EMBO reports

AMPK regulates cell shape of cardiomyocytes by modulating turnover of microtubules through CLIP-170

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1st Editorial Decision 14th Jul 2020

Dear Dr. Shintani,

Thank you for submitting your preliminary point-by-point response. I have now looked at your points carefully. I am glad to hear that you have another mouse strain with lower mutant CLIP-170 levels. I appreciate that you are willing to address the concerns raised and see that the proposed experiments will strengthen the manuscript.

Having looked at everything, I would like to invite you to submit a revised manuscript. However, I would like to point out that we need strong support from the referees to consider publication here. It is this aspect that is more difficult to assess at this stage.

You can submit the revised manuscript using the link below. Please see the guidelines for the revision below my signature.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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Please revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted in the editorial decision letter. Please contact the scientific editor handling your manuscript to discuss a revision plan should you need additional time, and also if you see a paper with related content published elsewhere.***

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1. A data availability section providing access to data deposited in public databases is missing (where applicable).
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Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines ().

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be

cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

9) Please make sure to include a Data Availability Section before submitting your revision - if it is not applicable, make a statement that no data were deposited in a public database. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

Reports:

Referee #1:

Yashirogi et al. present intriguing data on how AMPK and CLIP-170 localized to the intercalated disk may regulate cardiomyocyte size and eccentric remodeling. AMPK phosphorylates Ser311 of CLIP-170, which promotes its dissociation from the MT plus end, and exposes the plus end to depolymerizing factors (previous work). The authors find that pAMPK is enriched at the ITC, as is pCLIP-170. Treatment with a contractile inhibitor causes a loss of pAMPK at the ITC (but the ITC does not grossly disassemble as evidenced from connexin43/N-cad/plakoglobin staining). Inhibition of AMPK or KD causes CLIP to accumulate in comets at the ITC, as does non-phophorylatable CLIP170. Contractile inhibition also causes increased cell area (unclear if it's lengthening), and non-phosphorylatable CLIP170 is sufficient to phenocopy this affect, and phospho-mimetic CLIP does not increase area when contraction is inhibited. This data suggests a provocative mechanism whereby phosphorylation of CLIP170 at the ITC regulates eccentric remodeling through alterations in microtubule dynamics. The authors then aim to test the in vivo relevance of this hypothesis by characterizing a CLIP170-S311A overexpressing transgenic mouse. While the mouse has an interesting phenotype, the interpretation of this data is muddied by flaws in experimental design. Overall, the mechanism the authors propose is novel and potentially of considerable interest, but currently the data is not of sufficient quality to support their primary conclusions. While there is much to like about this work, there are two major limitations; 1) the quantification and replication of data is insufficient throughout the manuscript; 2) the design of the transgenic mouse studies appears fundamentally flawed in a way that obscures useful interpretation of the data.

Major:
1. Quantification and replication of data. There is a stark lack of quantification of essential data in the manuscript, and readers are left evaluating statements made from only single "representative" images (examples in Fig. 1 A, B, C, D; Supp. Fig. 1 A, 1 B; Fig. 2. A, B, C, D; Fig. 5 B, Fig. 6 C). Below are some specific points, but this is a general concern that significantly undercuts the work.
a. Lack of N and n values for many experiments.
b. Figure 2, please quantify pAMPKa co-localization with N-Cad, or intensity in N-Cad enriched ROIs. Same with negative controls (Connexin43, LKB1), in repeat experiments.

c. Knockdown of siAMPK appears to be a single qPCR reaction with no replication. Protein expression should also be quantified to ensure protein knockdown.
d. For cell size measurements (Figure 4C), please show data distributions, not bar graphs.
e. Cell area measurements in figure 4C are non-sensical. An area of 8um2 would reflect a 2x4 cell, which is far too small for any cardiomyocyte. If possible, please also report dimensions, not just area throughout- are cells lengthening or widening, or both?
f. The characterization of the TG mouse model is incomplete. Specifically:
i. Chamber remodeling and hypertrophy should be quantified. From echocardiographical measurements the authors should be able to quantify chamber and wall diameters, and LVMI or HW/BW should be reported.
ii. There is a claim of no tissue degeneration at 3 months. This should be quantified, as should fibrosis at 1 year of age.
iii. The enhanced tubulin accumulation in the mouse model should be quantified (and simple western blot from tissue lysis for total tubulin levels and detyrosinated or acetylated tubulin levels).
iv. In Figure 6, cell length and width (and area) should be displayed - from the rep image the cells appear to show generalized hypertrophy, not simply eccentric remodeling.
2. Interpretation of transgenic mouse data - The goal presumably was to produce a mouse expressing CLIP170 that could not be phosphorylated at a specific residue of interest - however, the mutation was not knocked in to the endogenous locus, but instead appears to be a massive overexpression of CLIP170. How do the others tease out effects of phosphorylation vs. overexpression? What is the consequence of overexpression of CLIP170 alone? Data with similar overexpression of either the phospho-mimic or WT CLIP170 are required to make conclusions about the in vivo effects of phosphorylation at that site. The authors conclude that "data indicate the physiological CLIP-170 phosphorylation by AMPK" leads to the phenotypes observed. This is not what the mouse data necessarily indicates. From the current study design, we can only confidently conclude that a large overexpression of a mutant CLIP170 mutant has deleterious consequences on the heart. This conclusion is of little use to the field, as, for example, considerable overexpression of GFP is also sufficient to induce cardiac remodeling, and proteotoxic effects of overexpression cannot be ruled out.
3. Confirmation of microtubule stabilization - The authors exclusively use CLIP-170 (both WT and mutants) as a proxy to measure microtubule dynamics or infer stability, though only CLIP-170 comet length is quantified. The authors should complement this indirect metric with other measures of microtubule stabilization, as inferred changes in dynamics is a major component of their mechanism. Simple western blots of quantification of immunofluorescence for detyrosinated or acetylated tubulin (markers of stable microtubules) would suffice, and should be done for both in vivo and in vitro studies.

Minor:
1. Please provide some background context on the different subunits/isoforms of AMPK. It is often unclear why at different times the authors interrogate AMPKB2, AMPKa2, or knock down AMPKa1a2.

Referee #2:

Summary: The manuscript entitled "AMPK regulates cell shape of cardiomyocytes by modulating turnover of microtubules through CLIP-170" describes the localization of microtubule protein, CLIP-

170, to intercalated discs where it may be phosphorylated by stimuli in which AMPK is activated. The data presented suggest that AMPK activation causes microtubule dissociation and AMPK inhibition causes microtubule formation. Data are collected in vitro for pharmacologic AMPK inhibition and AMPK inhibition via gene silencing. A mouse model was generated with inability of CLIP-170 phosphorylation via S311A mutation and transgenesis. Mice with CLIP-170 S311A transgene demonstrate cardiac myocyte elongation and decline in cardiac function with time. Strengths of the manuscript include the novelty and rigor of demonstrating CLIP-170/AMPK localization at the intercalated disc and development of a mouse model with supporting functional data. Overall, the authors provide novel data for CLIP-170 phosphorylation and the impact on microtubule association/dissociation in cardiomyocytes. However, the mechanistic data could be more convincing had additional in vivo data been generated either by rescue experiment with the transgenic mouse strain or if a physiological or pathological context been provided for CLIP-170 phosphorylation events by AMPK.

