JCB

Alternative splicing of clathrin heavy chain contributes to the switch from coated pits to plaques

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Re: JCB manuscript #201912061

Dr. Stéphane Vassilopoulos
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Dear Dr. Vassilopoulos,

Thank you for submitting your manuscript entitled "Alternative splicing of clathrin heavy chain exon 31 allows the switch from coated pits to plaques". 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 the reviewers find that your study addresses an important and interesting question. However, in addition to some technical concerns, they find that your conclusions and significance are overstated in places. Therefore, in addition to attending to all technical criticisms, including the minor points, your manuscript will need careful rewriting to address all of the interpretative caveats pointed out by the reviewers and all of the instances in which they found your data was over-interpreted.

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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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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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

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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.

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,

Pier Paolo Di Fiore, MD, PhD
Editor
Journal of Cell Biology

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

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

Although it is well known that clathrin molecules assemble at the plasma membrane in two main structures, namely clathrin-coated pits and flat clathrin-coated plaques, the mechanisms that control the two alternative assemblies are not completely understood. In this work Dr. Moulay and colleagues identified an alternative splicing in the clathrin heavy chain molecule (CLTC). The retention of an exon (named exon31) correlated with increased formation of flat clathrin lattices in cells, including in a model of muscle differentiation. Moreover, CLTC exon 31 skipping was found in the severe congenital form of myotonic dystrophy and was associated to a reduced number of clathrin plaques. The study is well conducted and of general interest.
Nevertheless, the reviewer thinks that the data do not fully support the authors' conclusions, which should be therefore dampened: indeed, since direct measurements of the two spliced versions of CLTC at the protein level cannot be provided, their relative enrichment in the pits and plaques cannot be verified. Moreover, the actual modification of the triskelion angle of incidence upon exon31 insertion is only speculative.
Major points:
1) The authors measured the inclusion of exon31 by PCR. No information on the relative abundance of the two alternative splicing isoforms of CLTC is provided at the protein level. Two experiments should be added: first calibrated PCR should be performed to assess if the intensities of the two PCR reactions are actually reflecting the relative concentration of template present in the samples. Second a mass spectrometry analysis of purified triskelia is essential to define the co-relation of the PCR results with the actual protein production.
2) Triskelia assemble by trimerization of clathrin heavy chains. This means that according to the

relative abundance of the two spliced variants the cells will retain a relative fraction of triskelia with 0, 1, 2, or 3 exon31-containing clathrin heavy chains. Let's examine one situation as an example: in figure S1 HeLa cells contain ~80% of CLTC-exon31- and 20% of CLTC-exon31+. Therefore the solution of the binomial model will result in: ~51% of triskelia containing only CLTC-exon31- molecules; ~38% of triskelia containing 2 CLTC-exon31- and 1 CLTC-exon31+; ~10% of triskelia containing 1 CLTC-exon31- and 2 CLTC-exon31+; ~1% of triskelia containing only CLTC-exon31+. This calculation already demonstrates that only 1% of triskelia are supposed to be substantially flatter according to the authors, but the extension of the plaques accounts for more extended surface which may suggest a dominant effect relative to the inclusion of exon31, meaning that triskelia with just one leg flat may already promote plaques formation.
Nevertheless this is not supported by the experiment in figure 1 (myoblast with ratio similar to HeLa do not show any flat array); Figure 2 (control myotube with 50% ratio can still form very narrow pits); Figure 3 (control myoblasts with a ratio similar to BSC-1 cells have flat clathrin arrays).
3) The experiments suggested in point 1 are important also in light of the results in Fig. 6. Here the authors depleted the clathrin heavy chain transcripts that include the exon31 by forcing exon skipping, but the effect at the protein level is not investigated. This experiment requires a western blot reporting that total clathrin heavy chain remains indeed constant, and a mass spectrometry analysis to assess the relative concentration of exon31-retaining and -skipping clathrin heavy chain.
4) Although a certain correlation of exon31 inclusion and flat array formation is present, there are other determinants that can affect the assembly of clathrin structures at the plasma membrane especially in light of the random incorporation of different heavy chains in the triskelia. One already investigated is the modification of adaptor concentration. For instance, PICALM depletion results in the increase of flat clathrin arrays (Meyerholz, A., Hinrichsen, L., Groos, S., Esk, P.-C., Brandes, G., and Ungewickell, E.J. (2005). Effect of Clathrin Assembly Lymphoid Myeloid Leukemia Protein Depletion on Clathrin Coat Formation. Traffic 6, 1225-1234) and in the decrease of the curvature of the clathrin coated pits (Miller, S.E., Mathiasen, S., Bright, N.A., Pierre, F., Kelly, B.T., Kladt, N., Schauss, A., Merrifield, C.J., Stamou, D., Höning, S., et al. (2015). CALM Regulates Clathrin-Coated Vesicle Size and Maturation by Directly Sensing and Driving Membrane Curvature. Dev. Cell 33, 163-175.). Finally AP2 increase correlates with the increase of plaques (Dambournet, D., Sochacki, K.A., Cheng, A.T., Akamatsu, M., Taraska, J.W., Hockemeyer, D., and Drubin, D.G. (2018). Genome-edited human stem cells expressing fluorescently labeled endocytic markers allow quantitative analysis of clathrin-mediated endocytosis during differentiation. J. Cell Biol 217, 3301-3311). Although the authors suggest that they observed a similar increase of AP2 as previously described in Daumbournet et al. 2018 upon myoblast differentiation, it is not possible for the reviewer to find this data. Providing an experiment where serial dilutions of the samples are loaded may resolve the ambiguity.
5) Finally the data presented in this paper do not support either the constant curvature or the constant area mode of clathrin assembly since according to the authors triskelia including exon31 cannot form curved structures. Therefore according to the authors' data the only potential speculation is that pits form following a constant curvature mode while plaques remain flat and cannot transition into curved surfaces.
In conclusion the finding are interesting but the data do not support the strong claims of the authors (...the capacity to form plaques requires the inclusion of exon 31), which should be dampened and discussed further. The formation of clathrin lattices are indeed governed by multifarious protein interactions and it is highly reductive to bring back all the effects to a single splicing event, especially in light of the randomness of heavy chain incorporation in triskelia, of the inability of the authors in monitoring the distribution of the two splicing variants of clathrin heavy chain and their relative localization in pits and plaques, and of previous published results that demonstrate a clear role of the relative adaptor concentration in the assembly of pits or plaques.

Minor comments:
1) The summary contains a repetition, revise it (A single alternatively spliced exon alternative splicing in the CLTC...)
2) In Fig.1 the authors show less transferrin internalization in myotubes when compared to myoblast. The actual distribution are very similar if excluded some outliers. Perhaps the myotube are more densely distributed (Figure 1A) and this might be the reason why transferrin internalization is less.
3) The triskelia is in general rigid, with the most flexible part of the heavy chain may be the links between the distal portion of the leg and the terminal domain (Kirchhausen T, et al., 1986). The insertion of exon31 may change the puckered shape, but the authors do not have evidence to support such conclusion, which may be investigated in future experiments, including negative staining and rotary shadowing of triskelia purified from brain or muscle cells or their ability to assemble in clathrin cages (as performed for example in Fotin et al., Nature 2004, Bocking et al., Nat Structural Biol. 2011 and many other experiments).
4) The authors refer to this exon as number 31. The reviewer is not sure that this is the correct nomenclature. The clathrin heavy chain assembly XM_005257012.2 corresponds to an X variant of the protein. Therefore the exon31 may correspond to a previously not described exon. This protein variant seems indeed not present in ensembl genome browser (https://useast.ensembl.org/Homo_sapiens/Info/Index). Can the authors comment on this? It is also possible that the author should cite Blue et al., 2018 at page 7 line 115 instead of Giudice et al., 2016.
5) Figure S1 B: the dashed rectangle does not correspond to the inset.
6) The +ex31 PCRs in Fig5C, Fig6B, FigS3A-B show two bands while in the other figures only 1 band. Can you please comment on this?

