Load adaptation by endocytic actin networks

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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.)

RE: Manuscript #E21-11-0589
TITLE: "Load adaptation of endocytic actin networks"

Dear David:

excellent study. Please attend to a few concerns and suggestions that the reviewers had (I find all of their comments very helpful), and I will initiate acceptance without a delay.

Sincerely,
Alexander Mogilner
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Drubin,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

This well written paper makes a substantial contribution to our understanding of how the assembly of actin networks helps drive clathrin-mediated endocytosis. Based on their data the authors argue that: (1) actin assembly drives structural changes in clathrin-coated membrane structures; (2) the nature of these structural changes (i.e. whether shallow-to-U or U-to-omega transitions) depends on the level of membrane tension; and (3) the amount of actin assembled around clathrin-coated structures increases with increased membrane tension, suggesting that load adaptation is important for this process. The super-resolution views of clathrin-coated pits and their associated actin networks are particularly interesting.

Specific comments/conderns:
1. The manuscript presents evidence that Arp2/3 activity assists pit completion, as CK666 causes longer clathrin lifetimes. The later data about clathrin coat height and actin height are less convincing, because it is unclear whether these results are from multiple rounds of treatment or from one experiment. The authors could strengthen their manuscript by bolstering those later results (in Figs 3-4). The authors claim that Arp2/3 inhibition has the opposite effect when under membrane tension than it does under isotonic conditions. This is very intriguing, but a reader cannot tell if the results are replicated sufficiently to support the claim. How many biological replicates are reported in Fig 3H? In other words, how many times was a sample exposed to the treatment (e.g. CK666)? The p-values should be calculated using the number of biological replicates, not the number of pits measured.
2. The authors make a distinction between previous studies of load adaptation in branded actin networks, where the networks generate "pushing" forces and the current work where they suggest that the networks are producing "pulling" forces. This is misleading, since, in both cases, the actin network is most likely under compression (and hence "pushing") even though the topology of the situation leads the authors to think of the membrane as being "pulled" inward.

Sam Lord and R. Dyche Mullins

Reviewer #2 (Remarks to the Author):

In this manuscript, Kaplan et al. use STORM to quantitatively investigate the structure of the actin network at sites of clathrin mediated endocytosis (CME) under varying levels of membrane tension. They observed that CME-associated actin structures with different morphologies and coverages exist with a cell, but that there is no correlation between the shape of the clathrin coat and the extent to which the coat is covered by actin. Notably, they found that in hypotonic solutions, which generate high membrane tension, CME slowed while actin structures had significantly higher heights and covered more of the clathrin coat, suggesting that CME dynamics and actin assembly change in response to increased membrane tension. They also discovered that Arp2/3 is important for the adaptation of CME to high membrane tension in experiments using the drug CK666, which is an inhibitor of Arp2/3.

This paper nicely defines how actin organization responds to changes in membrane tension, and thus fill a gap that exists within the field between existing theories and causal experiments. Their conclusions are supported by rigorous, well-controlled experiments and carefully quantified data which convey their findings. For example, they use membrane tether pulling experiments by atomic force microscopy (AFM) to prove that hypotonic solutions increase membrane tension. They also show that their dosage for CK666 does not mask the effects of high membrane tension, which gives the reader confidence in their experimental setup. Moreover, they are transparent about their reasons for using certain imaging techniques (i.e. AFM over EM) and excluding any data in their analyses. Finally, they comparatively quantify relevant changes in the actin network (i.e. actin height and coverage) as well as CME dynamics (i.e. initiation and completion) in response to changes in membrane tension which supports their aim to examine CME-specific actin networks and how they adapt to increased load.

I have a few addressable concerns with the experiments and interpretations, as well as comments to help clarify data presentation.

Concerns and Questions
• Figured S2 and S10 claims to differentiate the heights of clathrin-associated versus cortical actin, but only two examples of a non-clathrin-associated structure are shown, and no quantification is provided. How were the two "random" areas of the cell surface selected? Can more quantitative data be provided to show a comprehensive range of heights of cortical actin in the non-clathrin-positive regions of the cell (e.g. for regions at the 5% actin intensity threshold selected for the clathrin-positive structures?). This range is shown in the color-coded images but not quantified, and clearly there are many "tall" actin structures that are not clathrin-associated.
• When treating the cells with CK666 the authors investigate how CME dynamics and clathrin coat structure change. Do the authors already have data assessing actin height or coverage under the same conditions? Analyzing how CK666 disrupts the actin network at CME sites could provide additional confirmation that the drug is indeed altering actin assembly associated with the coat changes, and would elucidate the role that Arp2/3 has in the formation of the diverse actin structures defined in this paper. However, if these data are not in hand already it is not critical to do a new experiment.

