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Rac1, the actin cytoskeleton and microtubules are key players in clathrin-independent endophilin-A3-mediated endocytosis

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MS TITLE: Rac1, actin cytoskeleton and microtubules are essential molecular players in clathrin-independent endophilin-A3-mediated endocytosis

AUTHORS: François Tyckaert, Pierre Morsomme, and Henri- François Renard
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, both reviewers raise a number of criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Tyckaert dissects the contribution of cytoskeletal components to endophilin-A3-dependent endocytosis. Using the cell adhesion protein CD166 as a model cargo, the authors show the participation of the actin cytoskeleton in this pathway via the small GTPase Rac1. In addition, the authors also show that the microtubule-based motors kinesins potentiate this pathway, likely by providing forces required for vesicle scission.

The manuscript is well written and figures are clear and carefully composed. The results do provide interesting insights into this poorly understood endocytic pathway. In addition, the requirement of microtubule-based motors in this pathway is interesting and contributes to the establishment of the compelling concept of friction-driven scission.

Having said that, a few issues still need to be addressed to reinforce the author conclusions.

Comments for the author

Major comments:
1) The major issue with the manuscript regards the experiments with kinesins presented in figure 5. The magnitude of endocytic defects shown in figure 5C are similar for all KIFs, despite their significantly different expression levels in HeLa cells (Hein, Cell 2015). Moreover, assuming these KIFs action is independent, their sum far surpasses 100% inhibition. How do the authors explain these results? Do knockdown of one kinesin affects the others?
2) As suggested by the authors, the burst of actin accumulation during endoA3-mediated endocytosis (clearly visible in Movie 1) suggests the formation of an ARP2/3-dependent actin network via Rac1 ->
WAVE. The localisation of these molecules (and the likely exclusion of N-WASP) to EndoA3 sites would provide a stronger support to the suggested role of Rac1 provided by the authors in their working model (figure 6).

Minor comments:
3) The authors should be a bit more careful on their interpretation of the role of actin cytoskeleton and its regulators on TF uptake. The clathrin mediated endocytosis (CME) community has recently come to terms that actin inhibiting drugs are poor tools to study this pathway as they have little effect on actin at CME sites (as shown by Collins, Cur Biol 2011). Gene knockdowns/knockouts have proven to be far more effective tools to study actin regulators in this endocytic route and have firmly established actin as a bona fide CME component. Examples are: Myosin II (Wyat MBOC 2021 and Chandrasekar Traffic 2014), ARP3 and FCHSD2 (Almeida-Souza, Cell 2018).
4) The quantification of actin colocalisation with EndoA3-CD166 (shown in figure 3F) should be presented, to some extent, earlier in the manuscript while discussing the results in figure 1. As a reader we eager for this information soon after you show the actin-endoA3 colocalisation.
5) For the sake of transparency, the bar graphs would benefit from also showing the spread of data.
6) Figure S3B – The Y-axis is labelled incorrectly.
7) The last sentence of abstract is, in my opinion, misplaced. It feels awkward to display new information about the manuscript after a wrap up sentence.
Good luck on your experiments during revision. I hope the current Covid situation in your area do not impede you to perform them. Take care and be kind to each other.
Leonardo Almeida-Souza

Reviewer 2

Advance summary and potential significance to field

This paper contains a series of characterizations of the endophilin A3-mediated endocytosis. The study appears to contain a lot of experiments. I would like to point out some concerns about the experiments.