Major comments:
• Most importantly, the submitted manuscript entirely lacks Figure 4. And the title of this section should be fixed: AMPK controls cell size and shape by regulating MT dynamics through CLIP-170229 phosphorylation of in cardiomyocytes
• Additional MRI data should be included, perhaps in table format, including chamber dimensions and wall thickness. Fig. 5 refers to echocardiography, not MRI. Please ensure the method used to evaluate cardiac physiology.
• Fig. 6 fibrosis quantification is very subjective. Fibrosis should be quantified by % area to increase accuracy.
• Lack of physiologic context for AMPK phosphorylation of CLIP-170. Pharmacologic inhibition and gene silencing were provided but neither provide insight into the in vivo functional role of CLIP-170 phosphorylation in health or disease.
• The authors make a Cre-responsive CLIP-170 S311A overexpressing transgenic mouse line. Importantly, the authors should ensure cardiac specificity of this new transgenic line (no leakiness) by showing lack of Tamoxifen inducible CLIP-170 in other tissues by Western blot (Supp. Fig. 3). Also, it is well established that prolonged expression of Cre from this Cre line, and especially tamoxifen inducible Cre expression, can be cardiotoxic (Pugach et al., 2015; Lexow et al., 2013). An important control missing here is MHC-MerCreMer (+);CLIP-170 Tg(-) with Tamoxifen to rule out Tamoxifen/Cre dependent cardiotoxicity and fibrosis. Minor comment: please refer to "MerCreMer" as "a-MYC-MerCreMer" throughout the text, instead of just in the methods.
• The transgenic mouse line selected, line 3, greatly over-expresses CLIP-170 which, at first glance, may seem like the desirable choice. However, massive transgene overexpression may lead to cardiac dysfunction from massive overexpression alone rather than the specific effect of S311A mutation. The authors indicate the other transgenic mouse line, line 9, with lower level of expression was "phenotypically similar"; therefore please provide MRI data for the lower expressing line.

Minor comments:
Please check throughout for grammar.

Referee #3:

To the authors:

The paper covers an important problem, namely the link between AMPK, mechanical activity and heart failure. The authors employed neonatal rat cardiomyocytes, inhibited contractility via the use of MYK-461 and observed mechanical activity dependent AMPK translocation to the intercalated discs. Moreover, CLIP-170 phosphorylation dependent microtubule localization at this structure has also been observed.
MYK-461 also increased cardiomyocyte cell size.
Last but not least, the authors generated tamoxifen inducible, cardiomyocyte-specific CLIP-170 S311A overexpressing transgenic mice (TG), which develop heart failure.
The authors conclude that AMPK regulates the cell shape and aspect ratio of cardiomyocytes by modulating the turnover of microtubules through homeostatic phosphorylation of CLIP-170 at the intercalated discs.

I have a few points:

1. The authors refer several times to human heart failure and the need for the development of novel therapies. Therefore, the authors could possibly strengthen their case by linking AMPK and CLIP-170 even more to human heart failure, for example in patients with plakophilin mutations.
2. In addition, are CLIP-170 mutations known, and if, what is the phenotype?
3. The authors generated tamoxifen inducible, cardiomyocyte-specific CLIP-170 S311A overexpressing transgenic mice (TG), which develop heart failure.
It would be good if all the data related to the cardiac echocardiography could be provided and not only EF% (if possible, for left and right ventricles, figure 5C).
In addition, how was the line characterized? How many transgenes were inserted and if possible, it would be good to know, where the insertions took place? What level of overexpression has been observed? Did all lines generated have similar levels of overexpression, or could conclusions be drawn in regard to the levels of expression and phenotypes observed (i.e. gene dosage effects)?
4. AMPK mutations have been linked to the Wolff Parkinson White syndrome. Did the CLIP-170 S311A overexpressing TG mice develop additional phenotypes, such as arrhythmias (also often observed in patients with mutations in intercalated disc / desomosomal proteins)? Did the authors observe early lethality?
5. The authors focused on the CLIP-170 S311A overexpressing TG mice, but did the authors also generate the CLIP-170 S311D overexpressing TG line (i.e. the phosphomimetic line)?
6. What type of fibrosis has been observed in the TG animals (i.e. focal, replacement, perivascular types of fibrosis)?
7. The sentence in line 339 (page 10) is not supported by the data presented: "Our results suggest that the activity of the AMPK, which is specifically localized at the intercalated discs, does not correlate with energy metabolism (sensing AMP/ATP ratio) but it does correlate with mechanical stress."
8. In figure 1C, is pAMPKα also localized at sarcomeres, apart from intercalated discs?
9. It might be possible that the signal captured from intercalated discs in the pAMPKα channel is just a bleed-thorough signal from Plakoglobin channel to the pAMPKα channel. More information about the microscopy strategy is crucial to rule out this possibility.
10. In figure 1D, is pCLIP-170 also localized at sarcomeres, apart from intercalated discs?
11. It might be possible that the signal captured from intercalated discs in the pCLIP-170 channel is just a bleed-thorough signal from Plakoglobin channel to the pCLIP-170 channel. More information about the microscopy strategy is crucial to rule out this possibility.
12. No information regarding the culture condition (days in culture, ...) was reported for the fig. 1.
13. No information regarding the culture condition (days in culture, ...) was reported for the fig. 2.
14. Authors discussed that the outcomes observed in the figure 2 is due to the contraction of

cardiomyocytes, whereas no data was reported about the average of the contraction frequency after treatment with MYK-461 and after its removal. We know that MYK-461 suppresses the contraction, this suppression should be shown clearly in the manuscript.
15. Figure 3A at t = 0 (Pre), seems to be out of focus or in other words the z-plane of t = 0 (Pre) is not exactly the same plane of t = 15 min after treatment. Maybe if both images were taken from the same z-plane, the comet lengths were not differentially significant!
16. Moreover, the interpretation of results from figure 3 C and D are just based on comparing one cell, treated with control siRNA (siCL), with one siAMPKα1α2-targeted cell. Comparing just one treated-cell with just one control cell seems not enough at all for interpretation!
17. The approach used for measurement of comet length is only valid when the cardiomyocyte of interest is not spontaneously contracting at all and any small contraction makes the measurements unreliable.
18. Based on the outcomes of figure 6, authors concluded on lines 273 and 274 that physiological CLIP-170 phosphorylation by AMPK is important for homeostatic MT dynamics, thereby maintaining the cell shape and cardiac function of cardiomyocytes, whereas the reported data just indicates elongation of cardiomyocytes. Moreover, it is not unequivocally shown that this elongation changes the aspect ratio of cardiomyocytes or not.
19. It is essential to report how the cell length is measured in figure 6.
20. Could it be that figure 4 is missing in the manuscript?

Reviewer #1:

We thank Reviewer #1 for the critical and constructive comments, which allowed us to improve the manuscript. We addressed all of the reviewer's comments below *in italic*, added sentences in the revised manuscript are marked in red. We believe that the manuscript is now much improved and more solid.