Figure and Methods clarifications
• The authors should clarify in the first paragraph on p9 what fraction of CCS/CCP were excluded based on the stated criteria

(double, non-circular, small, or large structures)
• Figure 1F: Different y axes makes it difficult to compare the shape indices of distinct coat geometries. Suggest keeping them uniform.
• Figure S2: Suggest including a color bar for z position scale for actin.
• Figure S4B: Please add a label on the X axis e.g. "normalized pixel intensity"
• Figure S4E: Looks like erroneous symbols on the axes.
• Figure S4F: It's not entirely transparent how the overall asymmetry measurement is made from the XZ and YZ projections. Please clarify the calculation.
• Figure S6: What fraction of analyzed structures fall into the categories in A and B? The qualitative description is much less meaningful to the reader without information about the distribution.
• There is a typo in the column label in Fig 2B "hyptotonic".
• There are some "STROM" typos in figure legends.
• Figure S8B is missing a key to show what condition each color represents (presumably [CK666].
• Figure S8D is missing statistical symbols. These are important to show whether the sharp increase in the percentage of persistent associated tracks in cells treated with 100uM CK666 +150 mOsmo is significant or not. I believe these data are not represented there the supplemental tables though it was hard to relate the figures to the tables (see last point).
• In the text, the authors say that the average actin height "dramatically increased" for all endocytic geometries when treated with hypotonic media. First, the changes in actin height are actually fairly modest (mean goes from 130+/-30nm to 160+/140nm), so while they are significant and likely to be biologically important, perhaps they should not be described as "dramatic". In making this statement, they reference figures 4B and C and figures S9E-G, but they do not separate any of the actin height measurements by geometry in these figures. It seems that this idea is conveyed in figure 4E; thus, if the authors intend to use figure 4E to support this idea, they should also cite figure 4E when making this argument.
• In the text, there is a reference to figure S9G when discussing asymmetry of actin and clathrin that should instead reference Figure S9H.
• Please include a scale bar and time stamp (and perhaps an overlay of text describing the movie number and sample) on the supplementary movies in case the ultimate file names do not contain this information (the file names in the editorial management system do not)
• It would be nice to have associated figure panels noted in the tables.

RE: *MBoC* manuscript #E21-11-0589
Rebuttal:

Reviewer #1:

1. The manuscript presents evidence that Arp2/3 activity assists pit completion, as CK666 causes longer clathrin lifetimes. The later data about clathrin coat height and actin height are less convincing, because it is unclear whether these results are from multiple rounds of treatment or from one experiment. The authors could strengthen their manuscript by bolstering those later results (in Figs 3-4). The authors claim that Arp2/3 inhibition has the opposite effect when under membrane tension than it does under isotonic conditions. This is very intriguing, but a reader cannot tell if the results are replicated sufficiently to support the claim. How many biological replicates are reported in Fig 3H? In other words, how many times was a sample exposed to the treatment (e.g. CK666)? The p-values should be calculated using the number of biological replicates, not the number of pits measured.

We thank the reviewer for this comment. Unfortunately, while there are at least three biological replicates for the remaining work in this paper, this is not the case here. The STORM data in Fig. 3H are from three cells under a single treatment (e.g., exposed to CK666 or CK666 + hypotonic shock once). We note that this observation is entirely congruent with findings from three papers published previously, as cited below. While the large number of pits in the experiment gives us confidence about the effect of CK666 in that particular experiment, we agree that this measurement should be interpreted cautiously and presented transparently because of a deficiency in biological replicates. For greater transparency, we added additional qualifications to the main text and figure legend:

"Thus, when Arp2/3 complex activity was inhibited in these cells, more clathrin pits accumulated at a greater height. This observation suggests that more clathrin pits may stall at a later stage of progression, consistent with observations of accumulated coat geometries from actin inhibitor studies (Yarar et al., 2005; Boulant et al. , 2011; Yoshida et al., 2018)."

"Clathrin coat heights when cells were treated with DMSO (n = 154) or CK666 (n = 158) in isotonic media or CK666 in hypotonic media (n = 159). Clathrin coat images for quantitative analysis were collected from at least 3 different cells for each condition from a single experiment."

2. The authors make a distinction between previous studies of load adaptation in branded actin networks, where the networks generate "pushing" forces and the current work where they suggest that the networks are producing "pulling" forces. This is misleading, since, in both cases, the actin network is most likely under compression (and hence "pushing") even though

the topology of the situation leads the authors to think of the membrane as being "pulled" inward.

