of the endocytic structures. Every single one of their experiments is based on physiological, genetic or pharmacological perturbations that modify the cargo concentration presumably available for uptake at the plasma membrane. The connections made between cargo and fate of endocytic structures is by inference. Unless I missed the point, I believe the authors never determined whether cargo was present on the yeast endocytic structures. The standards in mammalian cells are different, where this is an important component for the studies and/or interpretations. I concede that interpretation of the experiments in yeast might one day be consistent with their current inferences, yet the way

the whole paper is written suggests they have now established a new important facts: that cargo determines the fate of ccps. I point as examples the headings " Maturation through the CME transition point is faster in cellular regions with concentrated endocytic cargo' and 'the delivery of cargo, rather than cell cycle, dictates maturation rate through the regulatory transition point'. This was a specific point made by Ehrlich et al, 2004, summarized at the end of the abstract by stating "Cargo incorporation occurs primarily or exclusively in a newly formed coated pit. Our data lead to a model in which coated pits initiate randomly but collapse unless stabilized, perhaps by cargo capture".

2. In my view it is essential to quantify data obtained by fluorescence microscopy. In the early days quantification meant 'accurate measurements'. Today, the standards have changed, particularly reflecting the availability of genome-edited cells. Drubin and others have been strong proponents for doing so, with effective experiments carried with yeast and now also including mammalian cells.

This is not a trivial point and let me expand: Every modern study in mammalian cells I am aware that traces formation of clathrin coated pits and vesicles finds abortive coated pits. Contrary to the assertion by Pedersen et al, this is true regardless of whether cells express fluorescently tagged clathrin or AP2 using ectopic or genome-editing procedures. To my knowledge there is only one group that failed to observe abundant short lived / abortive coats, and its from work by Drubin's lab (Doyon 2011, Hong, 2015). This problem might be related to a potential lack of sensitivity and/or coupled to proper signal quantification in their imaging conditions such that they missed weak signals (Srinivasan et al PLosBiol 2018).

3. The authors state they were not able to quantify their fluorescence signal, though this won't affect their interpretations. This might or not be true. My non trivial concern comes from the fact that most of their analysis is based on TIRF imaging. I have two problems: first, due to the evanescence field, the fluorescence intensity is strongly dependent on the relative position of the fluorescent emitter with respect to the coverslip. This matters because the estimated thickness of the yeast wall is ~ 110 nm, well within the range of distance dependence. In other words, a coat component at the base of the pit will be vastly more fluorescent than the same one as it moves lets say 100 nm away from the membrane. Others solved this problem by either using glancing illumination or by correcting with WF illumination. Second, unless the corrections are done, it is simply not possible to establish quantitative relationships between molecules, particularly if located at various locations along the optical axis.

4. Finally, while the authors have made efforts to clarify similarities and distinctions of behavior/mechanisms of ccps/ccvs between yeast and mammalian cells, it is my view this is still not correct. For example, the tittle makes no reference at all to the fact that their model, if valid, is specific for yeast and not for mammalian cells. I don't consider this a superficial comment, particularly since I believe there are clear similarities and distinctions between these organisms. Two perhaps non-trivial ones: (1) in contrast to mammalian cells, yeast cells fail to make fully enclosed endocytic clathrin coated vesicles; at best their endocytic coats contain about 13 clathrin triskelia - far less than the ~ 36-60 required for most mammalian endocytic coated vesicles. (2) In yeast, actin dynamics is 100% essential for the functionality of ccps. In mammalian cells, this dependence only shows up in very particular conditions exemplified by high membrane tension.

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We are glad that we have been able to more clearly report our findings having taken this reviewer's comments into account. We thank the reviewer for taking the time to make helpful, constructive suggestions and giving us the opportunity to improve our manuscript.

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We thank this reviewer for the kind words about our manuscript. We agree that this manuscript raises interesting questions, and we look forward to seeing these new mechanistic questions addressed by future research conducted by others and ourselves.

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We are sorry that the reviewer still feels that we have been disingenuous. We did our best to edit the manuscript to satisfy this reviewer's criticisms, but we also endeavored to support the thrust and novelty of our manuscript.

Unfortunately, this reviewer's second round of criticisms are not something that we feel we can or should address beyond what we have done already. Rather than providing counterarguments to our rebuttal, we find this reviewer to have simply re-stated their original concerns. We still feel that our original rebuttal arguments apply. We have nevertheless made additional minor edits to try to address the new concerns raised by this reviewer:

1) In concern 1, the reviewer questions whether cargo is present in yeast endocytic structures. We have added a citation to Toshima et al, *PNAS*, 2006, which demonstrates that, like in mammalian cells, cargo collects in budding yeast clathrin-mediated endocytosis sites early in the process.
2) Also in concern 1, the reviewer takes issue with two of the section titles in our manuscript. We had already edited these section titles in the previous round of revisions to make it clearer that our experiments *correlate* fast maturation of endocytic sites with regions of high cargo concentration. In response to this reiterated criticism, we have edited the second section heading further to read "Polarized cargo delivery, rather than cell cycle stage, dictates maturation rate through the regulatory transition point."
3) In response to the reviewer's 4th concern, we elected not to change the title of the manuscript. The *Journal of Cell Biology* normally discourages mention of the study organism in the title, and doing so would also make our title longer than the allowable length. However, in a previous revision we elected to make prominent mention of our model organism early in the abstract of our manuscript, and we hope that this will clear up any confusion for readers.