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

Moulay et al.
By modulating the splicing of endogenous clathrin heavy chain (CHC) exon 31, Moulay et al showed that alternative splicing of this exon regulates the formation of clathrin coated pits versus clathrin plaques in multiple cell lines. They concluded that 1) inclusion of exon 31 favors the formation of flat clathrin lattice while its exclusion favors the formation of clathrin coated pits for CME; 2) some cell lines contain high content of clathrin plaques due to their expression of exon 31-included CHC. This research is novel in providing a structural basis (i.e. inclusion or not of a 7 aa insert in the tripod region of CHC) for clathrin's propensity to form flat vs curved structures and most of the conclusions are clear and supported by the experiments presented. However, some speculation, describe below, is unwarranted and potentially weakens rather than strengthens the paper. My recommendation is that these unnecessary speculations be removed or clearly stated as speculation. The Graphical Abstract should be redrawn o reflect the conclusions and not the speculation and the Discussion should be rewritten to distinguish conclusions from speculation.
Specific major concerns:
1. 'Pucker angle' is mentioned several times in the main text and shown in the 'Graphic Abstract'; however, no clear structural basis for how the 7 aa insertion and changes in the amphiphilicity or trimerization propensity of the tripod domain would affect clathrin pucker is provided. Pucker angle is not mentioned in the results section that describes potential structural changes in the tripod domain as a result of the insertion. Its only mention occurs in the Discussion (Lines 240-241), where the authors statement that the increase in helix amphiphilicity 'would directly reduce the clathrin triskelion pucker angle and lead to the flattening of individual triskelia and subsequent plaque

assemble' is in conflict with their Graphic Abstract drawing, where reduced pucker angle would result in more curved structures.
Another possibility, which should also be discussed, is that changes in the packing of the tripod and the twist in the C-terminus will alter the ability of hsc70, the uncoating ATPase, to interact with clathrin at the downstream QPQLM motif (Rapoport et al, PMID 17978091) and reduce uncoating rates. Indeed, recent papers have suggested that hsc70 activity might be required to rearrange lattices to help generate curvature.
Lastly, in cells that express equal levels of the two splice isoforms, I would assume that these would be randomly assembled into triskelions that then express a mixture of these splice variants. How would that effect assembly?
My strong recommendation is to replace the Graphical Abstract with a less speculative model and one that more accurately reflects the conclusions that are supported by the data.

2. The first two paragraphs of the Discussion state conclusions that are not warranted by the data. For example, there is no evidence that 'the capacity to form plaques requires the inclusion of exon 31" (line 233), nor is there an rational given as to the authors ' prediction regarding changes in clathrin pucker (lines 240-241, see above). Similarly, they cannot conclude that "clathrin is genetically programmed...to produce two conformations.. a ubiquitous puckered form..etc" (line 247). These need to be rewritten to accurately reflect the conclusions that can be drawn and a clear distinction between speculation, which I welcome the authors to make, vs conclusions needs to be made.
3. The authors also over-interpret their conclusion on constant curvature vs. constant area models as described in Lines 283-284: 'This work also provides an explanation for why the constant curvature and the constant area models of clathrin assembly are both correct but depend on the genetic context'. This speculation is inconsistent with the expression of both splice variants in some cells that display both flat and curved structure, the effects of membrane tension and specific adaptors on the ratio of flat vs curved structures, evidence provided from polarized TIRF that flat lattices can gain curvature, etc. etc. Moreover, no evidence is presented that flat lattices composed almost exclusively by exon 31-containing clathrin can be converted to curved structures. Again,my strong recommendation is to remove this speculation, as it does not add to the findings regarding tissue-specific expression and functional differences in clathrin assemblies.
Minor concerns:

1. What does the area frequency plot (upper pane Fig. 2E, 3C) represent? What is the X axis? The authors state it does not change, but this depends on where you draw the dividing line, there is clearly a leftward shift. This needs to be explained.

2. Along the same lines, Supplemental Fig 4 should be moved up and presened along with the first morphological quantification (Fig. 2E) and better described in the main text (while referring the reader to methods).

3. CCPs are consistently larger in the myotubes and cell lines that express more exon 31. This should be mentioned. I assume both splice variants will be randomly incorporated into clathrin assemblies.
4. The Tfn CME endocytosis measurements are based on microscope imaging of Tfn-488. The authors should show some representative images to visually convince the readers that Tfn internalization was significantly changed after treatments. Moreover, based on the description in Materials and Methods, the surface bound Tfn-488 was not removed by acid wash. In this case, to accurately measure internalized Tfn-488, it is important to exclude cell edge area during Tfn-488 intensity measurements. However, this information is missing in the current manuscript. The

authors should clarify this.
5. There exist some typos. Below show some examples:
Line 83: 'highligh' should be 'highlight'
Line 152: 'CPPs' should be 'CCPs'
The Introduction can be rewritten to deliver the message more clearly.
The current very long paragraphs can be break into several paragraphs that focus on different sub-topics (e.g. new paragraph line 255 starting with "We also demonstrated....; new paragraph line 262 starting with "Interestingly, we...) etc. There may be more paragraphs that are obscured by the lack of indentation.
The story is focused on exon 31 splicing, but there is no background introduction of why they are motivated to study this exon 31 splicing on clathrin structures on the plasma membrane. The only background knowledge can be found is in a single sentence in Line 114-115.
The authors need to be consistent in symbol usage and be accurate in giving names throughout the manuscript. For the gene name of clathrin heavy chain, both CLTC and Cltc have been intermittently used. Moreover, the big and flat clathrin structures are defined as FCLs (flat clathrin lattices) in Figure 2; however, in Page 10 Line 198 and Line 201, they are inaccurately described as 'flat clathrin structures' and 'flat assemblies', which are broader concepts including those small and flat structures.

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

In this manuscript Moulay and colleagues demonstrate that the exon 31 of clathrin heavy chain has a critical role in determining the shape of clathrin coats on the plasma membrane of different cell types. The authors show that the inclusion of the exon 31 promotes flat clathrin coats and its exculsion promotes curved coats. The coat shapes allow different functions of the clathrin coats: budding of transport vesicles or the formation of flat adhesive structures.
The authors test their hypothesis comprehensively in several different ways in different cell types, focusing mainly on muscle cells where the flat clathrin coated adhesive structures are prevalent.
The main conclusions of the paper are strongly supported by the experimental data. The experiments are well performed and the data is of high quality.
The paper is very clearly written and the reasoning is easy to follow. I have some relatively minor comments and suggestions listed below. Overall, I think this work is a very important contribution to our understanding of the function of the clathrin coat, and especially to understanding how the same molecular machinery is adapted to be used in different cell types in a multicellular organism. This understanding of the tissue context is still largely missing from most cell biology studies.

The first sentence of the abstract is somewhat cumbersome to read. Maybe this is due to the abstract word limit, but it might be easier to read if expanded a bit.

The graphical abstract could indicate where the exon 31 is located in the clathrin structure. That would make it more obvious in the graphical abstract how the exon could alter the pucker angle.

In Fig 1A it would be nice to have the inset images shown also as separate channels. It is not so easy to see the distribution of the two proteins in the merged images.

Transferrin uptake assay is not a conclusive way to measure the CME rates in different cell types. Different cells could have different levels of the receptor or could recycle it back to the plasma

membrane more or less actively. However, I'm not sure there is a simple way to measure the CME rates in different cell types. The uptake of transferrin is still an interesting piece of information in this context. I would suggest that authors just tone down their conclusion about this assay determining the CME rate.

The Fig 2E and 3C have four quadrants, but only two of these are explained and labeled (CCP and FCL). I think the authors should also provide an interpretation for the structures that fall in the two remaining quadrants. Indeed, later on page 8 the lower left quadrant is mentioned and described as small plaques. Are both the small and large plaques FCL?

