EMBO reports

Muscle-specific Cand2 is translationally upregulated by mTORC1 and promotes adverse cardiac remodeling

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 7th Jan 2021

Dear Dr. Völkers,

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. We have now received the enclosed referee reports.

As you will see, all referees acknowledge that the findings are novel and potentially interesting. However, they also all point out that significant revisions will be required before the study can be considered for publication here. Given the delay due to the Christmas break, I thought to share the reports with you now instead of waiting for referee cross-comments. If you can address all points, I am happy to invite you to revise your manuscript. If you would like to discuss any of the comments, we can set a time for a video chat to do so. You can of course also send me an email. Please let me know how you would like to proceed.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns 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 major 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

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).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) 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: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- 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.

4) 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.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). 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 (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) 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 at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also 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 https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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

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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.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In this manuscript the authors identify Cullin-associated NEDD8-dissociated protein 2 (Cand2) to function downstream of mTOR. Via a set of both in vivo and in vitro experiments the authors are able to show that Cand2 blocks cardiomyocyte hypertrophy via Cul1 neddylation and Grk5 activity.

The manuscript describes an extensive set of experiments that support a link between mTOR/Cand2 and cardiomyocyte hypertrophy. Several additional datapoints would provide more comfort around the specificity and relevance of the proposed mechanism.

Specific comments:
- Torin 1 inhibits both mTORC1 and mTORC2 activity. How specific is Torin 1 for mTOR?
- EV1 shows dose-dependent inhibition of mTORC1 and 2 phosphorylation. Which dose was used for the Ribo-seq experiment and how long after PE treatment were the samples taken?
- Are the known mTOR targets regulated based on the Ribo-seq data?
- EV2C shows that Torin 1 inhibits the TAC-induced increase in HW/BW ratio. Can the protein changes observed in NRCM treated with PE and Torin 1also be confirmed in vivo? The blots in

Figure 1H should also include 2d TAC samples treated with Torin 1.
- The authors perform Ribo-seq on hearts at different time points after TAC, but this should have been done on TAC hearts with and without Torin 1 to really define the in vivo mTOR targets during stress induced remodeling.
- The authors take heart 2 days after pressure overlaod. However at this point the hypertrophy is still known to be compensatory.
- In EV1C Torin 1 has no effect on Nppa expression. However, inhibition of Cand2 blocks Nppa expression and overexpression of Cand2 enhances Nppa expression. Also Cand2 KO shows less Nppa 4 weeks after banding. The authors should explain this discrepancy
- While the authors show that Cand2 is induced 2 days after banding, it is unclear whether this is also the case 4 weeks after banding, when the authors perform the functional analysis. These data should be included.
- How significant are the transcriptomic changes in Cand2 KO hearts versus wildtype hearts?
- Why would Grk5 in vivo be regulated at the mRNA level under baseline conditions, but only under stress conditions at the protein level? And, in line with this, why would the regulation in vitro only be at the protein level?
- What is the effect of Torin 1 on NRCM under baseline conditions and does it regulate Cand2?
- Why do the authors say Cand2 regulates Grk5 at the protein level if they found Grk5 to be regulated by Cand2 by mRNA sequencing?
- Can the effects on MEF2 targets and the change in Cul1 neddylation be confirmed in vivo in the Cand2 KO mice?

Minor issues:
- The manuscript and figure legends would benefit from a spelling check as there are currently many typos.
- Legend Figure 6 Raptor is misspelled
- The legend of Figure 3C unclear and proteins should indicated in capitals

Referee #2:

This paper explores the mechanism through which mTORC1 enhances the translation of specific, not general, mRNAs to promote cardiomyocyte hypertrophy. Combined 2 Ribo-seq analyses using 1) rat neonatal cardiomyocytes in the presence or absence of Torin1, a mTOR inhibitor, under phenylephrine (PE) stimulus and 2) hearts in response to TAC or sham identified 17 mRNAs as mTOR targets in response to hypertrophic stimuli. Among them, the authors focused on Cand2, Cullin-associated NEDD8-associated protein 2, muscle-specifically expressed gene. Cand2 knockout (KO) attenuated TAC-induced hypertrophy and dysfunction, whereas AAV-mediated Cand2 overexpression increased PE-induced cardiomyocyte hypertrophy. Mechanistically, they showed that mTOR translationally, not transcriptionally, upregulates Cand2, which interacts with and inhibits Cullin 1, resulting in an increase in Grk5. Grk5 stimulates the MEF2 transcriptional activity and promotes hypertrophy.

