



# The role of APC-mediated actin assembly in microtubule capture and focal adhesion turnover

M.Angeles Juanes, Daniel Isnardon, Ali Badache, Sophie Brasselet, Manos Mavrakis, and Bruce Goode

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*Scientific Editor: Rebecca Alvania*

## Transaction Report:

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June 4, 2019

Re: JCB manuscript #201904165

Prof. Bruce L Goode  
Brandeis University  
Biology  
Rosenstiel Center  
415 South Street  
Waltham, MA 02454

Dear Bruce,

Thank you for submitting your manuscript entitled "The role of actin assembly in promoting focal adhesion turnover." The manuscript was assessed by two expert reviewers, whose comments are appended to this letter.

You will see that both are enthusiastic about your work. However, both all also have thoughtful suggestions for improvement. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here. In particular, Reviewer #1 suggests further support is needed to show that the observed effects on focal adhesion dynamics are due to a specific role for APC rather than a more general consequence of altered actin organization, and this seems straightforward to address. They also pose other questions that can likely be addressed by textual revisions. Reviewer #2 raises several points that should be addressed, either by additional experimentation or a textual response. My view is that Reviewer #2 points 1, 2, 5 and 6 seem most pertinent and straightforward to address. Please feel free to contact us with a revision plan if you would like further input after you have had a chance to review the reviewer's comments.

Once we receive a revised manuscript, I hope to be able to render a decision myself, but I may request additional reviewer feedback if necessary.

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.

#### GENERAL GUIDELINES:

Text limits: Character count for an Article 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.

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.

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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.\*\*\*

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

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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](mailto:cellbio@rockefeller.edu) or call (212) 327-8588.

Sincerely,

Matthew Welch  
Monitoring Editor  
JCB

Rebecca Alvania  
Executive Editor  
JCB

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

In this manuscript Juanes et al investigate the role of actin assembly in focal adhesion turnover using a combination of microscopy approaches. They find that actin assembly, which they hypothesize is mediated by APC, plays an important role in regulating the microtubule capture at disassembling focal adhesions. Microtubules are captured more often at focal adhesions in APC-m4 cells, where actin assembly is reduced, and this somehow delays the delivery of the autophagosome that ultimately triggers disassembly.

Overall this is an interesting manuscript which highlights the role of actin assembly in the process of adhesion disassembly. My biggest issue with the conclusions is the argument that this is a process mediated by APC driven assembly. The authors argue that actin density and organization are decreased at FAs in APC-m4 cells and then go on to show a number of defects in FA disassembly and FA dynamics. The connection to APC, however, is indirect. In the absence of other evidence, this might be compelling, but a number of previous papers have shown perturbations to actin

organization can also impact FA dynamics: crosslinking through alpha-actin and enzymatically dead myosin (Choi et al Nat Cell Bio 2008); inhibition of formin and knockdown of alpha-actinin (Oakes et al J Cell Biol 2012; Stricker et al PLoS ONE 2013); mutations to vinculin (Thievensen J Cell Biol 2013). These studies were primarily focused on adhesion assembly dynamics, but the larger point still stands I believe. Could the altered disassembly dynamics in the APC-m4 mutants simply be a product of disorganized actin at the FA? Put another way, if you altered actin in another way (e.g. by inhibiting formins using the SMIF compound, or looked at adhesion in myosin inhibited cells which don't form large stress fibers), would you see similar results? Or is this a specific response to changes in APC activity?

Additional comments and questions:

In the introduction, I disagree with the statement "In addition to stress fibers guiding microtubules to FAs, actomyosin tension appears to be important for FA growth and turnover". The best evidence against the role of tension comes from Choi et al Nat Cell Biol 2008 where they showed that they could recover FA growth using just crosslinking or enzymatically dead motors. There's also a nice discussion of other complementary work in this area discussed in the review by Burridge & Guilly Exp Cell Res 2016.

In Figure 1, the differences between conditions appear more subtle than the figure suggests. The histograms in panels D and E are confusing because they showed the inside and outside regions lumped together. Is there an actual difference in the molecular order between the two regions in a given cell type (it's unclear from panel G)? It would be helpful to distinguish the two regions in the histogram.