Comments for the author

1. The anti-CD166 antibody was tracked instead of CD166 in this study. Although it is highly likely that it binds to CD166, but it can be aggregated to non-specifically bind to the surface of the cells, and then it can be internalized.
Because galectin is suggested to increase the CD166 internalization by cross-linking CD166, it would be likely that the aggregated antibody would also be the target of endocytosis. Therefore, some validation of the antibody would be required. Those may include the co-localization of fluorescent-protein labeled CD166. Furthermore, the fluorescent intensity of the CD166 antibody should be quantified for their molecule numbers.
2. It appears that endophilin A3 dependency and endophilin A2 dependency would not be mutually exclusive. Because both endocytosis uses similar machinery although it would be distinct in regulators, the amino-acid sequence of endophilin A3 is very similar to that of endophilin A2. Therefore, this reviewer was not so convinced that endophilin A3 is so specifically involved in the observations. In the report in Nature Communications (2020) 11:1457 that they cited, the evidence of endophilin A3 specificity is from siRNA experiments which might not be perfect for other endorphins. The expression levels of endophilin A1, A2, and A3 might be better to be presented or discussed. The discussion for the specific involvement of endophilin A3 should be discussed. In this point of view, I guess the endophilin A2-dependent endocytosis would also be dependent on the machinery that is required for the endophilin A3-dependent endocytosis.
3. The inhibitors and the treatment will globally affect the cells and will induce the changes in the endophilin A3 dependent endocytosis in a non-specific manner. I feel at least one of the specific molecular interactions between endophilin A3 and the examined pathways should be presented. These interactions should be weaker with endophilin A2 than with endophilin A3.
4. Most of the figures are just graphs. The pictures showing the representative observation for the quantification should be.

First revision

Author response to reviewers' comments

First of all, we would like to thank both reviewers for their insightful comments, which helped us to considerably improve the quality of our manuscript.

For the ease of readers, we provide a Word file containing all modifications highlighted in yellow color. We also provide a PDF version of this rebuttal letter, which contains a figure.

Please, find below our point-by-point responses to the points raised by the reviewers.

Reviewer #1 comments:

Major comments

1) The major issue with the manuscript regards the experiments with kinesins presented in figure 5. The magnitude of endocytic defects shown in figure 5D are similar for all KIFs, despite their significantly different expression levels in HeLa cells (Hein, Cell 2015). Moreover, assuming these KIFs action is independent, their sum far surpasses 100% inhibition. How do the authors explain these results? Do knockdown of one kinesin affect the others?

As rightfully pointed out by the reviewer, the sum of kinesin hit effects on CD166 uptake far exceeds 100%, which could result from different phenomena. To address this question, we performed CD166 uptake assays using different combinations of siRNAs against kinesin hits (Fig. S8C). Interestingly, the combined depletion of all kinesin-14 members showed a decrease of CD166 uptake (-49%, Fig. S8C) to a similar extent than the depletion of KIF25 alone (-47%, Fig. 5D), which could indicate a cooperative (rather than individual) function of these motors in the process. However, given the lack of specific antibodies against minus-end kinesins, we could not exclude off-targeting or compensation effects due to siRNA knockdown. Regarding KIF5B, which had the

strongest effect on CD166 uptake (-50%, Fig. 5D), we propose that opposing forces resulting from both plus- and minus-end kinesin motors might be required for vesicle fission. This statement is supported by the combined depletion of KIF5B with KIFC1 or KIF25, which further accentuated, although not significantly, the inhibition of CD166 uptake (-60%, Fig. S8C).

These new data have been included in Fig. S8B-C. Additional text has been added in the manuscript (p10, §1, lines 279-290).

2) As suggested by the authors, the burst of actin accumulation during endoA3-mediated endocytosis (clearly visible in Movie 1) suggests the formation of an ARP2/3-dependent actin network via Rac1 -> WAVE. The localization of these molecules (and the likely exclusion of N-WASP) to EndoA3 sites would provide a stronger support to the suggested role of Rac1 provided by the authors in their working model (figure 6).