In the discussion section the authors make, in my opinion, unnecessarily strong conclusions, which I would suggest toning down. The authors state that the exon31 is required for the formation of flat clathrin lattices (lines 233, 242) and that the exon31 determines two different clathrin conformations (line 247). The flat lattices are not completely gone in assays where the exon31 is down regulated. Maybe this is because there is still some clathrin with exon31 left, or maybe because it does promote flat lattices, but is not the only determinant for them. Also, the different conformations being determined by the exon31 seem likely, but not definitively demonstrated here. Finally, the authors finish the discussion by saying that they provided an explanation for why both the constant curvature and constant area models are correct (lines 283-284). Their data is definitely highly relevant for these two models, but the data presented in this manuscript does not deal with possible shape transitions during vesicle budding and does not explain how a flat lattice can be shaped into a curved vesicle.

April 14th, 2020

Dear Dr. Di Fore and Dr Marat,

We herewith submit a revised version of our manuscript #201912061 entitled: "Alternative splicing of clathrin heavy chain exon 31 allows the switch from coated pits to plaques".

We were very pleased that our manuscript was taken into consideration and referees found it of interest, although some concerns were raised that preclude publication of the work in its primary version. We would like to thank the three Reviewers for the detailed assessment of our manuscript and helpful remarks and suggestions. Based on feedbacks from Reviewers and Editors, we have substantially revised our manuscript to address the points raised. The revised manuscript contains a number of new experimental results and analyses that we hope will significantly strengthen its conclusions. We feel that, despite the difficult times due to the coronavirus confinement, we have addressed all the major concerns that the referees raised and added new data that considerably substantiate our earlier conclusions. Please find below a detailed point by point response to the reviews indicating how we have addressed each point raised.

Briefly, we produced new data requested by the reviewers which are now presented in the form of additional panels in the main and supplementary figures (mass spectrometry in Fig. S2 and revised Fig. 1), additional AP2 and CHC immunoblots (new Fig. S2 and revised Fig. S5), as well as images illustrating the transferrin assays (revised Fig. S1, S4 and Fig. 5) and super-resolution light microscopy (revised Fig. S1). We extensively modified the text by adding new and clarifying statements in the paper, and amended the discussion and graphical abstract according to the referees' suggestions.

Additionally, we have added Florent Dingli and Damarys Loew as co-authors recognizing their assistance in the mass spectrometry analysis now included in the manuscript.

We also would like to thank JCB editors for the opportunity to submit this revised manuscript and for the Reviewers thoughtful comments and consideration.

Sincerely,

Stéphane VASSILOPOULOS

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The original comments from the reviewers are in Times font italics and our responses are in plain text. When a line, figure number, or panel is mentioned, our answer refers to the revised manuscript. Please, also excuse the length of our response. We appreciate the thought that went into the reviewer's comments and wanted to give them the considered response they deserve.

Response to the reviewers:

Reviewer #1 (Comments to the Authors):

Although it is well known that clathrin molecules assemble at the plasma membrane in two main structures, namely clathrin-coated pits and flat clathrin-coated plaques, the mechanisms that control the two alternative assemblies are not completely understood. In this work Dr. Moulay and colleagues identified an alternative splicing in the clathrin heavy chain molecule (CLTC). The retention of an exon (named exon31) correlated with increased formation of flat clathrin lattices in cells, including in a model of muscle differentiation. Moreover, CLTC exon 31 skipping was found in the severe congenital form of myotonic dystrophy and was associated to a reduced number of clathrin plaques. The study is well conducted and of general interest.

We would like to thank Reviewer #1 for his appreciation of our work and for deeming our overall conclusions convincing.

Nevertheless, the reviewer thinks that the data do not fully support the authors' conclusions, which should be therefore dampened: indeed, since direct measurements of the two spliced versions of CLTC at the protein level cannot be provided, their relative enrichment in the pits and plaques cannot be verified. Moreover, the actual modification of the triskelion angle of incidence upon exon31 insertion is only speculative.

Major points:

1) The authors measured the inclusion of exon31 by PCR. No information on the relative abundance of the two alternative splicing isoforms of CLTC is provided at the protein level. Two experiments should be added: first calibrated PCR should be performed to assess if the intensities of the two PCR reactions are actually reflecting the relative concentration of template present in the samples. Second a mass spectrometry analysis of purified triskelia is essential to define the co-relation of the PCR results with the actual protein production.

We agree with the reviewer's suggestion that the *CLTC* splicing isoforms should be validated at the protein level. While this is not a trivial question because exon 31 consists of only 7 amino acids and there is no specific antibody against this region, we have concentrated our efforts on proving its existence at the protein level. To this end, we performed quantitative label-free mass spectrometry analysis of immunoprecipitated clathrin heavy chain from myoblasts containing mostly CHC without exon 31 and myotubes expressing CHC with exon 31. The mass spectrometry results are now introduced in figure 1 (new panel J) and in a supplementary figure (Fig. S2). We tested several different types of enzymatic digestions which all demonstrate the existence of this exon and settled on Glu-C digestion that generates unique peptides covering the exon junctions, and therefore discriminates CHC isoforms arising from exon 31 splicing (Fig. S2 C). Figure S2 D displays representative MS/MS spectra

identifying both isoforms, confirming the existence of clathrin heavy chain with exon 31 inclusion at the protein level in human muscle cells. Further quantification of both unique peptides in the myoblast to myotube differentiation model is now presented in Fig. 1 J for comparison with RT-PCR data. Specific peptides quantification followed the trends seen at mRNA level as now stated in the manuscript at lines 140-145:
"We therefore performed a shotgun proteomic analysis enabling the detection of unique peptides covering the exon junctions with or without exon 31, in our myogenic differentiation model (Fig. S2 C and D). Quantifying the relative amounts of peptides ± exon 31 confirmed the RT-PCR results at the protein level (Fig. 1 I), with a replacement of CHC protein without exon 31 by CHC protein including exon 31 during human muscle cell differentiation (Fig. 1J)".
A "Proteomics and Mass Spectrometry Analysis" section was added to the materials and methods section, and the mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository. The dataset (identifier PXD018333) is accessible to reviewers using this url: https://www.ebi.ac.uk/pride/login (username: reviewer48651@ebi.ac.uk, password: qIh6RqWj).

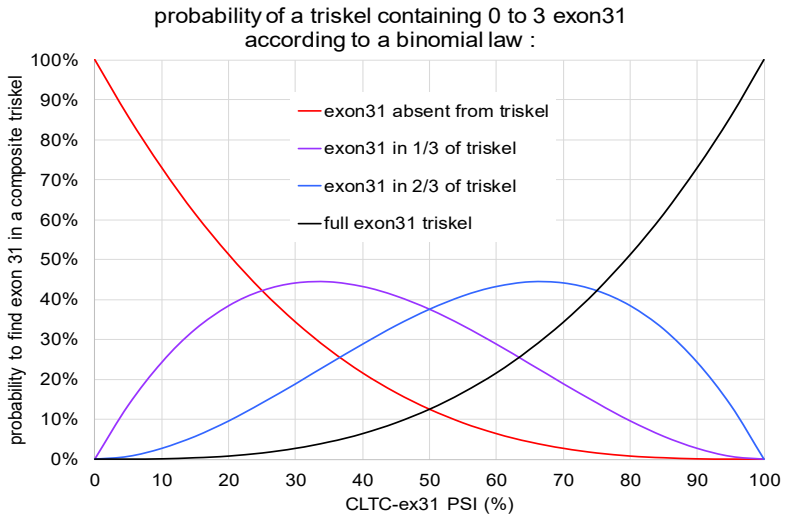
Concerning the analysis of relative ratio of the two *CLTC* isoforms (± exon 31), our semi-quantitative RT-PCR was performed with the use of a single pair of primers to amplify both amplicons (± exon 31) as illustrated in Fig. 1 I. This approach is routinely used in the splicing field to assess the relative ratio of two alternative spliced isoforms since both amplicons arise from a single PCR reaction. To strengthen our RT-PCR conclusions we confirm *CLTC*-ex31 progressive inclusion during muscle cell differentiation by extracting data from this particular locus in a publicly available transcriptome (see lines 137-139 and Fig. S2 B).