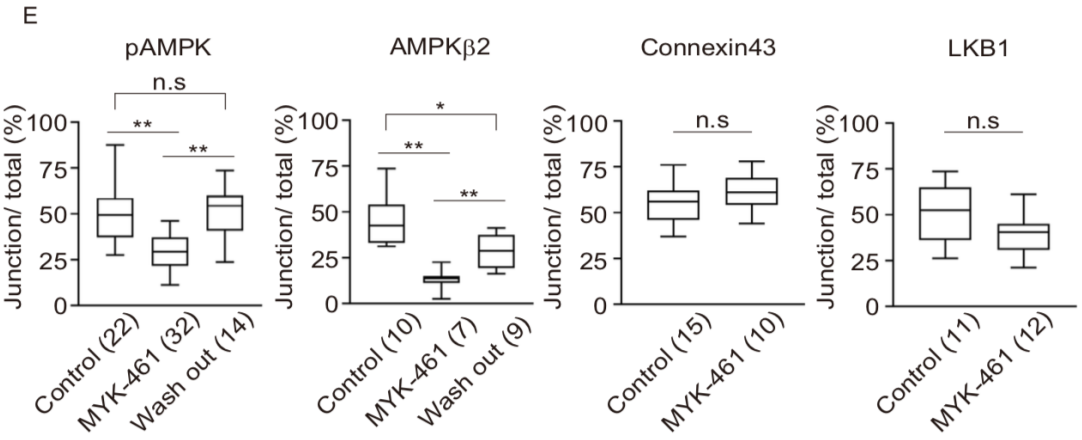
1- Quantification and replication of data. There is a stark lack of quantification of essential data in the manuscript, and readers are left evaluating statements made from only single "representative" images (examples in Fig. 1 A, B, C, D; Supp. Fig. 1 A, 1 B; Fig. 2. A, B, C, D; Fig. 5 B, Fig. 6 C). Below are some specific points, but this is a general concern that significantly undercuts the work.

a. Lack of N and n values for many experiments.

Thank you very much for the critical comments. We would like to apologize for the lack of experimental information and quantitative analysis. We added n number or number of individual experiments in the figure legends, if possible, in the figures.

b. Figure 2, please quantify pAMPKa co-localization with N-Cad, or intensity in N-Cad enriched ROIs. Same with negative controls (Connexin43, LKB1), in repeat experiments.

Thank you very much for the critical comment. We did confirm the results by 4 independent experiments. We added the quantification of pAMPK, AMPKβ_2, Connexin43 and Lkb1. Numbers in the brackets indicate the cells that were analyzed for quantification. We measured them from more than 2 independent experiments. As shown in the figure below, quantification confirmed that the signals of pAMPK, and APMKβ_2 decreased from Ncad positive cell-cell junctions after MYK treatment, while Connexin43 and LKB1 did not show any change in localization by MYK. We added the graphs in the revised Fig 2E and revised figure legends.



c. Knockdown of siAMPK appears to be a single qPCR reaction with no replication. Protein expression should also be quantified to ensure protein knockdown.

We apologized for poor presentation of the data. We did confirm qPCR data from 3 independent experiments and Western blotting with quantification. We added these data in the revised Fig EV3 and figure legend.

d. For cell size measurements (Figure 4C), please show data distributions, not bar graphs.

Thank you for the comment, we revised Fig 4C with data distribution and the figure legend.

e. Cell area measurements in figure 4C are non-sensical. An area of 8um2 would reflect a 2x4 cell, which is far too small for any cardiomyocyte. If possible, please also report dimensions, not just area throughout- are cells lengthening or widening, or both?

Thank you very much for pointing this out. When we used off-line image analysis, the magnification should have been converted. We apologize our mistake and revised Fig 4C as it should be. For cultured neonatal cardiomyocytes which are not rectangular, it is very difficult to measure length and width, therefore we analyzed its area (Fig 4C). For cardiomyocytes in tissue sections, we measured the length, width and aspect ratio, which was in the revised Fig 6G and H (please see below, f-iv).

f. The characterization of the TG mouse model is incomplete. Specifically:
i. Chamber remodeling and hypertrophy should be quantified. From echocardiographical measurements the authors should be able to quantify chamber and wall diameters, and LVMI or HW/BW should be reported.

Thank you very much for the comment. We added the tables containing echocardiographical measurements from 2 lines of S311A TG mice and 1 line from S311D TG mice (phosphomimetic mutant) in Fig EV5. We also added HW/BW measurements and MRI measurements from S311A line 3.
Systolic dysfunction in S311A TG became evident around 8-12 weeks after TG induction. Chamber remodeling (dilatation) were also found in S311A TG, but these were found 52 weeks after TG induction. Wall thickening was not found in S311A TG mice.
In contrast to S311A, S311D TG mice did not show any difference compared to the control assessed by echocardiography.
We revised the results section in page 8 line 259-282.

ii. There is a claim of no tissue degeneration at 3 months. This should be quantified, as should fibrosis at 1 year of age.

Thank you very much for the comment. According to the suggestion, we added the graph containing quantification of fibrosis in the revised Fig 6B.

Quantification was made as %area of fibrosis (in blue) in the left ventricle. As shown in the revised Fig 6B, there was no difference in fibrosis at 3 months after TG induction, but increased fibrosis was seen in S311A TG heart 1 year after TG induction.

iii. The enhanced tubulin accumulation in the mouse model should be quantified (and simple western blot from tissue lysis for total tubulin levels and detyrosinated or acetylated tubulin levels).

Thank you very much for the constructive comment. According to the suggestion, we added quantitation in the revised Fig 6D. Also, we analyzed tubulin in the tissue lysate by Western blot and found that tubulin was increased in the lysate from S311A TG mice. We added this in the revised Fig 6E-F. [Figures for referees not shown.]
Robison P, et al. reported that both α-tubulin and detyrosinated tubulin are accumulated in the tissues from several types of human heart diseases (Science. 2016;352:aaf0659-). Thus, we assume that acetylated or detyrosinated tubulin was probably increased in CLIP-170 S311A TG mice. However, the amount of acetylated tubulin varied among individuals of S311A TG, although the mean of S311A TG mice was greater than the control (shown in figure below). The reason for this is not known, but we speculate that post translational modification might be more fragile during tissue collection than the total amount of tubulin.

iv. In Figure 6, cell length and width (and area) should be displayed - from the rep image the cells appear to show generalized hypertrophy, not simply eccentric remodeling.