We thank the reviewer for his suggestions. We performed additional TIRF experiments to assess the association of ARPC2 (subunit of ARP2/3 complex) and WASF2 (WAVE isoform 2) proteins with endophilin-A3. To do so, we expressed mCherry-tagged constructs of each protein in HeLa cells stably expressing GFP-tagged endoA3. ARPC2 showed a strong and dynamic association with endoA3 at the plasma membrane (Fig.2C). Likewise, WASF2 (WAVE2) strongly colocalized with endoA3. Interestingly, overexpression of the latter resulted in a stabilization of endoA3 patches at plasma membrane (Fig. S5B, movie 5). To verify if this stabilization of the endocytic function of endoA3, we measured the endocytosis of CD166 in the same overexpression condition. As expected, WASF2 overexpression correlated with a decrease of CD166 uptake (-65%, Fig. S5C). This effect might result from reduced membrane deformability, as WASF2 overexpression was shown to induce the formation of an extremely dense actin meshwork, presumably increasing cell surface tension.

These new data have been added in Fig. 2C and Fig. S5B-C. The text of the manuscript has been modified accordingly (p8, §1, lines 216-224).

Minor comments:

3) The authors should be a bit more careful on their interpretation of the role of actin cytoskeleton and its regulators on TF uptake. The clathrin mediated endocytosis (CME) community has recently come to terms that actin inhibiting drugs are poor tools to study this pathway as they have little effect on actin at CME sites (as shown by Collins, Cur Biol 2011). Gene knockdowns/knockouts have proven to be far more effective tools to study actin regulators in this endocytic route and have firmly established actin as a bonafide CME component. Examples are: Myosin II (Wyat MBOC 2021 and Chandrasekar Traffic 2014), ARP3 and FCHSD2 (Almeida-Souza, Cell 2018).

We thank the reviewer for his comment. We performed additional CD166 and Tf uptake assays in cells transfected with siRNAs against the ARPC2 subunit of the ARP2/3 complex (Fig. 2B). This experiment showed an effect on CD166 and Tf uptake similar to the treatment with the ARP2/3 inhibitor (Fig. 2A). Of note, in the study, we also used other drugs that do not directly target actin polymerization. For instance, inhibition of ezrin, which links CD166 to the actin cytoskeleton, did significantly reduce CD166 endocytosis.

The new data have been added in Fig. 2B and textual additions have been made in the text (p6, §1, lines 153-158).

4) The quantification of actin colocalization with EndoA3-CD166 (shown in figure 3F) should be presented, to some extent, earlier in the manuscript while discussing the results in figure 1. As a reader, we eager for this information soon after you show the actin-endoA3 colocalization.

The data has been moved to Fig. 1B, as suggested.

5) For the sake of transparency, the bar graphs would benefit from also showing the spread of data.

We thank the reviewer for raising this important point. While we totally agree that displaying the spread data on each graph would provide better transparency, the sheer amount of data points displayed (≥100) would greatly affect the clarity of the results. This is why we only displayed the

spread data for graphs with less than 50 data points per condition (*e.g.* Fig. 1B). For more transparency, we added representative images, showing the effect of each treatment (siRNA or inhibitor) on protein uptake (see Fig. S1, S2, S3B, S4, S5C, S6, S7A and S8). In addition, we also provided a source data file that gathers all the data used to build the graphs.

6) Figure S3B – The Y-axis is labelled incorrectly.

Thanks, this has been corrected.

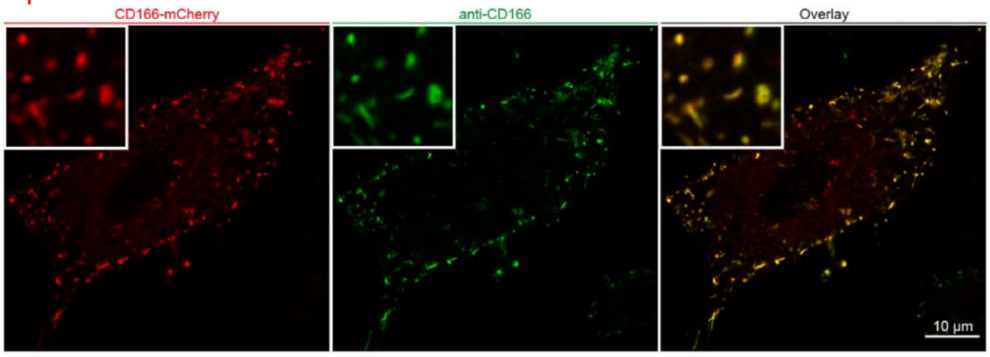
7) The last sentence of the abstract is, in my opinion, misplaced. It feels awkward to display new information about the manuscript after a wrap up sentence.