Also the choices for the representative images in Figure 1D and E seem slightly odd. The benefit of micropatterning the cells is that you can control the cell shape and thus produce structures that are very similar. In this case the APC-m4 FA is chosen from the center of the cell, while the WT is chosen from a region at the corner. This leads to a bigger point: Where were the FAs chosen for analysis taken from? Is there a spatial dependence on the results? And were cells mixed from the two patterns shown (i.e. the Y shape and the double sided anchor)? Why use two different patterns?

In Figure 3A-D there appears to be a strong difference in the size and shape of the cells, which will impact the total fluorescence intensity measurements. Are these measurements normalized by cell area? If not, could the micropatterns be used to enforce a normalization? Also, for B and D, do the intensity values include the nuclear regions which show a much stronger staining? Or was it restricted to FAs?

In Figure 4, which way are the cells moving in A and B? The cell in A has FAs chosen from a variety of different regions, but the cell in B has them all chosen from one side. Is there a difference in disassembly rates between adhesions at the leading edge and adhesions at the trailing edge? Also, does the difference in disassembly rate (i.e. longer life time) lead to a decrease in overall migration speed of the cells?

In Figure 5, the authors point out that "given that APC-m4 FAs have longer lifetimes, this does not necessarily reflect an increase in the frequency of microtubule visits". Presumably this would be easy to calculate and show? Is there a difference in the amount of time between microtubule visits in the two conditions?

The bias for zone 3 in the APC-m4 cells is intriguing. Is there something to make of that?

In Figure 6, what determined whether a LC3 molecule was actually targeted to the FA? In the APC-m4 FA the LC3 molecules in time points 3-6 appear near but not on the FA. Would these be counted as targeting events? Also this FA appears to show a dip in intensity (at time point 9) and then increase again. Is this a common feature of the APC-4 FAs?

In the conclusion the authors state "microtubules are captured at FAs and are able to sense the maturation state of the FA...". Giving the microtubules a sense of agency feels too strong. It's unclear the mechanism that allows for the delivery of the autophagosome, but it doesn't necessarily have to be an active process of the microtubule sensing something about the maturation state of the FA. It could instead be an indirect result of another interaction, such as altered binding dynamics.

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

In this manuscript, Juanes et al. extend their previous work demonstrating a role for APC actin nucleation activity in mediating FA turnover. Here they investigate with greater detail the spatio-temporal roles of APC-dependent actin assembly at focal adhesions and how this influences FA turnover. They demonstrate a critical role for APC mediated actin assembly in maintaining FA dynamics and the timely organization of F-Actin as well as FA components. Further, they identify a role for APC mediated actin assembly in promoting the efficiency of microtubule capture at mature FAs, an event that is necessary to facilitate efficient delivery of autophagosomes to mature FAs and subsequent disassembly. Overall, this is a straightforward and well written manuscript that extends the field of FA dynamics in that it links actin dynamics as a mechanism of FA disassembly in a way that includes and corroborates recent findings regarding turnover such as microtubule capture and autophagy. Some additional experiments are needed to more rigorously support the model that is proposed.

Comments:

- 1) In Figure 3, Focal Adhesion Kinase (FAK) is phosphorylated by SRC at numerous sites that impact its activity, presence at, and regulation of FAs. In addition to Phospho-Paxillin, what is the FAK phospho-status in the APC-m4 mutants in general and at FAs?
- 2) In Figure 4, the authors should calculate and compare the assembly/disassembly rate constants in addition to plotting average FA assembly/disassembly lifetime. In addition, it would be beneficial to see the effects on FA- turnover during directed migration in APC-m4 using a scratch wound assay.
- 3) For Figure 5, what is the spatio-temporal relationship between MT and Actin capture/presence and FA disassembly? Is the presence of Actin at MT capture sites more stable once FAs are mature? To get at this, it may be beneficial to image microtubule and actin dynamics at FAs with live cell microscopy in WT cells in addition to the genetic functional assays presented.
- 4) There is a growing body of evidence that actin dynamics and proteins that influence actin nucleation play an important role in the regulation of autophagosome formation and maturation.

What is the overall autophagy status (autophagosome formation and maturation, total LC3-II puncta) in the APC mutant cells? Does this influence the dynamics of LC3/autophagosomes at FAs?