2) Triskelia assemble by trimerization of clathrin heavy chains. This means that according to the relative abundance of the two spliced variants the cells will retain a relative fraction of triskelia with 0, 1, 2, or 3 exon31-containing clathrin heavy chains. Let's examine one situation as an example: in figure S1 HeLa cells contain ~80% of CLTC-exon31- and 20% of CLTC-exon31+. Therefore the solution of the binomial model will result in: ~51% of triskelia containing only CLTC-exon31- molecules; ~38% of triskelia containing 2 CLTC-exon31- and 1 CLTC-exon31+; ~10% of triskelia containing 1 CLTC-exon31- and 2 CLTC-exon31+; ~1% of triskelia containing only CLTC-exon31+.
This calculation already demonstrates that only 1% of triskelia are supposed to be substantially flatter according to the authors, but the extension of the plaques accounts for more extended surface which may suggest a dominant effect relative to the inclusion of exon31, meaning that triskelia with just one leg flat may already promote plaques formation.
Nevertheless this is not supported by the experiment in figure 1 (myoblast with ratio similar to HeLa do not show any flat array); Figure 2 (control myotube with 50% ratio can still form very narrow pits); Figure 3 (control myoblasts with a ratio similar to BSC-1 cells have flat clathrin arrays).

We thank reviewer #1 for raising this interesting point. As suggested, we agree that a binomial distribution probability model applied to the composition of an individual triskelion suggests a dominant effect of exon31 when it comes to forming flat structures.

probability that a triskel contains 0 to 3 exon31 according to a binomial law :

PSI (%)	CHC + exon31 per triskel 0	1	2	3
0	100%	0%	0%	0%
5	86%	14%	1%	0%
10	73%	24%	3%	0%
15	61%	33%	6%	0%
20	51%	38%	10%	1%
25	42%	42%	14%	2%
30	34%	44%	19%	3%
35	27%	44%	24%	4%
40	22%	43%	29%	6%
45	17%	41%	33%	9%
50	13%	38%	38%	13%
55	9%	33%	41%	17%
60	6%	29%	43%	22%
65	4%	24%	44%	27%
70	3%	19%	44%	34%
75	2%	14%	42%	42%
80	1%	10%	38%	51%
85	0%	6%	33%	61%
90	0%	3%	24%	73%
95	0%	1%	14%	86%
100	0%	0%	0%	100%



Indeed, once plotted against the spliced-in percentage that corresponds to exon 31 inclusion, we observe that the probability to obtain flat triskelia purely composed of exon 31 CHC isoform is only 1-2% with PSI values of 20-25%. Thus, composite triskelia with one or two exon 31 containing CHC may already favor or trigger flat structures assembly. This model is further supported by myotubes (forming mainly flat structures) tending toward 45-50% inclusion where an equivalent amount of triskelia fully composed of CHC - exon 31 or CHC + 31 are predicted to exist, and where triskelia composed of 1/3 or 2/3 CHC + exon 31 would tip the balance toward flat structures following the dominant effect hypothesis. This is now summarized in the discussion on lines 295-306.

However, we do notice in our comparison that several cell types behave differently around low PSI values of 6-7%. For instance, the reviewer mentions that myoblasts with ratio similar to HeLa cells do not show any flat array. We recognize that the electron microscopy pictures of myoblast and myotubes displayed in Fig. 1 illustrate only the main structure in such cells, but flat structures can indeed be seen in myoblasts as shown and quantified latter in Fig. 3. We now added the following sentence at line 117 in Fig. 1 description to account for that notion from the start of the manuscript: "Of note, although CCPs are the main clathrin structures encountered in myoblasts, some plaques are present albeit at much lower frequency (Fig. S1 E); conversely, myotubes displaying mostly large clathrin plaques still produce canonical coated pits and are capable of performing endocytosis as small puncta are visible using super-resolution light microscopy (Fig. 1 A)". Several other factors that could mitigate flat clathrin assembly predictions between different cell lines at low exon 31 inclusion ratio are now also discussed in the discussion on lines 309-322.

3) The experiments suggested in point 1 are important also in light of the results in Fig. 6. Here the authors depleted the clathrin heavy chain transcripts that include the exon31 by forcing exon skipping, but the effect at the protein level is not investigated. This experiment requires a western blot reporting that total clathrin heavy chain remains indeed constant, and a mass spectrometry analysis to assess the relative concentration of exon31-retaining and -skipping clathrin heavy chain.

We agree with Reviewer #1 and have now included additional results on the effect of *in vivo* exon skipping at the protein level by adding CHC immunoprecipitation experiments in Fig. S5 C to address this point. We have now updated the text to state (line 250) that *in vivo* exon

skipping occured “without changes in CHC protein amount or its associated light chain CLCa upon co-immunoprecipitation”.

4) Although a certain correlation of exon31 inclusion and flat array formation is present, there are other determinants that can affect the assembly of clathrin structures at the plasma membrane especially in light of the random incorporation of different heavy chains in the triskelia. One already investigated is the modification of adaptor concentration. For instance, PICALM depletion results in the increase of flat clathrin arrays (Meyerholz, A., Hinrichsen, L., Groos, S., Esk, P.-C., Brandes, G., and Ungewickell, E.J. (2005). Effect of Clathrin Assembly Lymphoid Myeloid Leukemia Protein Depletion on Clathrin Coat Formation. Traffic 6, 1225-1234) and in the decrease of the curvature of the clathrin coated pits (Miller, S.E., Mathiasen, S., Bright, N.A., Pierre, F., Kelly, B.T., Kladt, N., Schauss, A., Merrifield, C.J., Stamou, D., Höning, S., et al. (2015). CALM Regulates Clathrin-Coated Vesicle Size and Maturation by Directly Sensing and Driving Membrane Curvature. Dev. Cell 33, 163-175.). Finally AP2 increase correlates with the increase of plaques (Dambournet, D., Sochacki, K.A., Cheng, A.T., Akamatsu, M., Taraska, J.W., Hockemeyer, D., and Drubin, D.G. (2018). Genome-edited human stem cells expressing fluorescently labeled endocytic markers allow quantitative analysis of clathrin-mediated endocytosis during differentiation. J. Cell Biol 217, 3301-3311). Although the authors suggest that they observed a similar increase of AP2 as previously described in Daumbournet et al. 2018 upon myoblast differentiation, it is not possible for the reviewer to find this data. Providing an experiment where serial dilutions of the samples are loaded may resolve the ambiguity.

This point is important, and also made by Reviewer #3. We agree that other parameters than exon 31 alternative splicing certainly participate to the formation of canonical pits vs flat clathrin structures. In addition to the experiments describing an increased AP2 expression during differentiation into myotubes (Supplementary Fig. S2 A), we have now included additional references and amended our discussion to explicitly state this (see lines 309-322).

5) Finally the data presented in this paper do not support either the constant curvature or the constant area mode of clathrin assembly since according to the authors triskelia including exon31 cannot form curved structures. Therefore according to the authors' data the only potential speculation is that pits form following a constant curvature mode while plaques remain flat and cannot transition into curved surfaces.
In conclusion the finding are interesting but the data do not support the strong claims of the authors (...the capacity to form plaques requires the inclusion of exon 31), which should be dampened and discussed further. The formation of clathrin lattices are indeed governed by multifarious protein interactions and it is highly reductive to bring back all the effects to a single splicing event, especially in light of the randomness of heavy chain incorporation in triskelia, of the inability of the authors in monitoring the distribution of the two splicing variants of clathrin heavy chain and their relative localization in pits and plaques, and of previous published results that demonstrate a clear role of the relative adaptor concentration in the assembly of pits or plaques.

We agree with Reviewer #1 and have removed the sentence referring to the constant curvature or the constant area mode of clathrin assembly, to favor the following clarification on line 307:
“While there is a strong correlation of exon 31 inclusion and clathrin plaque formation, the data presented here does not deal with possible shape transitions during vesicle budding nor does it explain how a flat lattice can be shaped into a curved vesicle.”