We totally agree with the reviewer. We inverted the two last sentences of the abstract.

Reviewer #2 comments:

1. The anti-CD166 antibody was tracked instead of CD166 in this study. Although it is highly likely that it binds to CD166, but it can be aggregated to non-specifically bind to the surface of the cells, and then it can be internalized. Because galectin is suggested to increase the CD166 internalization by cross- linking CD166, it would be likely that the aggregated antibody would also be the target of endocytosis. Therefore, some validation of the antibody would be required. Those may include the co-localization of fluorescent-protein labeled CD166. Furthermore, the fluorescent intensity of the CD166 antibody should be quantified for their molecule numbers.

We thank the reviewer for this comment. The antibody used to label CD166 was previously validated in our study "Endophilin-A3 and Galectin-8 control the clathrin-independent endocytosis of CD166" (Renard et al., 2020). Here, as requested, we provide additional validation by showing the colocalization between a transiently expressed mCherry-tagged version of CD166 and the antibody used in our studies. As one can see in the figure below, the overlap between both signals is perfect.



We strongly agree that antibody-mediated crosslinking of CD166 might accentuate/induce endocytosis similarly to galectin-8 molecules. To bypass this putative clustering effect, we have tried to perform uptake experiments using a fluorescently labeled monovalent antibody fragment (ScFv). However, unfortunately, this antibody did not succeed to label endogenous CD166. On the other hand, CD166 still strongly colocalized with endoA3 even without preincubation with primary antibody (see Fig. 5B), which demonstrates that this association is not an artifact due to antibody recognition and clustering.

2. It appears that endophilin A3 dependency and endophilin A2 dependency would not be mutually exclusive. Because both endocytosis uses similar machinery, although it would be distinct in regulators, the amino-acid sequence of endophilin A3 is very similar to that of endophilin A2. Therefore, this reviewer was not so convinced that endophilin A3 is so specifically involved in the observations. In the report in Nature Communications (2020) 11:1457 that they cited, the evidence of endophilin A3 specificity is from siRNA experiments, which might not be perfect for other endorphins. The expression levels of endophilin A1, A2, and A3 might be better to be presented or discussed. The discussion for the specific involvement of endophilin A3 should be discussed. In this point of view, I guess the endophilin A2-dependent endocytosis would also be dependent on the machinery that is required for the endophilin A3-dependent endocytosis.

The specificity of endoA3 function in CD166 endocytosis has been extensively studied in our previous 2020 publication (Renard et al., 2020) that you mention. In that paper, we showed in Fig. S2 that the siRNAs used to deplete the three endoA provide an efficient and specific depletion of their target protein (confirmed in the current manuscript, Fig. S3C). In this same 2020 study, we also performed uptake assays in conditions where endoA isoforms are overexpressed (Fig. S3D). In accordance with siRNA-based uptake assays, overexpressing endoA3 in HeLa cells increased CD166 uptake, in contrast to endoA2 overexpression which did not have any effect. We also demonstrated that endoA2 and endoA3 do not strongly overlap and control the uptake of distinct cargoes (Fig. 2). This was shown by colocalization assays, uptake assays, and pull-down experiments. Finally, isoform specific functions within the endoA subfamily have been reported by other studies (Boucrot et al., 2015; Sugiura et al., 2004; Tian et al., 2012). For instance, we can mention the study conducted by Boucrot et al (2015), in which pull-down experiments revealed that the SH3 domain of each endoA isoform shows preferential binding to specific cargoes. Collectively, these observations indicate that endoA2 and endoA3 have distinct endocytic functions and are not redundant.