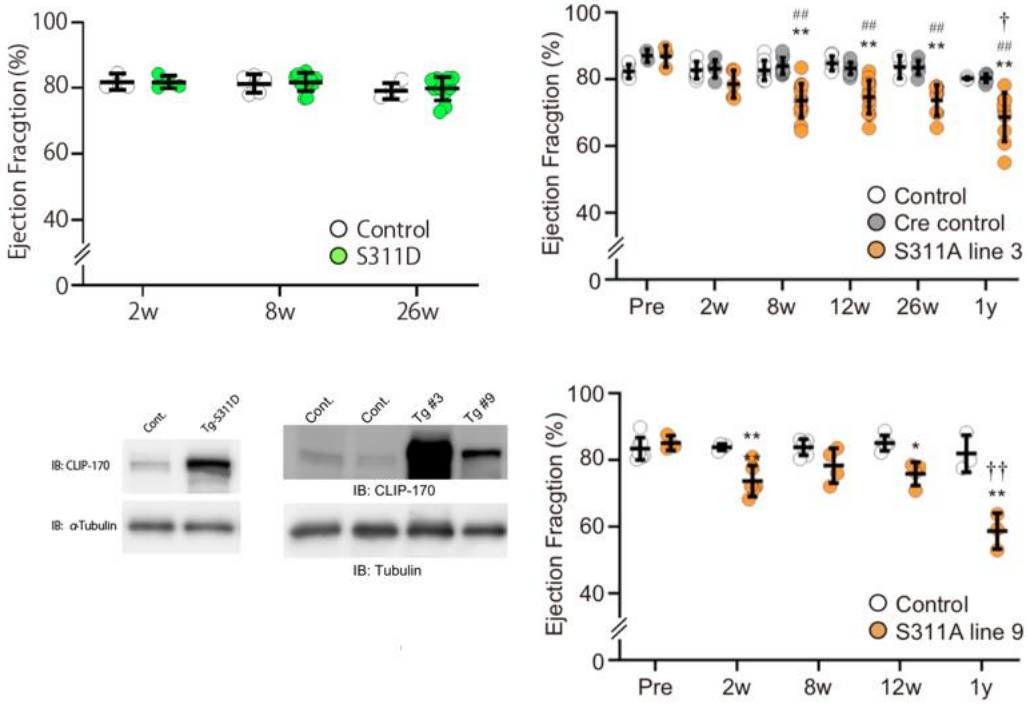
Thank you very much for the comment. We replaced the images in the revised Fig 6G, so that the images properly represent the quantitative analysis shown in the graph (Fig 6H). As the reviewer suggested, we added a graph containing cell length, width and aspect ratio in the revised Fig 6H. It showed significantly increased cell length in S311A TG mice heart, but not in cell width, resulted in the increased aspect ratio.

2. Interpretation of transgenic mouse data - The goal presumably was to produce a mouse expressing CLIP170 that could not be phosphorylated at a specific residue of interest - however, the mutation was not knocked in to the endogenous locus, but instead appears to be a massive overexpression of CLIP170. How do the others tease out effects of phosphorylation vs. overexpression? What is the consequence of overexpression of CLIP170 alone? Data with similar overexpression of either the phospho-mimic or WT CLIP170 are required to make conclusions about the in vivo effects of phosphorylation at that site. The authors conclude that "data indicate the physiological CLIP-170 phosphorylation by AMPK" leads to the phenotypes observed. This is not what the mouse data necessarily indicates. From the current study design, we can only confidently conclude that a large overexpression of a mutant CLIP170 mutant has deleterious consequences on the heart. This conclusion is of little use to the field, as, for example, considerable overexpression of GFP is also sufficient to induce cardiac remodeling, and proteotoxic effects of overexpression cannot be ruled out.

Thank you very much for the critical comment. We agree with the reviewer. Actually, we established CLIP-170 phosphomimetic mutant (S311D) TG mice and analyzed them until 6 months after induction. Unfortunately, we needed to shut down the animal experiments for several reasons at that time. We reluctantly stopped our CLIP S311D TGs. We did not include S311D mouse data in the initial submission because it lacked 1y echo, MRI, or histology.

We analyzed echocardiography of S311D TG, and as shown in the figure below, there was no difference in parameters assessed by echo in S311D TG compared to the control.

In addition, we had another line of S311A, which was line 9. As shown in the figure below, the expression levels of CLIP-170 were significantly lower than that of line 3, but echocardiography showed almost as similar phenotype as line 3 as shown in the figure below. Other parameters for these mice were shown in Fig EV5.



With these data, although not perfectly performed, we believe that the phenotype seen in CLIP-170 S311A line 3 TG mice was not from overexpression of the mutant protein, but from the inhibition of CLIP-170 S311 phosphorylation.

We added the data from both S311A and D TG mice in Fig 5A, B, E and EV5. We added sentences about this topic in the results, in page 8 line 259-282.

3. Confirmation of microtubule stabilization - The authors exclusively use CLIP-170 (both WT and mutants) as a proxy to measure microtubule dynamics or infer stability, though only CLIP-170 comet length is quantified. The authors should complement this indirect metric with other measures of microtubule stabilization, as inferred changes in dynamics is a major component of their mechanism. Simple western blots of quantification of immunofluorescence for detyrosinated or acetylated tubulin (markers of stable microtubules) would suffice, and should be done for both in vivo and in vitro studies.

Thank you very much for the comment. Please see the answer in earlier comment, 1-F-iii.

Minor

1. Please provide some background context on the different subunits/isoforms of AMPK. It is often unclear why at different times the authors interrogate AMPKB2, AMPKa2, or knock down AMPKa1a2.

Thank you very much for the comment. We added the background information about subunit of AMPK in page 5 line 154-158.

AMPK is a holoenzyme consisting of three subunits: α, β, and γ. Catalytic domain exists in the α subunit, while the β and γ are regulatory subunits. Further, there are two isoforms for each α and β subunit and three for the γ subunit. The heart expresses α_1 and α_2, β_1 and β_2, and γ_1 and γ_2 isoforms.

Reviewer #2:

We thank Reviewer #2 for the critical and constructive comments, which allowed us to improve the manuscript. We addressed all of the reviewer's comments below *in italic*, added sentences in the revised manuscript are marked in red. We believe that the manuscript is now much improved and more solid.

1- Most importantly, the submitted manuscript entirely lacks Figure 4. And the title of this section should be fixed: AMPK controls cell size and shape by regulating MT dynamics through CLIP-170229 phosphorylation of in cardiomyocytes

We would like to apologize that the merged data lacked Fig 4. Despite this, thank you very much for your effort on evaluating our manuscript. We will make sure, this time, to upload the revised version with the entire figures.

2- Additional MRI data should be included, perhaps in table format, including chamber dimensions and wall thickness. Fig. 5 refers to echocardiography, not MRI. Please ensure the method used to evaluate cardiac physiology.

Thank you very much for the comment. We added the additional parameters from MRI measurements of S311A TG mice in supplementary Fig EV5. For most of the functional assessment, we used echocardiography. To further confirm the phenotype and evaluate the right ventricle, we used cardiac MRI for 3 mice from each genotype and showed the data in Fig 5C and D. To avoid confusion, we indicated cardiac MRI in the revised Figure.

3- Fig. 6 fibrosis quantification is very subjective. Fibrosis should be quantified by % area to increase accuracy

Thank you very much for the comment. According to the suggestion, we added the graph containing quantification of fibrosis in the revised Fig 6B. Quantification was made as %area of fibrosis (in blue) in the left ventricle. As shown in the revised Fig 6B, there was no difference in fibrosis at 3 months after TG induction, but increased fibrosis was seen in S311A TG heart 1 year after TG induction.

4- Lack of physiologic context for AMPK phosphorylation of CLIP-170. Pharmacologic inhibition and gene silencing were provided but neither provide insight into the in vivo functional role of CLIP-170 phosphorylation in health or disease.

Thank you very much for the comment. As the other reviewer (3) suggested, we speculate that alteration of CLIP-170 phosphorylation by AMPK might be linked to heart failure. Cardiac MTs have drawn attention in cardiovascular research. we wrote in the discussion, α-tubulin and detyrosinated MTs are found to be elevated in human or rodent heart failure models, suggesting that cardiac MTs has a relevance in the pathology of heart diseases (Caporizzo

MA, et al. Exp Biol Med. 2019;244:1255-1272, Robinson P, et al. Science. 2016;352:aaf0659). Importantly, it has been shown that suppression of detyrosinated MTs can be a novel treatment for heart failure (Chen CY, et al. Nat Med. 2018;24:1225-1233). However, the mechanisms that are responsible for the accumulation of tubulins or PTM in heart diseases are not well understood.