5) Is actin targeted to autophagosomes once they are recruited to mature FAs that have maximum MT capture? What are the temporal dynamics here?

6) NBR1 can interact with LC3 at multiple sites in the cell in addition to focal adhesions, such as peroxisomes, midbodies, and ubiquitinated protein aggregates. Thus, the lack of differences in the immunoprecipitation assay of NBR1 with LC3 in Figure 6G is not sufficient to rule out that the observed disassembly defect is not the result of decreased NBR1 localization to FAs. This should be more rigorously addressed with directed approaches using live cell microscopy with a fluorescent-tagged NBR1 to discern the number/dynamics of FAs that interact with NBR1 in WT versus APC-m4 cells.

Reviewer #1

In this manuscript Juanes et al investigate the role of actin assembly in focal adhesion turnover using a combination of microscopy approaches. They find that actin assembly, which they hypothesize is mediated by APC, plays an important role in regulating the microtubule capture at disassembling focal adhesions. Microtubules are captured more often at focal adhesions in APC-m4 cells, where actin assembly is reduced, and this somehow delays the delivery of the autophagosome that ultimately triggers disassembly.

Overall this is an interesting manuscript which highlights the role of actin assembly in the process of adhesion disassembly. My biggest issue with the conclusions is the argument that this is a process mediated by APC driven assembly. The authors argue that actin density and organization are decreased at FAs in APC-m4 cells and then go on to show a number of defects in FA disassembly and FA dynamics. The connection to APC, however, is indirect. In the absence of other evidence, this might be compelling, but a number of previous papers have shown perturbations to actin organization can also impact FA dynamics: crosslinking through alpha-actin and enzymatically dead myosin (Choi et al Nat Cell Bio2008); inhibition of formin and knockdown of alpha-actinin (Oakes et al J Cell Biol 2012; Stricker et al PLoS ONE 2013); mutations to vinculin (Thievensen J Cell Biol 2013). These studies were primarily focused on adhesion assembly dynamics, but the larger point still stands I believe. Could the altered disassembly dynamics in the APC-m4 mutants simply be a product of disorganized actin at the FA?

Yes, this is correct. This is our model, as described in the paper:

Page 5 (end of Intro): “Our results show that actin assembly by APC plays a critical role in maintaining proper F-actin organization and dynamics at FAs in migrating cells, and that its loss results in severe delays in FA disassembly stemming from an inability of FAs to respond properly to microtubule capture events”.

Page 15 (last sentence in opening paragraph of Discussion): “these results demonstrate that actin assembly by APC is critical for maintaining proper levels, organization, and dynamics of F-actin at FAs”.

Page 16-17 (end of Discussion): “Our current results combined with our previous observations (Juanes et al., 2017) demonstrate that human APC’s actin nucleation activity plays a critical role in maintaining proper F-actin levels, organization, and dynamics at FAs, which is required for FA turnover and directed cell migration. Thus, APC is a *bona fide* actin nucleator in vivo, and this may be one of its chief cytoskeletal roles”.

Put another way, if you altered actin in another way (e.g. by inhibiting formins using the SMIF compound, or looked at adhesion in myosin inhibited cells which don't form large stress fibers), would you see similar results? Or is this a specific response to changes in APC activity?

Our data reveal a critical requirement for APC-mediated actin nucleation in promoting FA turnover, but do not rule out contributions from additional actin regulators. FAs are immense and complex actin-based structures, so it would not be surprising if a

number of actin assembly-promoting factors are involved, as seen in other cellular actin structures (e.g., lamellipodia, filopodia, endocytic sites).

Dia1 is a well characterized formin that localizes to FAs and has an established role in FA assembly and maturation. Further, Dia1 silencing is known to cause a ~30% reduction in F-actin levels in cells (Rao and Zaidel-Bar, 2016; Carramusa et al., 2007; Oakes et al., 2012), similar to what we have reported for APC-m4 expression (Juanes et al., 2017; JCB). Therefore, to address the concern of the Reviewer about the specificity of the APC effects, we used live TIRF microscopy to compare the effects of Dia1 silencing and APC-m4 expression, in parallel, in migrating MDA-MB-231 cells. Our FAAS analysis of FA assembly and disassembly rates shows that Dia1 silencing causes only a very modest decrease in FA disassembly rate, whereas APC-m4 caused a striking reduction in FA disassembly rate (Fig. 4E and H). Further, we compared total cellular F-actin levels in APC-m4 and si-Dia1 cells, and confirmed that both genetic perturbations cause a similar ~ 30% reduction in total F-actin levels (Fig. 4I). Thus, the effects of APC-m4 on FA turnover do not appear to arise from a general loss of actin assembly in cells, but rather from a specific disruption of APC-mediated actin nucleation at FAs. Finally, we wish to again stress that while the effects of APC are specific, they do not rule out the possibility of additional actin assembly-promoting factors (e.g., Arp2/3 complex, ENA/VASP, or other formins) contributing to FA turnover.