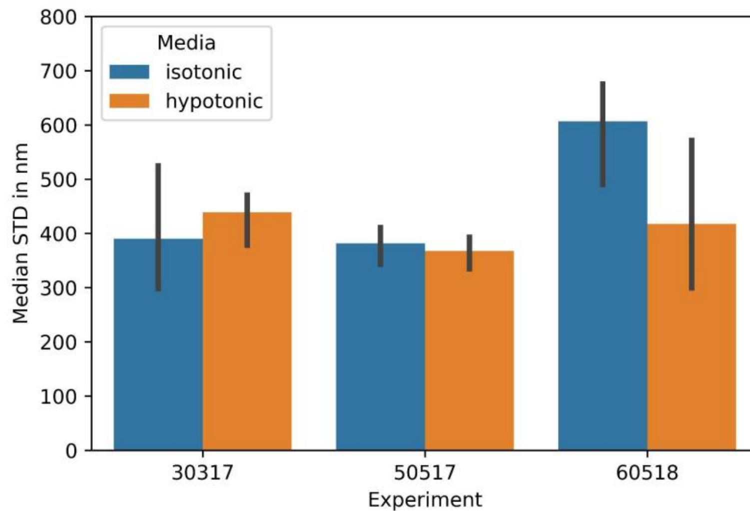
We thank the reviewer for pointing out our ambiguous use of the terms pushing vs. pulling. We have revised the text to distinguish CME from lamellipodia in terms of topology (flat vs changing curvature), rather than pushing vs. pulling, which as the reviewer points out changes depending on the frame of reference (actin vs membrane). The section now reads as follows:

“This phenomenon has been demonstrated in the context of actin networks producing force on the lamellipodium, whose membrane is essentially flat on the length scale of an individual actin branch. However, actin networks can also produce force on membranes that change shape over time, for example during vesicle formation. However, whether actin networks in this context show load adaptation has not been investigated.”

Reviewer #2:

1. Figured S2 and S10 claims to differentiate the heights of clathrin-associated versus cortical actin, but only two examples of a non-clathrin-associated structure are shown, and no quantification is provided. How were the two "random" areas of the cell surface selected? Can more quantitative data be provided to show a comprehensive range of heights of cortical actin in the non-clathrin-positive regions of the cell (e.g. for regions at the 5% actin intensity threshold selected for the clathrin-positive structures?). This range is shown in the color-coded images but not quantified, and clearly there are many "tall" actin structures that are not clathrin-associated.

We thank the reviewer for suggesting that we generate quantitative data reflecting cortical actin heights in non-clathrin-positive regions, which we now do, as described here. The example images in Figure S2 and S10 now serve to better represent visually the accumulation of actin at clathrin vs. the surrounding cortical actin. We performed an analysis of a larger region of interest (approx. 6 um x 6um) from the data set of super-resolved clathrin and actin in cells treated with isotonic and hypotonic media. The larger selected region of interest should better serve as an unbiased representative of the actin cortex for height measurements. A detailed description of the analytical procedure can be found in Materials and Methods/Image Analysis. In Figure S10 we included, among other additional quantitative information, the following plot:



We used the standard deviation of the Z actin coordinates as a metric to report the overall actin height in the selected region of interest. Based on this analysis, we found that the overall height of the actin cortex did not change in isotonic versus hypotonic conditions. We changed the main text and reference to Figure S10 as follows:

"When we quantified actin cell cortex height in randomly selected regions of the cell cortex, we did not observe any increase in cortical actin height, reinforcing the conclusion that any observed effects are specific for CME sites (Figure S10, A - J and P - R)."

2. When treating the cells with CK666 the authors investigate how CME dynamics and clathrin coat structure change. Do the authors already have data assessing actin height or coverage under the same conditions? Analyzing how CK666 disrupts the actin network at CME sites could provide additional confirmation that the drug is indeed altering actin assembly associated with the coat changes, and would elucidate the role that Arp2/3 has in the formation of the diverse actin structures defined in this paper. However, if these data are not in hand already it is not critical to do a new experiment.

We appreciate the reviewer's suggestion. We agree that monitoring actin height under CK666 treatment would be informative. We did immunostain CK666-treated cells for clathrin, but due to technical bottlenecks we did not simultaneously immunostain cells for clathrin and actin in this condition. This would be very interesting to follow up on in future studies and appreciate that the reviewer comments that "it is not critical to do a new experiment".

3. The authors should clarify in the first paragraph on p9 what fraction of CCS/CCP were excluded based on the stated criteria (double, non-circular, small, or large structures).

Thank you for the suggestion. We have included a new sentence on page 10 describing what fraction (86%) of the total clathrin structures were excluded based on uncertainty in the ability to identify a single clathrin-coated structure/pit.

4. Figure 1F: Different y axes makes it difficult to compare the shape indices of distinct coat geometries. Suggest keeping them uniform.