CLIP-170 S311A TG mice showed the accumulation of α-tubulin (Fig 6C-F) and CLIP-170 affect the turnover of MTs (Fig 3), suggesting that AMPK-CLIP-170 may be involved in the cause of increased tubulin, which is often associated with human heart failure. Of course, further investigation, especially using human samples, is required to clarify this possibility.

Also, regarding the relevance to the heart failure, the pathological relevance of desmosomal proteins including plakophilin 2 (PKP2), desmoplakin (DSP), and desmoglein 2 (DSG2) has been well established. Mutations in these intercalated disc proteins are frequent among patients not only with arrhythmogenic right ventricular cardiomyopathy, but also with dilated cardiomyopathy with left ventricular dysfunction (Rosenbaum AN, et al. Nat Rev Cardiol. 2020;17:286–297.). In these patients, the structure of intercalated disc including desmosomes is irregular or not well formed (Posch MG, et al. Mol Genet Metab. 2008;95:74–80). The ill-structured intercalated disc could alter the regulation of AMPK-CLIP-170, suggesting a possibility that alteration of CLIP-170 phosphorylation may modulate the disease course.

Although we did not show the direct evidence for relevance of CLIP-170 phosphorylation, we sincerely hope that the reviewer will agree with these point and possible relevance of CLIP-170 phosphorylation in health and disease. We added these points in the discussion, page10 line 322-344, 364-371.

5- The authors make a Cre-responsive CLIP-170 S311A overexpressing transgenic mouse line. Importantly, the authors should ensure cardiac specificity of this new transgenic line (no leakiness) by showing lack of Tamoxifen inducible CLIP-170 in other tissues by Western blot (Supp. Fig. 3). Also, it is well established that prolonged expression of Cre from this Cre line, and especially tamoxifen inducible Cre expression, can be cardiotoxic (Pugach et al., 2015; Lexow et al., 2013). An important control missing here is MHC-MerCreMer (+);CLIP-170 Tg(-) with Tamoxifen to rule out Tamoxifen/Cre dependent cardiotoxicity and fibrosis. Minor comment: please refer to "MerCreMer" as "a-MHC-MerCreMer" throughout the text, instead of just in the methods.

Thank you very much for pointing this out. First of all, we revised "MerCreMer" to "α-MHC-MerCreMer" throughout the manuscript.
As the reviewer suggested, we have added α-MHC-MerCreMer (+);CLIP-170 TG(-) line as an additional control group. We added echocardiography measurement in the revised Fig 5A and EV5. There was no difference among the control groups in cardiac function. Our α-MHC-MerCreMer line did not

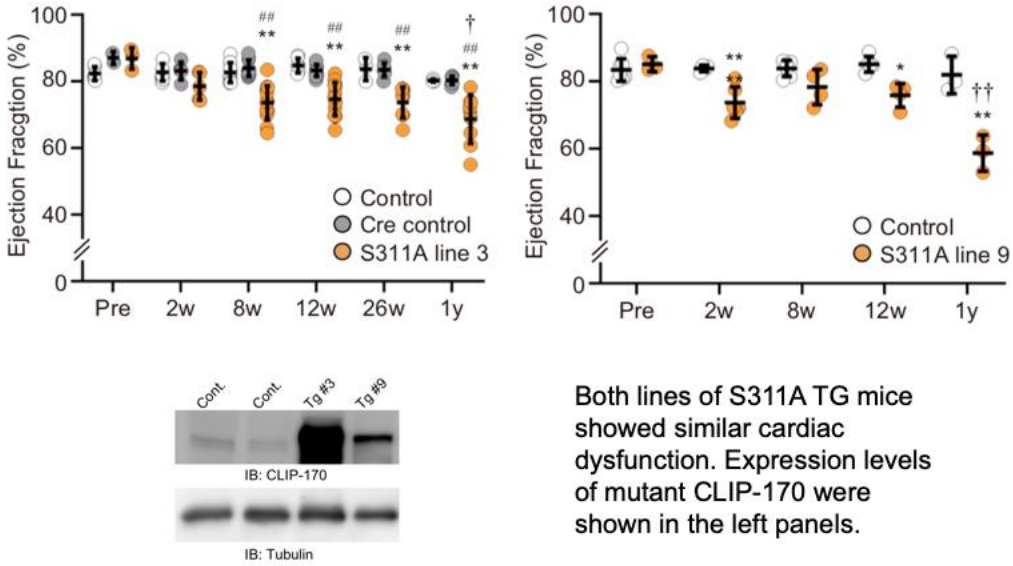
show cardiotoxicity in our experimental settings, it may be due to the difference in TG generations, housing or food, etc.

As our floxed TG line was established in our facility, we used α-MHC-MerCreMer (-);CLIP-170 TG(+) as the control for transgene insertion. We also added the data from another S311A TG line (line 9), and the line 9 also showed similar depression in cardiac function as well as the line 3 (relating to the next point).

Judging from these control data, we conclude that our Cre-responsive CLIP-170 S311A overexpression led to cardiac dysfunction.

6- The transgenic mouse line selected, line 3, greatly over-expresses CLIP-170 which, at first glance, may seem like the desirable choice. However, massive transgene overexpression may lead to cardiac dysfunction from massive overexpression alone rather than the specific effect of S311A mutation. The authors indicate the other transgenic mouse line, line 9, with lower level of expression was "phenotypically similar"; therefore please provide MRI data for the lower expressing line.

Thank you very much for the critical comment. We had another line of S311A, which was line 9. As shown in the figure below, the expression level was significantly lower than that of line 3, but echocardiography showed almost similar phenotype with line 3, shown in Fig 5 A, B (shown in below) and supplementary Fig. S5. Unfortunately, we used MRI to only line 3, because it required mouse transportation and additional cost. But, we believe that echocardiography is enough to draw the conclusion.



Both lines of S311A TG mice showed similar cardiac dysfunction. Expression levels of mutant CLIP-170 were shown in the left panels.

Minor comments:
Please check throughout for grammar.

Thank you for the comment. Our revised manuscript has been edited by a native speaker of English.

Reviewer #3:

We thank Reviewer #3 for the critical and constructive comments, which allowed us to improve the manuscript. We addressed all of the reviewer's comments below *in italic*, added sentences in the revised manuscript are marked in red. We believe that the manuscript is now much improved and more solid.

1. The authors refer several times to human heart failure and the need for the development of novel therapies. Therefore, the authors could possibly strengthen their case by linking AMPK and CLIP-170 even more to human heart failure, for example in patients with plakophilin mutations.

Thank you for the constructive comment. As the reviewer pointed out, the pathological relevance of desmosomal proteins including plakophilin 2 (PKP2), desmoplakin (DSP), and desmoglein 2 (DSG2) has been well established. Mutations in these intercalated disc proteins are frequent among patients not only with arrhythmogenic right ventricular cardiomyopathy, but also with dilated cardiomyopathy with left ventricular dysfunction (Rosenbaum AN, et al. Nat Rev Cardiol. 2020;17:286–297.). In these patients, the structure of intercalated disc including desmosomes is irregular or not well formed (Posch MG, et al. Mol Genet Metab. 2008;95:74–80). The ill-structured intercalated disc could alter the regulation of AMPK-CLIP-170, suggesting a possibility that alteration of CLIP-170 phosphorylation may modulate the disease course.