The take home message and our conclusions have been dampened throughout the text, and we now discuss several other factors needed to produce clathrin's structural landscape observed in different cell types as answered above to major points 2) and 4).

Minor comments:

1) The summary contains a repetition, revise it (A single alternatively spliced exon alternative splicing in the CLTC...).

This sentence has now been corrected.

2) In Fig.1 the authors show less transferrin internalization in myotubes when compared to myoblast. The actual distribution are very similar if excluded some outliers. Perhaps the myotube are more densely distributed (Figure 1A) and this might be the reason why transferrin internalization is less.

Contrary to cell lines such as C2C12 that may need confluent myoblasts to obtain myotubes exceedingly close to each other, this work used both human and mouse myoblasts with a high myogenic potential that we differentiate before having reached confluency. As a result, a good portion of myotubes is more spread and rare contacts between myotubes should not account for a substantial surface of each myotube membrane that would still be mainly accessible to soluble transferrin. This is now explicitly shown in the transferrin assay picture added in Fig. S1 F (or Fig. S4 G). Although some myoblasts display low transferrin uptake similar to that of myotubes, a significant portion of them has higher transferrin uptake. We would also like to emphasize that myoblasts are a heterogeneous population with some probably more mature than others. This reflects in terms of transferrin uptake (see picture added in Fig. S1 E), and different quartile distributions are observed between myoblasts and myotubes in Fig. 1 H box plot.
It is worth noting that in agreement with our results, although we are the first to publish quantitative results (myotubes internalize less transferrin than myoblasts), previous work from K Metsikko's group also mention a drop of transferrin internalization during differentiation (Kaisto et al., Exp Cell Res, 1999).

3) The triskelia is in general rigid, with the most flexible part of the heavy chain may be the links between the distal portion of the leg and the terminal domain (Kirchhausen T, et al., 1986). The insertion of exon31 may change the puckered shape, but the authors do not have evidence to support such conclusion, which may be investigated in future experiments, including negative staining and rotary shadowing of triskelia purified from brain or muscle cells or their ability to assemble in clathrin cages (as performed for example in Fotin et al., Nature 2004, Bocking et al., Nat Structural Biol. 2011 and many other experiments).

We agree with Reviewer #1 that the puckered shape change is speculative and is only suggested by *in silico* analysis in Fig. 4 E where an increased potency to form coiled-coil interactions in the tripod could consequently flatten the triskelion (assuming general triskelion rigidity as mention by the reviewer). We modified the text at lines 278-280 where the hypothetical nature of the pucker angle change could be misunderstood. We absolutely agree that future experiments investigating precise triskelion molecular composition and structure are needed to demonstrate this point and we now clearly state this in the discussion on line 303.

4) The authors refer to this exon as number 31. The reviewer is not sure that this is the correct nomenclature. The clathrin heavy chain assembly XM_005257012.2 corresponds to an X variant of the protein. Therefore the exon31 may correspond to a previously not described exon. This protein variant seems indeed not present in ensembl genome browser (https://useast.ensembl.org/Homo_sapiens/Info/Index). Can the authors comment on this? It is also possible that the author should cite Blue et al., 2018 at page 7 line 115 instead of Giudice et al., 2016.

The European database Ensembl does not currently reference transcripts containing *CLTC* exon 31. The American database NCBI has updated or removed transcripts containing exon 31 several times in the last few years based on their Gnomon algorithm. Gnomon predicted for instance the human XM_005257012.2 nucleotide sequence containing exon 31 that we cited in the text, but it has recently been removed following their regular genome annotation process. A substantial part of observed splicing variations are ignored in common classification pipelines, and to our knowledge there is no nomenclature univocally assigned for newly described alternative splicing events and databases use different ones. We refer to exon 31 using this number in agreement with two previously published works in mice cited in our manuscript (Giudice et al., 2016 and Blue et al., 2018), and based on mouse and human genomic sequence comparison. Independently of one's favorite nomenclature, no ambiguity will appear to the reader since several key features uniquely identifying this exon are mentioned in the manuscript (21 nucleotide size, full nucleotide and amino acid sequences in Fig. S2, sequence of PCR primers in materials section etc). Of note a mouse RNA reference sequence containing exon 31 was annotated in october 2019 under accession number NM_001356393.1, we therefore corrected Fig. 1 legend at lines 696-698 to cite this more persistent annotation in replacement of previously predicted sequence XM_006533983.1. Currently no human reference sequence contains exon 31, although it can be found expressed in some available transcriptomes (see Fig. S2 B). Our mass spectrometry data deposited alongside this article to the ProteomeXchange Consortium via the PRIDE partner repository under dataset identifier PXD018333 is the first experimentally validated human protein sequence containing this exon in a protein database (accessible to reviewers using url: https://www.ebi.ac.uk/pride/login and username reviewer48651@ebi.ac.uk, password: qIh6RqWj).

5) Figure S1 B: the dashed rectangle does not correspond to the inset.

This point has now been corrected in the revised version of figure S1 B.

6) The +ex31 PCRs in Fig5C, Fig6B, FigS3A-B show two bands while in the other figures only 1 band. Can you please comment on this?

Indeed, we observed an additional band specific to the mouse mRNA appearing both *in vivo* (Fig. 6 B and newly numbered Fig. S5 A-B) and *in vitro* (newly numbered Fig. S4 A) above the expected band. This feature is common in the field of alternative splicing research, and is especially visible when trying to resolve very close isoforms such as our 21 nucleotides exon. Usually such multiplication of RT-PCR inclusion top bands is attributed to either PCR artifact or the use of a cryptic splicing site on top of the more frequently used one. Both hypothesis were tested, first with the use of different Taq polymerase and master mix that sometime solves such issues, but it did not in this case. Second, we cloned both upper bands generated by RT-PCR on mouse muscle tissue and managed to sequence 11 clones before our lab shutdown. All 11 clones turned out to be regular 21 nucleotides exon 31 inclusions. In

addition *in silico* analysis did not predict the presence of cryptic splicing sites in the murine sequence. However, exon skipping was very efficient in both murine *in vitro* and *in vivo* experiments, and significantly reduced both bands.

Reviewer #2 (Comments to the Authors):

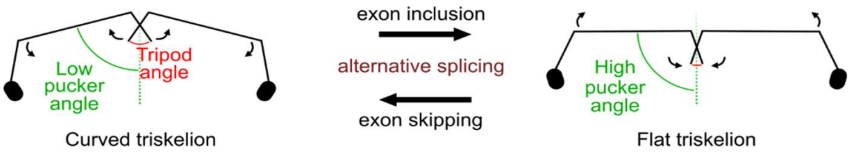
By modulating the splicing of endogenous clathrin heavy chain (CHC) exon 31, Moulay et al showed that alternative splicing of this exon regulates the formation of clathrin coated pits versus clathrin plaques in multiple cell lines. They concluded that 1) inclusion of exon 31 favors the formation of flat clathrin lattice while its exclusion favors the formation of clathrin coated pits for CME; 2) some cell lines contain high content of clathrin plaques due to their expression of exon 31-included CHC. This research is novel in providing a structural basis (i.e. inclusion or not of a 7 aa insert in the tripod region of CHC) for clathrin's propensity to form flat vs curved structures and most of the conclusions are clear and supported by the experiments presented. However, some speculation, describe below, is unwarranted and potentially weakens rather than strengthens the paper. My recommendation is that these unnecessary speculations be removed or clearly stated as speculation. The Graphical Abstract should be redrawn to reflect the conclusions and not the speculation and the Discussion should be rewritten to distinguish conclusions from speculation.

We would like to thank Reviewer #2 for this appreciation of our work.