Additional comments and questions:

In the introduction, I disagree with the statement "In addition to stress fibers guiding microtubules to FAs, actomyosin tension appears to be important for FA growth and turnover". The best evidence against the role of tension comes from Choi et al Nat Cell Biol 2008 where they showed that they could recover FA growth using just crosslinking or enzymatically dead motors. There's also a nice discussion of other complementary work in this area discussed in the review by Exp Cell Res 2016.

Thank you for pointing this out. We agree, and have deleted this statement.

In Figure 1, the differences between conditions appear more subtle than the figure suggests. The histograms in panels D and E are confusing because they showed the inside and outside regions lumped together. Is there an actual difference in the molecular order between the two regions in a given cell type (it's unclear from panel G)?

We had failed to point out that within a given cell type (WT or APC-m4) grown on micropatterns, there is no significant difference between the molecular order of the 'in' and 'out' regions. However, the data were (and still are) shown in Fig. 1G; we have now added stat bars in Fig. 1G highlighting this point, i.e., that there is no significant difference ("n.s.") between in and out regions of WT, or between in and out regions of APC-m4. Similarly, we added n.s. bars to Fig. S2B, which shows similar data for non-patterned cells.

It would be helpful to distinguish the two regions in the histogram.

This is a great suggestion. We have added this (see Fig. 1D and 1E).

Also the choices for the representative images in Figure 1D and E seem slightly odd. The



benefit of micropatterning the cells is that you can control the cell shape and thus produce structures that are very similar. In this case the APC-m4 FA is chosen from the center of the cell, while the WT is chosen from a region at the corner. This leads to a bigger point: Where were the FAs chosen for analysis taken from? Is there a spatial dependence on the results?

Thank you for bringing this to our attention. We have replaced the image in the lower right hand panel (APC-m4 rescue, triangular pattern) to show a cell where the FA was analyzed from the same region as the cell above in upper right panel (APC-WT, triangular pattern). These data now appear in Fig. 1C-E.

In regard to the larger point raised, all of the FAs we analyzed were selected from the cell periphery at the ends of ventral stress fibers. There are three major types of stress fibers in cells: ventral, dorsal, and arcs. We focused on ventral stress fibers given that most mature FAs are located at their two ends. Also, ventral stress fibers do not 'cross over' (like dorsal stress fibers and arcs do, which would interfere with the polarization-resolved microscopy analysis). We now discuss the rationale for this selection, briefly in the Results (and in more detail in the Methods).

And were cells mixed from the two patterns shown (i.e. the Y shape and the double sided anchor)? Why use two different patterns?

Yes, we used two different patterns (Y and H) to help ensure that different patterns do not change the outcome, i.e., the differences between WT and APC-m4. We elected to pool the data from both patterns and analyzed them together in order to increase our n. For WT cells, we analyzed n = 25 FA-stress fibers from patterned cells (17 from Y patterns, 8 from H patterns). For APC-m4 cells, we analyzed n = 31 FA-stress fibers from patterned cells (18 from Y patterns, 13 from H patterns). We added this information to the Methods.

In Figure 3A-D there appears to be a strong difference in the size and shape of the cells, which will impact the total fluorescence intensity measurements. Are these measurements normalized by cell area? If not, could the micropatterns be used to enforce a normalization? Also, for B and D, do the intensity values include the nuclear regions which show a much stronger staining? Or was it restricted to FAs?

This is an excellent point. To address this, we reanalyzed our data to normalize for cell area, and graphed 'density' (fluorescence intensity  $\mu\text{m}^{-2}$ ) for phospho-Src and phospho-Paxillin (and phospho-FAK, which was requested by Reviewer #2, comment 1). These data now appear in Fig. 3D.