We added this in the discussion, page 11 line 364-371.

2. In addition, are CLIP-170 mutations known, and if, what is the phenotype?

Thank you for the comment. We were able to find 41 variants of CLIP-170 by searching for rare variants (MAF<0.001) in the database, 'Clin var' (7 out of 41 are shown in Table).

Table. Detection of CLIP-170 SNV in human genome database

SNP ID	Clinical significance and condition	Chr12pos	Variation	AA Info	Type
rs138030919	Uncertain Significance: not specified	122,377,621(-)	G/A	p.Ala142Val	MISSENSE_VARIANT
rs140092191	Uncertain Significance: not specified	122,380,446(-)	T/C	p.Met3Val	MISSENSE_VARIANT
rs1555264879	Uncertain Significance: not specified	122,328,099(-)	T/A	p.Thr1022Ser	MISSENSE_VARIANT
rs201297279	Uncertain Significance: not specified	122,274,063(-)	C/T	p.Gly1345Arg	MISSENSE_VARIAN
rs201330075	Uncertain Significance: not specified	122,341,597(-)	T/C	p.Asn525Ser	MISSENSE_VARIANT
rs875989820	Uncertain Significance:	122,328,135(-)	G/A	p.Gln1010Ter	NONSENSE

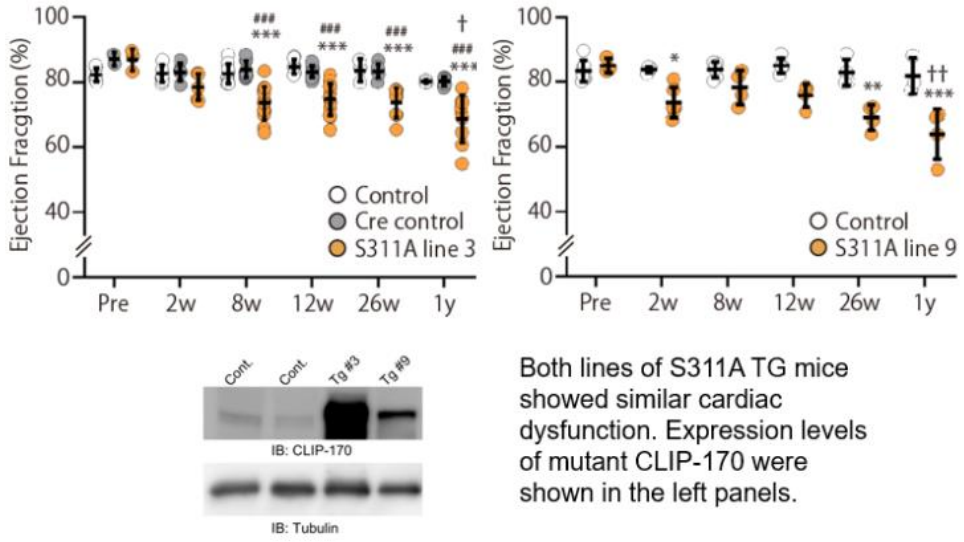
	not provided				

As shown in Table, the status of clinical significance is "Uncertain Significance or not specified". The only variant found in OMIM is rs875989820, which is shown in red. According to Larti et al. (Larti F, et al. Eur J Hum Genet. 2015;23(3):331-336.), they identified a homozygous c.3028C-T transition in the CLIP-170 gene, resulting in p.Gln1010Ter (Q1010X) substitution, that segregated with mental retardation in the family.
So far, there has been no direct link found between CLIP-170 and heart diseases, we would like to pursuit this point in the future study.

3. The authors generated tamoxifen inducible, cardiomyocyte-specific CLIP-170 S311A overexpressing transgenic mice (TG), which develop heart failure.
It would be good if all the data related to the cardiac echocardiography could be provided and not only EF% (if possible, for left and right ventricles, figure 5C).
In addition, how was the line characterized? How many transgenes were inserted and if possible, it would be good to know, where the insertions took place? What level of overexpression has been observed? Did all lines generated have similar levels of overexpression, or could conclusions be drawn in regard to the levels of expression and phenotypes observed (i.e. gene dosage effects)?

Thank you very much for the comment. First of all, we added the tables containing echocardiographical measurements from 2 lines of S311A TG mice, and 1 line from S311D (phosphomimetic) TG mice in Fig EV5. We also added HW/BW measurements and MRI measurements from S311A line 3.

Regarding TG lines, we have not identified the positions where the transgene was inserted, however, we had established 2 lines of S311A expressing TG lines; line 3 and 9. As shown in the figure below, the expression level of CLIP-170 in line 9 was significantly lower than that of line 3, but echocardiography showed that line 9 was almost similar phenotype to line 3.



opic in the results, in page 8 line 259-282.

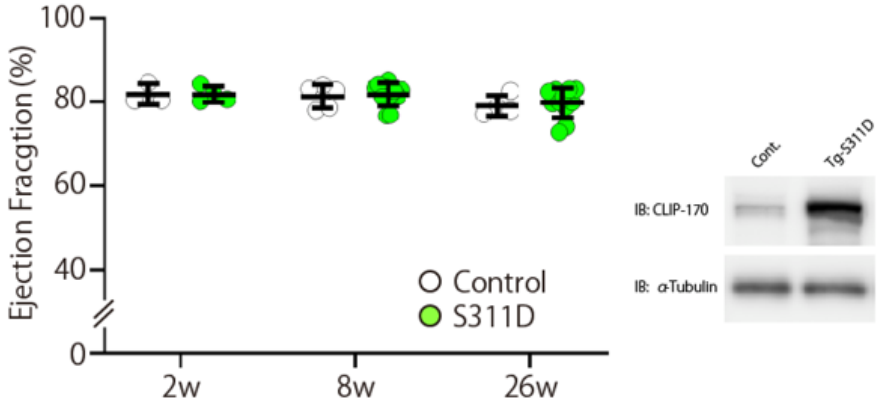
4. AMPK mutations have been linked to the Wolff Parkinson White syndrome. Did the CLIP-170 S311A overexpressing TG mice develop additional phenotypes, such as arrhythmias (also often observed in patients with mutations in intercalated disc / desmosomal proteins)? Did the authors observe early lethality?

Thank you for the comment. We checked ECG when we assessed them with echocardiography, however, there was no arrhythmia found in S311A TG mice. Also, we did not find early lethality in them. The AMPK mutation that has been linked to WPW syndrome is mutations in gamma 2 subunit. Glycogen accumulation found in heart tissues suggested that the mutations results in the dysregulated metabolism. AMPK has versatile functions and varieties of downstream substrates. CLIP-170 is one its many substrates, and we believe that CLIP-170 represents the function of AMPK outside the metabolism.

5. The authors focused on the CLIP-170 S311A overexpressing TG mice, but did the authors also generate the CLIP-170 S311D overexpressing TG line (i.e. the phosphomimetic line)?

Thank you very much for pointing this out. Actually, we established CLIP-170 phosphomimetic mutant (S311D) TG mice and analyzed them until 6 months after induction. Unfortunately, we needed to shut down the animal experiments for several complicated reasons at that time. We reluctantly stopped our CLIP
mutant TGs. We did not include S311D mouse data in the initial submission because it lacked 1y echo, MRI, or histology.