Specific major concerns:

1. 'Pucker angle' is mentioned several times in the main text and shown in the 'Graphic Abstract'; however, no clear structural basis for how the 7 aa insertion and changes in the amphiphilicity or trimerization propensity of the tripod domain would affect clathrin pucker is provided. Pucker angle is not mentioned in the results section that describes potential structural changes in the tripod domain as a result of the insertion. Its only mention occurs in the Discussion (Lines 240-241), where the authors statement that the increase in helix amphiphilicity 'would directly reduce the clathrin triskelion pucker angle and lead to the flattening of individual triskelia and subsequent plaque assemble' is in conflict with their Graphic Abstract drawing, where reduced pucker angle would result in more curved structures.
Another possibility, which should also be discussed, is that changes in the packing of the tripod and the twist in the C-terminus will alter the ability of hsc70, the uncoating ATPase, to interact with clathrin at the downstream QPQLM motif (Rapoport et al, PMID 17978091) and reduce uncoating rates. Indeed, recent papers have suggested that hsc70 activity might be required to rearrange lattices to help generate curvature.
Lastly, in cells that express equal levels of the two splice isoforms, I would assume that these would be randomly assembled into triskelions that then express a mixture of these splice variants. How would that effect assembly?
My strong recommendation is to replace the Graphical Abstract with a less speculative model and one that more accurately reflects the conclusions that are supported by the data.

In agreement with Reviewer #2 suggestion, the speculative model of triskelia pucker angle was removed from the Graphical abstract to only display the main conclusion of this article.



We realized thanks to the reviewer's comment concerning what we wrote in the initial manuscript: "As a result, the helices amphiphilicity was increased....which we predict would directly **reduce** the clathrin triskelion pucker angle and lead to the flattening of individual triskelia and subsequent plaque assemble" was the opposite to what we meant. We greatly appreciate Reviewer #2 for the careful reading and for pointing out this mistake. We have now corrected the sentence on line 278 accordingly: "As a result, the helices amphiphilicity was increased along with their potency to form coiled-coil interactions, which we predict could reduce the clathrin triskelion tripod angle, **increase** the pucker angle, and lead to the flattening of individual triskelia and subsequent plaque assembly."
As suggested by this reviewer we now also discuss the hypothesis of exon 31 inclusion impacting the ability of hsc70 chaperone to uncoat assembled triskelia which could contribute to increased plaque formation (lines 322-325). Lastly, we added discussion over triskelia assembly as heterotrimers and the possibility that exon 31 has a dominant effect on flat clathrin assembly in a new paragraph at lines 295-304 of the revised manuscript.

2. The first two paragraphs of the Discussion state conclusions that are not warranted by the data. For example, there is no evidence that 'the capacity to form plaques requires the inclusion of exon 31" (line 233), nor is there any rational given as to the authors ' prediction regarding changes in clathrin pucker (lines 240-241, see above). Similarly, they cannot conclude that "clathrin is genetically programmed...to produce two conformations.. a ubiquitous puckered form..etc" (line 247). These need to be rewritten to accurately reflect the conclusions that can be drawn and a clear distinction between speculation, which I welcome the authors to make, vs conclusions needs to be made.

The puckered shape change is a hypothesis that is supported by *in silico* analysis in Fig. 4 E where an increased potency to form coiled-coil interactions in the tripod is predicted (with the possible consequent angle modifications corrected in the previous answer). We agree with Reviewer #2 that some of our conclusions, in particular those in the discussion were exaggerated. We have toned down these claims in the discussion.

3. The authors also over-interpret their conclusion on constant curvature vs. constant area models as described in Lines 283-284: 'This work also provides an explanation for why the constant curvature and the constant area models of clathrin assembly are both correct but depend on the genetic context'. This speculation is inconsistent with the expression of both splice variants in some cells that display both flat and curved structure, the effects of membrane tension and specific adaptors on the ratio of flat vs curved structures, evidence provided from polarized TIRF that flat lattices can gain curvature, etc. etc. Moreover, no evidence is presented that flat lattices composed almost exclusively by exon 31-containing clathrin can be converted to curved structures. Again,my strong recommendation is to remove this speculation, as it does not add to the findings regarding tissue-specific expression and functional differences in clathrin assemblies.

We agree and have now removed this unsupported speculation. We added the following clarification on line 307: "While there is a strong correlation of exon 31 inclusion and clathrin

plaque formation, the data presented here does not deal with possible shape transitions during vesicle budding nor does it explain how a flat lattice can be shaped into a curved vesicle."
Several other factors influencing clathrin's structural landscape observed in different cell types are now also discussed on lines 309-321.

Minor concerns:

1. What does the area frequency plot (upper pane Fig. 2E, 3C) represent? What is the X axis? The authors state it does not change, but this depends on where you draw the dividing line, there is clearly a leftward shift. This needs to be explained.

The upper frequency plot represents the frequency of objects distributed along the X area axis in one or the other color-coded experimental condition (similarly the frequency plot on the right is a projection of objects frequency along the Y curvature axis).
For Fig. 2 E we do state on line 169 that "the size of CCPs remained unchanged" so this comment refers to the left quadrant of the upper frequency plot where no lateral shift of the frequency curve appears in control or skip below the area threshold attributed to CCPs (as mentioned in the methods section we used "an area cut-off threshold of 40,000 nm^2 corresponding to the size of the larger CCPs encountered with a diameter of ~200 nm").
We indeed observe a leftward shift of the area frequency curve from Fig. 3 C that we now comment as follow in the result section at line 190: "However, we observed in this experimental procedure that the upper frequency plot is shifted towards smaller structures (Fig. 3 C), likely because *CLTC*-ex31 inclusion is not the sole splicing event mis-regulated by CELF depletion."

2. Along the same lines, Supplemental Fig 4 should be moved up and presented along with the first morphological quantification (Fig. 2E) and better described in the main text (while referring the reader to methods).

We moved the supplemental figure up to go along with fig. 2 E, and now describe the first morphological quantification in more details in the result section according to Reviewer #2 suggestion by adding the following sentences at lines 159-164: "We performed a morphometric analysis in which clathrin structures present on PREM images were plotted according to their area and curvature (Fig. 2 E). We defined an area cut-off threshold of 40,000 nm2 corresponding to the size of the largest CCPs encountered with diameters of ~200 nm, while a curvature threshold was set manually to segregate flat clathrin lattices (FCL) from CCP in control myotubes after measuring each structure's electron opacity as described in Fig. S3 A."

3. CCPs are consistently larger in the myotubes and cell lines that express more exon 31. This should be mentioned. I assume both splice variants will be randomly incorporated into clathrin assemblies.

This point raised by Reviewer #2 is interesting however we have not consistently observed this phenomenon. Some cells such as neurons have low exon 31 inclusion and very small coated pits, while myoblasts with equivalent exon 31 inclusion do form larger coated pits. Also, the size of CCPs in myotubes containing less exon 31 due to skipping or DM1 disease was not altered in Fig. 2 F and Fig. 5 G. Thus, additional parameters might be involved here and we prefer to be cautious by not suggesting a correlation between inclusion of exon 31 and size of coated pits.

4. The Tfn CME endocytosis measurements are based on microscope imaging of Tfn-488. The authors should show some representative images to visually convince the readers that Tfn internalization was significantly changed after treatments. Moreover, based on the description in Materials and Methods, the surface bound Tfn-488 was not removed by acid wash. In this case, to accurately measure internalized Tfn-488, it is important to exclude cell edge area during Tfn-488 intensity measurements. However, this information is missing in the current manuscript. The authors should clarify this.

Representative images of each transferrin assay were added in Fig. S1 E-F, Fig. S4 E-H and Fig. 5 J-K to visually substantiate our claims that Tfn internalization was significantly changed after treatments. In our method the surface bound Tfn-488 is washed twice with ice-cold PBS. We do trace areas right below the cell edge to avoid quantifying overlapping areas near cell contacts that happen in myotube cultures, and no border effects are visible in our pictures.

5. There exist some typos. Below show some examples:
Line 83: 'highligh' should be 'highlight'
Line 152: 'CPPs' should be 'CCPs'

These errors were corrected.