We thank the reviewer for the suggestion. For better data visualization, we pooled the data into one plot and color coded the shallow, U-shaped and omega shape indices.

5. Figure S2: Suggest including a color bar for z position scale for actin.

We thank the reviewer for the suggestion. We added a color bar. We used the same color bar as in main Figure 1 since the ROIs are from the same cell/data.

6. Figure S4B: Please add a label on the X axis e.g. "normalized pixel intensity"

We thank the reviewer for pointing out this accidental omission of a label for the X axis. We have fixed this in the revised version of the manuscript.

7. Figure S4E: Looks like erroneous symbols on the axes.

We thank the reviewer for pointing out this problem and have now corrected the axis labels.

8. Figure S4F: It's not entirely transparent how the overall asymmetry measurement is made from the XZ and YZ projections. Please clarify the calculation.

We thank the reviewer for catching this omission. We include a detailed description of the analysis in three additional pages of methods on quantitative analysis of our STORM and live-cell imaging data. The section on measuring actin asymmetry reads as follows:

"Asymmetry of actin signal around clathrin coat: **To evaluate the asymmetry of the spatial actin organization around the clathrin coat, we determined the difference in the positions of the peak actin and clathrin signals on both x-z and y-z projections of our images (Fig. S1 E). We obtained the center of the clathrin coat in nm by rendering the clathrin super-resolved image into a diffraction limited image, as explained in paragraph 'Selection of clathrin-coated super-resolved structures for image analysis'. We first identified the position of the actin maximum intensity in the x-z projection profile and y-z projection profile in nm. Then we measured the distance of the obtained position to the**

middle position of the clathrin intensity profile. This distance measurement is proportional to the asymmetry of the actin position with respect to clathrin; namely a low distance corresponds to high symmetry and a high distance corresponds to high asymmetry in the position of the actin signal."

9. Figure S6: What fraction of analyzed structures fall into the categories in A and B? The qualitative description is much less meaningful to the reader without information about the distribution.

We thank the reviewer for their interest in the N-WASP STORM data. Unfortunately, because of the low signal of N-WASP in these datasets, obtaining sufficient replicates for quantitative analysis was not possible in this study. Our aim in imaging N-WASP and clathrin in two-color STORM was to reinforce our finding of the actin growth direction from the base of the pit to the tip of the pit. Future studies should investigate the role/function of N-WASP in greater detail.

10. There is a typo in the column label in Fig 2B "hyptotonic".

Thank you for catching this typo. We corrected it in the revised manuscript.

11. There are some "STROM" typos in figure legends.

Thank you for catching this typo. We corrected it in the revised manuscript.

12. Figure S8B is missing a key to show what condition each color represents (presumably [CK666].

We apologize that the color code legend was missing in the figure and in the legend. We added a color-coded legend in the figure and mentioned the color code in the figure legend.

13. Figure S8D is missing statistical symbols. These are important to show whether the sharp increase in the percentage of persistent associated tracks in cells treated with 100uM CK666 +150 mOsmo is significant or not. I believe these data are not represented there the supplemental tables though it was hard to relate the figures to the tables (see last point).

We apologize for the missing statistics symbols and have now included them in the Figure. We also provide the data in an Excel table.

14. In the text, the authors say that the average actin height "dramatically increased" for all endocytic geometries when treated with hypotonic media. First, the changes in actin height are actually fairly modest (mean goes from 130+/-30nm to 160+/140nm), so while they are significant and likely to be biologically important, perhaps they should not be described as "dramatic". In making this statement, they reference figures 4B and C and figures S9E-G, but they do not separate any of the actin height measurements by geometry in these figures. It seems that this idea is conveyed in figure 4E; thus, if the authors intend to use figure 4E to support this idea, they should also cite figure 4E when making this argument.

W*e thank the reviewer for this suggestion. We removed the word “dramatically”. Also, we now reference Fig. 4E as the reviewer suggested.*

15. In the text, there is a reference to figure S9G when discussing asymmetry of actin and clathrin that should instead reference Figure S9H.

We thank you for catching this. We corrected it in the revised manuscript.

16. Please include a scale bar and time stamp (and perhaps an overlay of text describing the movie number and sample) on the supplementary movies in case the ultimate file names do not contain this information (the file names in the editorial management system do not)

We apologize to the reviewer for the missing information in the movies. We now added the missing information as suggested above.

17. It would be nice to have associated figure panels noted in the tables.

We thank the reviewer for the suggestion and added the information of the associated figure panels to the tables.

RE: Manuscript #E21-11-0589R
TITLE: "Load adaptation by endocytic actin networks"

Dear David:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Alexander Mogilner
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Drubin:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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