We analyzed echocardiography of S311D TG, and as shown in the figure below, there was no difference in parameters assessed by echo in S311D TG compared to the control.



d
ata and another line of S311A TG mice (please see the answer above (4)), although not perfectly performed, we believe that the phenotype seen in CLIP-170 S311A TG mice is not from overexpression of the mutant protein, but from the inhibition of CLIP-170 S311 phosphorylation.

We added the data from both S311A and D TG mice in Fig. 5A, B, E and EV5. We added this topic in the results, in page 8 line 259-282.

6. What type of fibrosis has been observed in the TG animals (i.e. focal, replacement, perivascular types of fibrosis)?

Thank you for the comment. The fibrosis found in the S311A TG mice were not focal or perivascular types, rather was replacement fibrosis.

7. The sentence in line 339 (page 10) is not supported by the data presented: "Our results suggest that the activity of the AMPK, which is specifically localized at the intercalated discs, does not correlate with energy metabolism (sensing AMP/ATP ratio) but it does correlate with mechanical stress."

Thank you very much for the comment. To avoid overstatement, we changed the sentences, as follows in page 13, line 372-375.

Increased levels of pAMPK and pCLIP-170 in the heart, 8 weeks after birth, did not match with the levels of pACC that were almost unchanged in the heart (Fig 1A). ACC is a crucial substrate in the regulation of metabolism. It is of note that specific localization of AMPK at the intercalated discs in the heart was regulated by mechanical stress (Fig 2).

8. In figure 1C, is pAMPKα also localized at sarcomeres, apart from intercalated discs?

We agree with the comment. From the quantitative analysis that we added in the revised Fig 2E, signals found in cell-cell junction was estimated about 40-50 % at the baseline (pAMPK and AMPKβ2). In Fig 2B, beating-responsive population of AMPK were localized at the cell-cell junction, but non-responsive population of AMPK were found in sarcomeres. It suggests that AMPK is a versatile kinase which has a variety of substrates that might be regulated by spatiotemporal manner.

9. It might be possible that the signal captured from intercalated discs in the pAMPKα channel is just a bleed-thorough signal from Plakoglobin channel to the pAMPKα channel. More information about the microscopy strategy is crucial to rule out this possibility.

Thank you very much for the critical comment. We verified the localization of pAMPK and pCLIP-170 by single antibody staining to exclude the possibility of a bleed-through signal from plakoglobin. We also confirmed the localization of pAMPK and pCLIP-170 with phosphatase treatment on the slide, as shown in Fig EV2E and F. In addition, for pAMPK, we confirmed its signal by siRNA experiment (Fig EV2G). With these added data, we conclude that pAMPK and pCLIP-170 localized at the cell-cell junction. We hope that our data become more solid thanks to the reviwer's suggestion.

10. In figure 1D, is pCLIP-170 also localized at sarcomeres, apart from intercalated discs?

Thank you for the critical comment. Please refer to the answer above (9).

11. It might be possible that the signal captured from intercalated discs in the pCLIP-170 channel is just a bleed-thorough signal from Plakoglobin channel to the pCLIP-170 channel. More information about the microscopy strategy is crucial to rule out this possibility.

Thank you for the critical comment. Please refer to the answer above (9).

12. No information regarding the culture condition (days in culture, ...) was reported for the fig. 1.

There was no cell culture used for Fig 1. We are sorry for the confusion it might have caused. And for Fig 2, please refer to the next comment.

13. No information regarding the culture condition (days in culture, ...) was reported for the fig. 2.

Thank you very much for the comment. We apologize that there was no cell culture information regarding Fig 2. For MYK-461 experiment, cardiomyocytes were plated on the glass-bottom dish (IWAKI). On the next day after plating, the cells were washed twice with medium. On day-3 after plating, spontaneous beating of cardiomyocytes was about 60 beats per minute. A MYK-461, myosin ATPase inhibitor, was added at 2 μM, the heartbeat was immediately stopped until removal of MYK-461. After 2 hours of MYK-461 treatment, the medium was replaced with the fresh one without MYK-461, the heartbeat restores quickly after the replacement. We added this information in Results section, page 6, line 189-196, and Methods section, page 14, line 447-452.

14. Authors discussed that the outcomes observed in the figure 2 is due to the contraction of cardiomyocytes, whereas no data was reported about the average of the contraction frequency after treatment with MYK-461 and after its removal. We know that MYK-461 suppresses the contraction, this suppression should be shown clearly in the manuscript.

Thank you very much for the comment. The beating rate of primary cardiomyocytes was about 60 per min, though it varied. Please see the comment above.

15. Figure 3A at t = 0 (Pre), seems to be out of focus or in other words the z-plane of t = 0 (Pre) is not exactly the same plane of t = 15 min after treatment. Maybe if both images were taken from the same z-plane, the comet lengths were not differentially significant!

Thank you for the comment. Z-plane might not be exactly the same. However, if we change the focus, comet length in the same cells was not significantly changed. Also, we measured comet length from multiple cells from different fields (number of comets analyzed; 32 (pre), 35 (Cpd. C), 68 (s311A)). It means that the plane was randomly varied in each group, therefore, we conclude that the comet length in cardiomyocytes treated with compound C or transfected with CLIP-170 S311A was significantly elongated at the intercalated disc. We added this information in the legend for Fig 3.

16. Moreover, the interpretation of results from figure 3 C and D are just based on comparing one cell, treated with control siRNA (siCL), with one siAMPKα1α2-targeted cell. Comparing just one treated-cell with just one control cell seems not enough at all for interpretation!

Thank you for the comment. We apologize for poor presentation of the data. In fact, we confirmed our findings by 3 independent experiments. And we analyzed comet length from multiple cells, 5-10 comets from each cell.

We added sentences in the figure legend,
The comet length from multiple cells from different fields was analyzed. Data means ±S.D. Numbers of comets analyzed, siCL: n=178, siAMPKα1α2: n=184.

17. The approach used for measurement of comet length is only valid when the cardiomyocyte of interest is not spontaneously contracting at all and any small contraction makes the measurements unreliable.

Thank you for the comment. We agree with that point. To measure comet length in cardiomyocytes, it is important to acquire images from still cardiomyocytes. The beating rate of primary cardiomyocytes in our setting was about 60 per min, one cardiac cycle is about 1sec each. We set the exposure time for 200 msec, thus we believe that there was enough margin to acquire image from diastolic phase (cardiomyocytes with minimum movement). In addition, we eliminated images which had motion artifact from measurement. We added this information in Methods section, page 16, line 502-507.

18. Based on the outcomes of figure 6, authors concluded on lines 273 and 274 that physiological CLIP-170 phosphorylation by AMPK is important for homeostatic MT dynamics, thereby maintaining the cell shape and cardiac function of cardiomyocytes, whereas the reported data just indicates elongation of cardiomyocytes. Moreover, it is

not unequivocally shown that this elongation changes the aspect ratio of cardiomyocytes or not.

Thank you for the comment. According the reviewer's suggestion, we added the quantitative analysis of cell length, width and aspect ratio for immunostaining. It showed that cell length significantly was increased in S311A TG mice heart, but not cell width, thereby leading to the increased aspect ratio. We think that our data become more solid thanks to the reviewer's suggestion.