Yes, signals (now densities) include nuclear staining; however, we also measured density of signals at FAs (thus, unaffected by nuclear staining) - see Fig. 3H and 3I.

In Figure 4, which way are the cells moving in A and B? The cell in A has FAs chosen from a variety of different regions, but the cell in B has them all chosen from one side. Is there a difference in disassembly rates between adhesions at the leading edge and adhesions at the trailing edge?

The APC-WT cell in Fig. 4A moves directionally (to the left), while the APC-m4 cell in Fig. 4B has lost directionality and mostly wanders (though over time, it experiences net

movement toward the right). We now provide movies of these cells in the Supplement (Video 1). In the movies, we have also boxed the exact FAs analyzed in the montages in Fig. 4A and 4B.

We also thank the reviewer for raising the question of whether there is a difference in FA disassembly rates at the front versus rear of cells. To address this, we performed new experiments, with the data appearing in Fig. 4F and G. These new data show there is no statistical difference in FA disassembly rates between FAs found at the leading versus trailing edges within a particular cell type (APC-WT or APC-m4).

Also, does the difference in disassembly rate (i.e. longer life time) lead to a decrease in overall migration speed of the cells?

This is something we addressed in our previous study (see Juanes et al., JCB 2017, Fig. 3). We found that APC-m4 cells, in both wound healing assays and single cell chemotaxis assays exhibited severe defects in directed cell migration. This is mentioned in the Introduction.

In Figure 5, the authors point out that "given that APC-m4 FAs have longer lifetimes, this does not necessarily reflect an increase in the frequency of microtubule visits". Presumably this would be easy to calculate and show? Is there a difference in the amount of time between microtubule visits in the two conditions? The bias for zone 3 in the APC-m4 cells is intriguing. Is there something to make of that?

This is another excellent point. Indeed, we have the data to make these calculations, and now have done so, and state the results in the text. We find that the frequency of microtubule visits is strikingly reduced in APC-m4 cells ( $0.85 \text{ min}^{-1}$  for APC-WT and  $0.35 \text{ min}^{-1}$  for APC-m4). Further, the average time between microtubule visits is about three times longer in APC-m4 cells (24 sec in APC-WT versus 76 sec in APC-m4). Thus, in APC-m4 cells, microtubules visit FAs less frequently, but stay longer. These data suggest that microtubule capture may be less efficient in APC-m4 cells, which (as the Reviewer pointed out) may be related to our observation that microtubules preferentially pause in zone 3 in APC-m4 cells. We now raise these points in the Discussion.

In Figure 6, what determined whether a LC3 molecule was actually targeted to the FA? In the APC-m4 FA the LC3 molecules in time points 3-6 appear near but not on the FA. Would these be counted as targeting events? Also this FA appears to show a dip in intensity (at time point 9) and then increase again. Is this a common feature of the APC-4 FAs?

We only scored autophagosomes as being delivered to FAs when we could clearly see that GFP-LC3 signal overlapped with the FA marker (mCherry-Zyxin). Images were captured every 10 sec, whereas the montages show images at 1 min intervals. Thus, we have more time points available than what was shown in the montages. We reexamined the movies, and saw that the contact (GFP-LC3 with FA) was made in frames that were not part of the montage. Therefore, for clarity, we replaced the APC-m4 montage with a different example (lower panels in Fig. 6A), which better displays the contacts made.

The dip in intensity (time point 9 in the previous version) is not a common feature, and is likely due to the FA being partially out of the focal plane at specific time points in the TIRF imaging, a consequence of live imaging. We also replaced the video (now called

Video 4; previously Video 3) showing several GFP-LC3 contacting at FAs. In this video, one of the GFP-LC3 spots contacting the FA corresponds to the example shown in the montages for APC-WT and APC-m4 in Fig. 6.

To help readers see when GFP-LC3 contacts FAs during the Videos, we have incorporated yellow arrows highlighting a few examples of contacts. Yellow arrows appear from the time of first contact of the autophagosome with the FA to complete disassembly of the FA.