Ribo-seq combined with RNA-seq analyses unbiasedly identified Cand2 as a target of mTOR signaling for cardiac hypertrophy. The authors' finding of mTOR specific translational targets under hypertrophic stimuli is interesting. Furthermore, RNA-seq analysis using Cand2 KO mouse hearts identified Grk5 as a potential downstream of Cand2. These findings are novel and strengths of this paper. However, there are some weaknesses with regard to mechanistic studies.

Although the authors' findings that Cand2 upregulation mediates pathological hypertrophy is novel and interesting, the underlying mechanism is investigated in a superficial manner. The involvement of GRK5 is not very convincing. My suggestion is that the authors should show that GRK5 is critically mediated the effect of Cand2 upon cardiac hypertrophy in vivo by conducting rescue experiments (downregulated GRK5 in the presence of Cand2 overexression and restore the level of GRK5 in the presence of Cand2KO). In vivo analyses of cardiac hypertrophy and heart failure are insufficient. For example, although Cand2 is upregulated 2 days after pressure overload, the analysis of cardiac hypertrophy and heart failure was conducted 2weeks later when Cand2 is no longer upregulated. In addition, the authors used systemic Cand 2 KO mice. It is unclear whether the cardiac phenotype observed in Cand 2 KO mice was mediated through the effect in cardiomyocytes.

Major issues

1. Although the authors propose that stabilization of GRK5 plays a major role in mediating the effect of Cand 2, considering the fact that Cul1 may degrade many other substrates, it is unclear whether the proposed mechanism really mediates the effect of mTORC1 stimulation. Only data shown in Figure 6 is very shaky and hard to prove the critical involvement of GRFK5 in vivo.

2. The authors show that Cand2 is transiently activated at 2days after TAC but not at 2weeks. The effect TAC upon hypertrophy and LV function was investigated only after 2weeks. The authors should show how pressure overload affects the function of the heart in Cand2 KO mice.

3. The authors used systemic Cand2 KO mice. This raises the question of whether the effect of Cand2 KO is mediated through the effect in cardiomyocytes.

4. Considering that Cand2 KO mice had no apparent phenotype at baseline but protected against TAC, transcriptome analysis should be done in the hearts of mice subjected to pressure overload, not baseline hearts, in Figure 5A. The effect of Cand2 knock-down upon gene expression in vitro was also evaluated at baseline in Figure 6. The analysis should be conducted in the presence of stress.

Minor issues:
1.In Figure 5E, the results require quantification and statistical analyses.
2. In Figure 6H, why does Raptor downregulation downregulate Nppa, which is different from other results where Nppa expression is not affected.
3. In Figure 7B, why is Cabd2 upregulated in the presence of MLN? In C, why wasn't siCand2-induced decrease in cell size rescued by MLN?

Referee #3:

In this study, Gorska et al. focused on the role of Cand2 in cardiac pathological remodeling in the heart under pressure overload. The authors explored the possibility that Cand2 is critically mediating the action of mTORC1. Importantly, the authors identified the downstream effectors of Cand2, GRK5 and MEF2, in cardiac remodeling. The main hypothesis is Cand2 is upregulated by mTORC1 signaling and stabilizes GRP5, and the latter migrates to nucleus and enhances MEF2. Additionally, the authors showed that Cand2 suppresses Cullin 1 neddylation and this is a potential

mechanism underlying GRP5 stabilization. Overall, the study was thoughtfully designed and elegantly executed. A few issues remain before the final conclusions may be firmly drawn.
1. mTORC1 is a master regulator of cardiac cell growth under pressure overload. mTORC1 exerts its roles largely via general actions on protein translation with certain transcriptional regulations. While it is interesting and also important to identify one target of mTORC1 and explore its relevance, Cand2 is likely one of many mechanisms for mTORC1. Nevertheless, this study is comprehensive and convincing. I would however recommend adjusting the tone in the manuscript, stating Cand2 is one of mediators of complex actions of mTORC1. Current version sounds like Cand2 is the most important mediator, if not the only one, in this process.
2. The authors did great work in linking mTORC1, Cand2, GRP5, and MEF2 in pathological remodeling. However, the authors have spent more efforts on correlations, rather mechanistic explorations. A few questions remain: Does overexpression of MEF2 rescue the effect of Cand2 silencing? Does knockdown of GRK5 reverse the effects of Cand2 overexpression? What about overexpression GRK5 under the condition of Raptor silencing?
2. Does Cand1 change by TAC or PE treatment in vitro?
3. In Figure 4, the authors determined cardiomyocyte cross-sectional area 2 weeks after TAC. Have the authors tried different time points, such as 2 days and longer time (e.g. 4 weeks) after TAC?
4. In Figure 5, the authors showed GRK5 is increased by TAC at 4 weeks after the surgery. Since the induction of Cand1 is early, it would be interesting to know the time course of change for GRP5.
5. Figure 7E, the WB is not representative, for time 0 of Cand2 OE.
6. Figure 7F, GRK5 is heterogenous in the siCul1 group.
7. Although PE is a classical drug to induce mTORC1, more specific activation of mTORC1 would be preferred, such as silencing TSC2. Under this condition, what about the changes for Cand2, GRK5, and MEF2.