Nevertheless, as the reviewer rightfully mentions, the high sequence similarity shared by endoA isoforms (~78%) raises the question for the origin of such differences. The region that bridges the BAR domain to the SH3 domain concentrates a lot of the variability within endoA isoforms, which could potentially explain isoform specificity. Also, as mentioned above, isoform specificity might originate from the slight sequence variability within the SH3 domain. A deeper structural and mutational study of endoA isoforms would bring more insight into these questions, but is out of the scope of the current study.

3. The inhibitors and the treatment will globally affect the cells and will induce the changes in the endophilin A3 dependent endocytosis in a non-specific manner. I feel at least one of the specific molecular interactions between endophilin A3 and the examined pathways should be presented. These interactions should be weaker with endophilin A2 than with endophilin A3.

We thank the reviewer for this comment. Actually, in our previous 2020 publication (Renard et al., 2020), we performed pull-down experiments using the cytosolic tail of CD166 as a bait. Interestingly, we could pull down endoA3, but not endoA2 (Fig. 2D). Importantly, in the current study, we do not base our interpretations only on inhibitors and siRNA treatments, but also on colocalization data (on fixed and living cells), which confirm the specific involvement of the molecular actors in the endoA3- mediated CIE of CD166.

4. Most of the figures are just graphs. The pictures showing the representative observation for the quantification should be.

We thank the reviewer for this remark. For more transparency, we added representative images as well as the protein cell surface levels for each condition on the graphs (see Fig. S1, S2, S3B, S4, S5C, S6, S7A and S8).

References:

Boucrot, E., A.P. Ferreira, L. Almeida-Souza, S. Debard, Y. Vallis, G. Howard, L. Bertot, N. Sauvonnet, and H.T. McMahon. 2015. Endophilin marks and controls a clathrin-independent endocytic pathway. *Nature*. 517:460-465.

Renard, H.F., F. Tyckaert, C. Lo Giudice, T. Hirsch, C.A. Valades-Cruz, C. Lemaigre, M. Shafaq-Zadah, C. Wunder, R. Wattiez, L. Johannes, P. van der Bruggen, D. Alsteens, and P. Morsomme. 2020. Endophilin-A3 and Galectin-8 control the clathrin-independent endocytosis of CD166. *Nat Commun*. 11:1457.

Sugiura, H., K. Iwata, M. Matsuoka, H. Hayashi, T. Takemiya, S. Yasuda, M. Ichikawa, T. Yamauchi, P. Mehlen, T. Haga, and K. Yamagata. 2004. Inhibitory role of endophilin 3 in receptor-mediated endocytosis. *J Biol Chem*. 279:23343-23348.

Tian, Q., J.F. Zhang, J. Fan, Z. Song, and Y. Chen. 2012. Endophilin isoforms have distinct characteristics in interactions with N-type Ca2+ channels and dynamin I. *Neurosci Bull*. 28:483-492.

Second decision letter

MS ID#: JOCES/2021/259623

MS TITLE: Rac1, actin cytoskeleton and microtubules are key players in clathrin-independent endophilin-A3-mediated endocytosis

AUTHORS: François Tyckaert, Natacha Zanin, Pierre Morsomme, and Henri- François Renard
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Tyckaert et al. describes the participation of actin and microtubules in endophilin-A3-dependent endocytosis. Their findings represent a significant advance in the understanding of this recently described endocytic pathway. Furthermore, they also provide further evidence for the compelling concept of friction-driven scission.

Comments for the author

The authors have addressed all my comments satisfactorily. I have no further suggestions.

Reviewer 2

Advance summary and potential significance to field

The manuscript was significantly improved.

Comments for the author

They describe CIE, but it contains a wide spectrum of endocytosis. The abstract and introduction should clarify what kind of CIE was analyzed for readers. I think it should be the cross-link-dependent CD166 endocytosis or CD166-aggregation endocytosis or galectin-dependent CD166 endocytosis.