In the conclusion the authors state "microtubules are captured at FAs and are able to sense the maturation state of the FA...". Giving the microtubules a sense of agency feels too strong. It's unclear the mechanism that allows for the delivery of the autophagosome, but it doesn't necessarily have to be an active process of the microtubule sensing something about the maturation state of the FA. It could instead be an indirect result of another interaction, such as altered binding dynamics.

Our data show that microtubule capture events at FAs prior to the FA reaching peak maturity do NOT result in delivery of an autophagosome, but once a FA has reached peak maturity, the next microtubule capture event results in rapid delivery of an autophagosome. These observations suggest that the microtubule (somehow) sense or detect a change(s) at mature FAs. We don't consider this to be an active process. In an attempt to reach a compromise on this, we have changed the sentence to read: "microtubules are captured at FAs and somehow detect its maturity state...".

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Reviewer #2

In this manuscript, Juanes et al. extend their previous work demonstrating a role for APC actin nucleation activity in mediating FA turnover. Here they investigate with greater detail the spatio-temporal roles of APC-dependent actin assembly at focal adhesions and how this influences FA turnover. They demonstrate a critical role for APC mediated actin assembly in maintaining FA dynamics and the timely organization of F-Actin as well as FA components. Further, they identify a role for APC mediated actin assembly in promoting the efficiency of microtubule capture at mature FAs, an event that is necessary to facilitate efficient delivery of autophagosomes to mature FAs and subsequent disassembly. Overall, this is a straightforward and well written manuscript that extends the field of FA dynamics in that it links actin dynamics as a mechanism of FA disassembly in a way that includes and corroborates recent findings regarding turnover such as microtubule capture and autophagy. Some additional experiments are needed to more rigorously support the model that is proposed.

Comments:

1) In Figure 3, Focal Adhesion Kinase (FAK) is phosphorylated by SRC at numerous sites that impact its activity, presence at, and regulation of FAs. In addition to Phospho-Paxillin, what is the FAK phospho-status in the APC-m4 mutants in general and at FAs?

To address this, we performed new experiments, measuring phospho-FAK density at FAs. We confirm that the density of this FA component is reduced in APC-m4 cells compared to APC-WT cells, similar to what we observed for the densities of phospho-Src and phospho-Paxillin (Fig. 3C, 3D, 3G and 3H). In addition, the reviewer asked about the general (total) levels of phospho-FAK in APC-WT versus APC-m4 cells. We measured this in our previous study, which showed that total levels of phospho-FAK are significantly reduced in APC-m4 cells (Juanes et al., 2017; JCB; Fig. 5).

2) In Figure 4, the authors should calculate and compare the assembly/disassembly rate constants in addition to plotting average FA assembly/disassembly lifetime.

This was an excellent suggestion, since FAs in APC-m4 cells are enlarged, and FA disassembly rates are independent of FA size. We used the published FAAS method to measure FA assembly and disassembly rates in APC-WT and APC-m4 cells (n = 208-354 FAs each), and found that FA disassembly (but not assembly) is significantly slower in APC-m4 cells (Fig. 4E). Further, as mentioned above, we compared rates of FA assembly and disassembly for FAs at the leading versus trailing edge of cells (Fig. 4F and G) and found no significant differences (within a given cell type).

In addition, it would be beneficial to see the effects on FA- turnover during directed migration in APC-m4 using a scratch wound assay.

Our previous study demonstrated that APC-m4 expression leads to severe defects in directionality of cell movement in scratch assays (Fig. 3A-E, Juanes et al., JCB 2017). Individual trajectories of mutant cells at the edge of the wound were random and erratic, in contrast to WT cells which moved with persistent directionality. Similar results were obtained for individual (single) cells undergoing chemotaxis (Fig. 3F and 3G, Juanes et al., JCB 2017). Analyzing the turnover of individual FAs in cells within a tightly connected sheet (undergoing collective cell migration) is extremely challenging, and we anticipate that this would yield similar results to what we have already shown for individual cells undergoing migration.

3) For Figure 5, what is the spatio-temporal relationship between MT and Actin capture/presence and FA disassembly? Is the presence of Actin at MT capture sites more stable once FAs are mature? To get at this, it may be beneficial to image microtubule and actin dynamics at FAs with live cell microscopy in WT cells in addition to the genetic functional assays presented.