The Introduction can be rewritten to deliver the message more clearly.
The current very long paragraphs can be break into several paragraphs that focus on different sub-topics (e.g. new paragraph line 255 starting with "We also demonstrated...:; new paragraph line 262 starting with "Interestingly, we...) etc. There may be more paragraphs that are obscured by the lack of indentation.
The story is focused on exon 31 splicing, but there is no background introduction of why they are motivated to study this exon 31 splicing on clathrin structures on the plasma membrane. The only background knowledge can be found is in a single sentence in Line 114-115.
The authors need to be consistent in symbol usage and be accurate in giving names throughout the manuscript. For the gene name of clathrin heavy chain, both CLTC and Cltc have been intermittently used. Moreover, the big and flat clathrin structures are defined as FCLs (flat clathrin lattices) in Figure 2; however, in Page 10 Line 198 and Line 201, they are inaccurately described as 'flat clathrin structures' and 'flat assemblies', which are broader concepts including those small and flat structures.

We appreciate this reviewer's help to improve the quality of the manuscript. We have now expanded the introduction to clearly present the rationale and background for the study of alternative splicing and exon 31 on lines 75-85. In agreement with this reviewer's suggestion, we have also separated the introduction into different sub-topics to make it easier for the reader.
CLTC and *Cltc* gene names are used on purpose according to gene nomenclature for human and mouse species respectively when it applies in the text.
We agree that “flat clathrin structures” and “flat assemblies” are broad concepts. We therefore introduced the following clarification in the result section that completes our answer to Reviewer #2 Minor concern 2 on line 165: “It is worth noting that we chose to use the term FCL to describe flat structures comprising both small objects below the area threshold and the big and flat structures corresponding to clathrin plaques above this threshold and found in the

lower-right quadrant." And we changed references to flat assemblies throughout the text and figures to conform to that notion.

Reviewer #3 (Comments to the Authors):

In this manuscript Moulay and colleagues demonstrate that the exon 31 of clathrin heavy chain has a critical role in determining the shape of clathrin coats on the plasma membrane of different cell types. The authors show that the inclusion of the exon 31 promotes flat clathrin coats and its exculsion promotes curved coats. The coat shapes allow different functions of the clathrin coats: budding of transport vesicles or the formation of flat adhesive structures.
The authors test their hypothesis comprehensively in several different ways in different cell types, focusing mainly on muscle cells where the flat clathrin coated adhesive structures are prevalent.
The main conclusions of the paper are strongly supported by the experimental data. The experiments are well performed and the data is of high quality.
The paper is very clearly written and the reasoning is easy to follow. I have some relatively minor comments and suggestions listed below. Overall, I think this work is a very important contribution to our understanding of the function of the clathrin coat, and especially to understanding how the same molecular machinery is adapted to be used in different cell types in a multicellular organism. This understanding of the tissue context is still largely missing from most cell biology studies.

We thank Reviewer #3 for his appreciation of our work and for pointing out the importance of the tissue context for understanding the structure/function relationship.

The first sentence of the abstract is somewhat cumbersome to read. Maybe this is due to the abstract word limit, but it might be easier to read if expanded a bit.

We have modified the first sentence in the abstract for clarity.

The graphical abstract could indicate where the exon 31 is located in the clathrin structure. That would make it more obvious in the graphical abstract how the exon could alter the pucker angle.

Although we agree with Reviewer #3's suggestion, in agreement with the other reviewers comments we decided to remove this part which was deemed too speculative. Thus, the graphical abstract no longer describes the structural transition occurring in triskelia with and without exon 31 and focuses on the rearrangement from pits to plaques.

In Fig 1A it would be nice to have the inset images shown also as separate channels. It is not so easy to see the distribution of the two proteins in the merged images.

We have now included the separate channels for the light microscopy imaging in a supplemental figure (Fig S1 A) for clarity.

Transferrin uptake assay is not a conclusive way to measure the CME rates in different cell types. Different cells could have different levels of the receptor or could recycle it back to the plasma membrane more or less actively. However, I'm not sure there is a simple way to measure the CME rates in different cell types. The uptake of transferrin is still an interesting

piece of information in this context. I would suggest that authors just tone down their conclusion about this assay determining the CME rate.

Although Transferrin uptake is the gold standard for monitoring clathrin-mediated endocytosis, we absolutely agree with Reviewer #3 that there is no simple way to measure CME rates, especially in differentiated cell types such as neurons and myotubes. All the transferrin assays reported in this article however focus on one cell type (muscle cells). Specifically measuring rates would require plotting the results of assays realized at different incubation times or to adapt all presented experiments to live imaging. We have followed Reviewer #3 suggestion and toned down our conclusions by changing the word "rate" to the broader term "activity" in the result section to account for that limitation. We have added representative images of transferrin uptake in Supplementary Fig. S1 E-F, Fig. S4 E-H and Fig. 5 J-K to visually substanciate the claim that Tfn internalization was significantly changed after treatments. Also, in agreement with our results, it is worth noting that previous work from K Metsikko's group has also observed a drop of transferrin internalization during differentiation of muscle cells (Kaisto et al., Endocytosis in skeletal muscle fibers Exp Cell Res,1999).

The Fig 2E and 3C have four quadrants, but only two of these are explained and labeled (CCP and FCL). I think the authors should also provide an interpretation for the structures that fall in the two remaining quadrants. Indeed, later on page 8 the lower left quadrant is mentioned and described as small plaques. Are both the small and large plaques FCL?

The description and interpretation of the 2 other quadrants (lower left and upper right) in the graphs depicting clathrin structures have been amended in the result section of Fig. 2 E, Fig. 3 C and Fig. 5 F. We also clarified the broad concept of FCL at the first mention of morphometric analysis in the result section by adding the following sentence at line 165: “It is worth noting that we chose to use the term FCL to describe flat structures comprising both small objects below the area threshold and the big and flat structures corresponding to clathrin plaques above this threshold and found in the lower-right quadrant”. Lastly, we changed references to flat assemblies throughout the text and figures to conform to that notion.

In the discussion section the authors make, in my opinion, unnecessarily strong conclusions, which I would suggest toning down. The authors state that the exon31 is required for the formation of flat clathrin lattices (lines 233, 242) and that the exon31 determines two different clathrin conformations (line 247). The flat lattices are not completely gone in assays where the exon31 is down regulated. Maybe this is because there is still some clathrin with exon31 left, or maybe because it does promote flat lattices, but is not the only determinant for them. Also, the different conformations being determined by the exon31 seem likely, but not definitively demonstrated here. Finally, the authors finish the discussion by saying that they provided an explanation for why both the constant curvature and constant area models are correct (lines 283-284). Their data is definitely highly relevant for these two models, but the data presented in this manuscript does not deal with possible shape transitions during vesicle budding and does not explain how a flat lattice can be shaped into a curved vesicle.

We agree with Reviewer #3 that some of the conclusions in the discussion were unwarranted and too strong. This was also a concern raised by both Reviewer #1 and #2. We have now rewritten the discussion to take this suggestion into account.

May 6, 2020

RE: JCB Manuscript #201912061R

Dr. Stéphane Vassilopoulos
Institute of Myology, Sorbonne Université
47, boulevard de l'Hopital
PARIS F-75 561
France

Dear Dr. Vassilopoulos:

Thank you for submitting your revised manuscript entitled "Alternative splicing of clathrin heavy chain exon 31 allows the switch from coated pits to plaques". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below), as well as the following final text edits.

Title: replaces "allows" with "contributes to" - "Alternative splicing of clathrin heavy chain exon 31 contributes to the switch from coated pits to plaques"

Abstract: edit "determines" to "helps determine" in the following sentence "Here, we show that the alternative splicing of a single exon of the clathrin heavy chain gene (CLTC exon 31) helps determine the clathrin coat organization."

In the two instances mentioned by Reviewer #2, please use similarly attenuated statements.