19. It is essential to report how the cell length is measured in figure 6.

Thank you for the comment. For cell length and width measurements from immunohistology in the revised Fig 6G and H, 5-10 cells from each image that was picked randomly, and average 10 images from 1 heart were analyzed. Thus, about 100 cells from 1 heart were analyzed. We measured 3 hearts from each genotype group, the number of the cells analyzed were Ctr vs. S311A 259 vs. 326. We added this information in the method section, page 17, line 522-524.

20. Could it be that figure 4 is missing in the manuscript?

We would like to apologize that the merged data lacked Fig 4. Despite this, thank you very much for your effort on evaluating our manuscript. We will make sure, this time, to upload the revised version with the entire figures.

1st Revision - Editorial Decision 5th Oct 2020

Dear Dr. Shintani

Thank you for submitting your revised manuscript. It has now been seen by all of the original referees.

As you can see, the referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address some minor points below:

• Please address the remaining minor concerns of referee #1.
• Please ZIP the movies with their legends and update the callouts to 'Movie EV#'.
• Please rename the 'Disclosures' section to 'Conflict of Interests'.
• We noticed that there are authors with the same initials in the 'Author Contributions' section. Please SYash and SYam, and HKi and HKa to differentiate the authors.
• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors have responded admirably to my concerns, including a large amount of new data in this revised version that substantially strengthens their primary conclusions, most notably with more robust and transparent replication and quantification of data, and the addition of two complementary mouse models that considerably strengthen their in vivo studies. Further, I think the less thorough characterization of the phosphomimetic model is permissible, given the inability to carry these animals out to 1 year of age in current times. I feel the paper has been substantially strengthened, and have only minor remaining concerns.

Minor:
1. There are still a number of grammatical errors and misspellings throughout the manuscript. For example, ejection fraction, brightfield, plakoglobin, and relative are all misspelled in the figures, to name a few. There are also a number of missing articles, particularly in the introduction. Please revise the text closely and carefully.
2. Line 171 refers to extended figure 2g, yet there is no panel g in this figure.
3. I recommend that the western blots quantifying the data in figure 1 should be included in figure 1 and not relegated to the supplementary information.

Referee #2:

The re-submitted manuscript entitled "AMPK regulates cell shape of cardiomyocytes by modulating turnover of microtubules through CLIP-170" describes the localization of microtubule protein, CLIP-170, to intercalated discs where it may be phosphorylated by stimuli in which AMPK is activated. The data presented suggest that AMPK activation causes microtubule dissociation and AMPK inhibition causes microtubule formation. Data are collected in vitro for pharmacologic AMPK inhibition and AMPK inhibition via gene silencing. A mouse model was generated with inability of CLIP-170 phosphorylation via S311A mutation and transgenesis. Mice with CLIP-170 S311A transgene demonstrate cardiac myocyte elongation and decline in cardiac function with time. Strengths of the manuscript include the novelty and rigor of demonstrating CLIP-170/AMPK localization at the intercalated disc and development of a mouse model with supporting functional data. Overall, the authors provide novel data for CLIP-170 phosphorylation and the impact on microtubule association/dissociation in cardiomyocytes. The revised manuscript does provide an expanded discussion with speculative mechanism for cardiac dysfunction in CLIP-170 S311A transgenic mice. The inclusion of mechanistic discussion and addition of functional data for another transgenic mouse line strengthen the overall manuscript.

Referee #3:

My points have been adequately addressed.

Reviewer #1:

We thank Reviewer #1 for the positive and constructive comments. We addressed all of the reviewer's comments below *in italic*, added sentences in the revised manuscript are marked in green. We hope that the manuscript is now acceptable for publication.

1. There are still a number of grammatical errors and misspellings throughout the manuscript. For example, ejection fraction, brightfield, plakoglobin, and relative are all misspelled in the figures, to name a few. There are also a number of missing articles, particularly in the introduction. Please revise the text closely and carefully.

Thank you very much for the critical comments. We would like to apologize for the grammatical errors and misspellings. We thoroughly checked the revised manuscript and figures, and made corrections as much as possible, which are shown in green in the revised manuscript.
Also, we added several articles that we thought important in this research field, but were missed in the initial submission. Thank you very much for your thoughtful suggestion.

2. Line 171 refers to extended figure 2g, yet there is no panel g in this figure.

Thank you very much for pointing this out. We made sure to delete it this time from the main text.

3. I recommend that the western blots quantifying the data in figure 1 should be included in figure 1 and not relegated to the supplementary information.

Thank you very much for the comments. We included the quantification data in the revised Fig 1.

2nd Revision - Editorial Decision 23rd Oct 2020

Dear Dr. Shintani,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice study!

In order to increase the clarity and accessibility of your work, I have taken the liberty of performing some changes in the below items and writing bullet points. Please take a look and confirm, or feel free to make changes.

- I would like to add 'cardiomyocyte' as the fifth keyword of the study.

- Synopsis blurb: Mechanical stress elicited by the heart contractility promotes AMPK localization to the intercalated discs, where AMPK regulates microtubule dynamics and cardiomyocyte function.

- Bullet points:
• Activated AMPK localizes at the intercalated discs in the heart, a specific cell-cell junction present between cardiomyocytes.
• Cardiomyocyte contractility is required for AMPK localization to the intercalated discs.
• Inhibition of CLIP-170 phosphorylation by AMPK leads to the accumulation of microtubules at the intercalated discs.
• Heart-specific phospho-deficient CLIP-170 transgenic mice results in elongation of cardiomyocytes along with accumulated MTs, leading to progressive decline in cardiac contraction.

- Abstract:

AMP-activated protein kinase (AMPK) is a multifunctional kinase that regulates microtubule (MT) dynamic instability through CLIP-170 phosphorylation; however, its physiological relevance in vivo remains to be elucidated. In this study, we identify an active form of AMPK localized at the intercalated discs in the heart, a specific cell-cell junction present between cardiomyocytes. A contractile inhibitor, MYK-461, prevents the localization of AMPK at the intercalated discs, and the effect is reversed by the removal of MYK-461, suggesting that the localization of AMPK is regulated by mechanical stress. Time-lapse imaging analysis reveals that the inhibition of CLIP-170 Ser-311 phosphorylation by AMPK leads to the accumulation of MTs at the intercalated discs. Interestingly, MYK-461 increases the individual cell area of cardiomyocytes in CLIP-170 phosphorylation-dependent manner. Moreover, heart-specific CLIP-170 S311A transgenic mice demonstrates elongation of cardiomyocytes along with accumulated MTs, leading to progressive decline in cardiac contraction. In conclusion, these findings suggest that AMPK regulates the cell shape and aspect ratio of cardiomyocytes by modulating the turnover of MTs through homeostatic phosphorylation of CLIP-170 at the intercalated discs.

Kind regards,

Deniz Senyilmaz Tiebe
--
Deniz Senyilmaz Tiebe, PhD
Editor

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Corresponding Author Name: Yasunori SHINTANI
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Manuscript Number: EMBOR-2020-50949V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We have not estimated sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Yes, line 528
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA (no exc;lusion)
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Yes, line 528
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NO
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes, line 528
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. We performed a normal distribution test by the Kolmogorov–Smirnov test to select a statistical analysis method.
Is there an estimate of variation within each group of data?	Yes

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes. When we compare two groups, we carry out the F-test with each variance value. In the case of multi-group comparison, ANOVA is used to evaluate the variance value.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Done
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Done
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirmed

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Understood
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No