We have not been able to perform simultaneous live cell imaging of three colors: MT dynamics in one color, actin dynamics by FRAP in another color, and FAs in a third color. Further, even if we could overcome this technical hurdle, it would be extremely challenging to get the timing right for this experiment, i.e., to assess changes in actin dynamics (FRAP) at FAs during relatively short (25 sec) visits by MTs.

4) There is a growing body of evidence that actin dynamics and proteins that influence actin nucleation play an important role in the regulation of autophagosome formation and maturation. What is the overall autophagy status (autophagosome formation and maturation, total LC3-II puncta) in the APC mutant cells? Does this influence the dynamics of LC3/autophagosomes at FAs?

We agree that this is an important question, and have added a new Supplemental figure to address this point (Fig. S5). Our data show that APC-m4 expression does not significantly alter general autophagy. Specifically, APC-m4 does not alter:

- (a) Total cellular levels of LC3-I and LC3-II (in cells untreated and treated with Bafilomycin 1, a drug that blocks autophagy) (Fig. S5 A and B).
- (b) The maturation state of autophagosomes trafficking from ER to fuse with lysosomes (also known as 'autophagy flux'), using a pH-sensitive two-color Premo autophagy tandem sensor (RFP-GFP-LC3B) (Fig. S5 C).
- (c) The percentage of autophagosomes in cells undergoing fusion with lysosomes (late step in autophagy), as determined by live imaging of GFP-LC3 and mCherry-Lamp1 (lysosome marker), and by co-immunoprecipitation of LC3 and GFP-LAMP1 (Fig. S5 D and E).

5) Is actin targeted to autophagosomes once they are recruited to mature FAs that have maximum MT capture? What are the temporal dynamics here?

We were curious about this too, given that actin is recruited to LC3 during starvation conditions (Aguilera et al., *Autophagy* 2012; Kast et al., *CB* 2015). However, in these studies, LC3 localization was more obvious near the nucleus, where actin is less dense. At FAs, it would be almost impossible to test for actin recruitment to LC3, given that FAs are themselves actin-based structures, and given the general abundance of actin in this region of the cell. Put another way, once an LC3-autophagosome arrives at a FA, it is by definition surrounded by actin, making it virtually impossible to detect the transfer of new actin to LC3.

6) NBR1 can interact with LC3 at multiple sites in the cell in addition to focal adhesions, such as peroxisomes, midbodies, and ubiquitinated protein aggregates. Thus, the lack of differences in the immunoprecipitation assay of NBR1 with LC3 in Figure 6G is not sufficient to rule out that the observed disassembly defect is not the result of decreased NBR1 localization to FAs. This should be more rigorously addressed with directed approaches using live cell microscopy with a fluorescent-tagged NBR1 to discern the number/dynamics of FAs that interact with NBR1 in WT versus APC-m4 cells. We have done live-imaging in WT and m4 cells and investigated.

To address this, we have now performed live TIRF imaging, comparing GFP-NBR1 dynamics in APC-WT and APC-m4 cells. Our data show that there is no significant difference between APC-WT and APC-m4 cells in the percentage of mature FAs contacted by GFP-NBR1, or in the dwell time of GFP-NBR1 at mature FAs (Fig. 6 G-H).

July 18, 2019

RE: JCB Manuscript #201904165R

Prof. Bruce L Goode  
Brandeis University  
Biology  
Rosenstiel Center  
415 South Street  
Waltham, MA 02454

Dear Prof. Goode:

Thank you for submitting your revised manuscript entitled "The role of APC-mediated actin assembly in microtubule capture and focal adhesion turnover". We assessed your manuscript revisions editorially and feel that the additions nicely address the original reviewer concerns. The reviewers were both quite positive in the first round of peer review, and editorially we are satisfied with the revisions and feel the work is now appropriate for JCB. Therefore, in the interest of providing a timely decision we do not feel further external peer review is necessary. We would be happy to publish your paper in JCB pending any final minor 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.\*\*

#### 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](mailto:lhollander@rockefeller.edu)).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- 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.

**\*\*It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.\*\***

**\*\*The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.\*\***

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

Please contact the journal office with any questions, [cellbio@rockefeller.edu](mailto: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,

Matthew Welch  
Monitoring Editor  
JCB

Rebecca Alvania  
Executive Editor  
JCB

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