Please attend to the comment by Reviewer #2 on discussing alternative interpretations of some of the data, as well as the editorial changes suggested by Reviewer #2.

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 (e.g. 1A, you may alternatively indicate the width of the inset). * Molecular weight or

nucleic acid size markers must be included on all gel electrophoresis.

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

5) Abstract and title: need to be edited as indicated.

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

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

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.

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). 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. * Your suggested eTOC needs to be edited in line with the reviewers' concerns.

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).

-- 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. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

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,

Pier Paolo Di Fiore, MD, PhD
Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

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

In this revised versions, the authors answered the major concerns of the reviewer. In particular he thinks it is interesting that the splice variants can be identified at the protein level.
He thinks also that the authors agreed that this alternative splicing of clathrin heavy chain is only one of the multiple determinants that control the formation of pits or plaques. This is clear from the authors' binomial distribution analysis and how this does not reflect the frequency of pit and plaques in their data. As consequence the reviewer believes that the title and the abstract should be revised taking into consideration this conclusion (splicing of clathrin heavy chain is NOT the switch from pits to plaques), stating therefore that this alternative splicing is involved in the formation of plaques and pits but it is not the major determinant of this switch.
Otherwise, he thinks that is an interesting descriptive work that adds novel information to the field of clathrin mediated endocytosis.

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

The authors have address most of my concerns. However, I still think they need to tone down their conclusions (e.g. line 84-86, exon31 inclusion in CHC "determiners" the presence of plaques, and "its inclusion induces the formation of plaques". Don't get me wrong, I like the paper and the findings are novel and interesting; however, they remain correlative. I also think the mechanistic speculation is a bit too clathrin-centric. For example, if the 7 aa insert co-assembles with exon17-skipped heavy chains, then a longer CC region could not be made, indeed there might a 'bulge' created by the extra 7 aa that could become a binding site for different adaptors/curvature generators. It seems that the 'dominant effect' suggested is much more readily explained by recruitment or displacement of a curvature generating factor, than it is by the clathrin lattice structure. For example, the conversion from CCPs to plagues by expression of Dab2 has been reported in Cos7 cells (PMID 19000037) and BSC1 cells (PMID 2023186).

I also found some typos:

Line 107 cell types
Line 108 and throughout the text HeLa cells should have two capital letters
Line 130-133, the sentence beginning with "Exlusion of CLTC-ex31... " is awkward.
Line 150; "In order to demonstrate the direct control of CLTC-ex31" is too strong. I would say "a role for f CLTC-ex31 alternate splicing in plaque vs pit formation"
Line 158 "skipping induced a reduction " (maybe correlated with)

Line 170 3 'p's in skipping and Interestingly
Line 276 in silico
Line 278 helices'
Line 281 'participates in clathrin plaque formation

May 14th, 2020

Dear Dr Di Fiore and Dr Marat,

Thank you for your reply regarding our manuscript #201912061R entitled: "Alternative splicing of clathrin heavy chain contributes to the switch from coated pits to plaques".

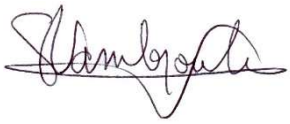
We are grateful for the positive evaluation of our work following its revision. We have now amended the text with the final reviewers' suggestions and corrections as described below.

All authors would like to take this opportunity to thank the reviewers for their truly constructive comments and suggestions that we feel have greatly strengthened the manuscript. We would also like to thank the *JCB* editors who handled the manuscript swiftly and all the editorial team at *JCB* who run such a wonderful journal despite the difficult times we are facing.

Merci!

Sincerely,

Stéphane VASSILOPOULOS



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The original comments from the reviewers are in Times font italics and our corrections are in plain text. When a line is mentioned, our answer refers to the revised manuscript.

Final edits:

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

In this revised version, the authors answered the major concerns of the reviewer. In particular he thinks it is interesting that the splice variants can be identified at the protein level.

He thinks also that the authors agreed that this alternative splicing of clathrin heavy chain is only one of the multiple determinants that control the formation of pits or plaques. This is clear from the authors' binomial distribution analysis and how this does not reflect the frequency of pit and plaques in their data. As consequence the reviewer believes that the title and the abstract should be revised taking into consideration this conclusion (splicing of clathrin heavy chain is NOT the switch from pits to plaques), stating therefore that this alternative splicing is involved in the formation of plaques and pits but it is not the major determinant of this switch.
Otherwise, he thinks that is an interesting descriptive work that adds novel information to the field of clathrin mediated endocytosis.

We replaced "allows" with "contributes to" in the title that is now: "Alternative splicing of clathrin heavy chain exon 31 contributes to the switch from coated pits to plaques".
The abstract was edited by replacing "determines" to "helps determine" in the following sentence at line 31: "Here, we show that the alternative splicing of a single exon of the clathrin heavy chain gene (*CLTC* exon 31) helps determine the clathrin coat organization."

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

The authors have address most of my concerns. However, I still think they need to tone down their conclusions (e.g. line 84-86, exon31 inclusion in CHC "determines" the presence of plaques, and "its inclusion induces the formation of plaques". Don't get me wrong, I like the paper and the findings are novel and interesting; however, they remain correlative. I also think the mechanistic speculation is a bit too clathrin-centric. For example, if the 7 aa insert co-assembles with exon-skipped heavy chains, then a longer CC region could not be made, indeed there might be a 'bulge' created by the extra 7 aa that could become a binding site for different adaptors/curvature generators. It seems that the 'dominant effect' suggested is much more readily explained by recruitment or displacement of a curvature generating factor, than it is by the clathrin lattice structure. For example, the conversion from CCPs to plagues by expression of Dab2 has been reported in Cos7 cells (PMID 19000037) and BSC1 cells (PMID 2023186).

The message was toned down by replacing “determines” with “contributes to” at lines 25 (eTOC summary) and 84. Similarly “controls” was replaced with “participates to” at line 26 (eTOC summary), and “induces” was replaced with “participates to” at line 86.
We now mention at lines 306-310 a less clathrin-centric hypothesis: “we cannot exclude that it could also modify the interaction with clathrin adaptors” and “the mixed coil-coiled region could be altered in a way that recruits different clathrin adaptors or reduces binding to curvature generators”. Finally, Dab2 is now added to our discussion on adaptors involved in plaque

formation along with previously mentioned AP2 and PICALM at lines 322-324 and we have included the two additional references suggested by reviewer #2.

Reviewer #2 also found some typos:

Line 107 cell types
Line 108 and throughout the text HeLa cells should have two capital letters
Line 130-133, the sentence beginning with "Exclusion of CLTC-ex31... " is awkward.

The sentence was re-written.

Line 150; "In order to demonstrate the direct control of CLTC-ex31" is too strong. I would say "a role for CLTC-ex31 alternate splicing in plaque vs pit formation"
Line 158 "skipping induced a reduction " (maybe correlated with)
Line 170 3 'p's in skipping and Interestingly
Line 276 in silico
Line 278 helices'
Line 281 'participates in clathrin plaque formation

These typos and modifications are now corrected.

Additional modifications:

Scale bars were added in the inset magnifications of Fig. 1 A and Fig. S1 A.
Nucleic acid size markers were included in all figures containing RT-PCR gel electrophoresis.
Details regarding Fig. 2 H and Fig. 5 I box plots were added in the figure legends.
The number of independent data points (n) was added in the legend for Fig. 6 B.
A precision was added on line 464 stating that the CELF siRNA sequence given corresponds to the passenger strand.
The numerical apertures of two objectives were added at line 473 and 529, and microscope image acquisition temperatures and mounting medium were added in the materials and methods section when omitted.
An image acquisition software was added at line 529 of materials and methods.
We specified in the materials and methods section that primers used for human and mouse *CLTC*-ex31 PCR were the same as the one used for vervet monkey (BS-C-1 cells) and rat respectively at lines 567-571.
The method used to quantify AP2 western blot in Fig. S2 A was added at lines 594-596.
The author contribution section was separated from the acknowledgments section and all authors full names were mentioned.