Response to reviewers

Reviewer #1 comments:
In this manuscript the authors identify Cullin-associated NEDD8-dissociated protein 2 (Cand2) to function downstream of mTOR. Via a set of both in vivo and in vitro experiments the authors are able to show that Cand2 blocks cardiomyocyte hypertrophy via Cul1 neddylation and Grk5 activity.
The manuscript describes an extensive set of experiments that support a link between mTOR/Cand2 and cardiomyocyte hypertrophy. Several additional datapoints would provide more comfort around the specificity and relevance of the proposed mechanism.

Thank you for your very positive comments about our manuscript. Below are our responses to your comments

Specific comments:

1. **Torin 1 inhibits both mTORC1 and mTORC2 activity. How specific is Torin 1 for mTOR?**

The reviewer asked a very important question and we added additional data into the revised manuscript. We completely agree that Torin 1 inhibits both mTORC1 and mTORC2 activity. However, when used at 100nM in isolated cardiomyocytes, mTORC2 activity (measured by phosphorylation of Akt1) is less inhibited compared to the inhibitory effect of Torin 1 on mTORC1 (see revised Fig. EV1). We analyzed additional signaling pathways and could confirm that Torin 1 does not inhibit Erk1 phosphorylation, p38 phosphorylation when used at 100nM. We cannot completely exclude that some of the effects of Torin 1 is mediated through mTORC2 inhibition or other signaling pathways. However, a very strong inhibition of mTORC1 after short term incubation with Torin 1 was observed.
We included this information into the revised manuscript as follows:

Page 4, line 22
Specific inhibition of mTOR-dependent protein signaling by Torin 1 was confirmed by immunoblots (**EV1A**), and other signaling pathways such as Erk1/2 and p38 signaling were unaffected by Torin 1

2. **EV1 shows dose-dependent inhibition of mTORC1 and 2 phosphorylation. Which dose was used for the Ribo-seq experiment and how long after PE treatment were the samples taken?**

Again, this is an important question. We used Torin 1 at 100nM for 3h for the Ribo-Seq experiments. We added this information into the manuscript as follows:

Page 4, line 18
To define mRNAs translationally regulated by mTOR signaling in response to pathological cell growth, cultures of neonatal rat ventricular cardiomyocytes (NRCMs) were acutely treated for 3h with the α-1 adrenoreceptor agonist phenylephrine (50uM, PE) and with Torin 1 (100nM), which inhibits mTOR by binding to the ATP-binding site in the kinase domain

Page 15, line 28
For analysis of hypertrophy, cells were treated with 50 uM PE for indicated time points. mTORC1 was pharmacologically inhibited with 100 nM Torin 1 for indicated timepoints. For generation of the Ribo-Seq libraries, mTORC1 was pharmacologically inhibited with 100 nM Torin 1 for 3h.

3. **Are the known mTOR targets regulated based on the Ribo-seq data?**

We appreciate the opportunity to clarify this important point. We used a previously published dataset that defined mTORC1 targets with a TOP motif in the 5'UTR in MEF cells(Thoreen *et al*, 2012). This dataset defined 66 mTOR targets with a TOP-motif and we used three (rpS5, rpS3 and eEF2) for the initial validation of our Torin 1 treatment.

4. **EV2C shows that Torin 1 inhibits the TAC-induced increase in HW/BW ratio. Can the protein changes observed in NRCM treated with PE and Torin 1 also be confirmed in vivo? The blots in Figure 1H should also include 2d TAC samples treated with Torin 1.**

We followed the suggestion of the reviewer and added new data to the revised manuscript. We confirmed that Torin 1 treatment after TAC surgery (2days), blocked protein expression of Desmin, eEF2, 4epb1 and rpS20, whereas protein expression of another ribosomal protein, rpS5, was unaffected. Successful inhibition of mTOR was confirmed by strong inhibition of phosphorylated rpS6. In line with our Ribo-Seq data *in vitro*, mRNA levels of those mTORC1 targets quantified by RT-PCR was unaffected both by TAC surgery as well as by Torin 1 treatment.
We included this information into the revised manuscript as follows:

Page 5, line 27
Consistent with these results, protein levels of eEF2, desmin as well as 4epb1 increased after TAC, but Torin 1 blocked the induction (Fig 1J). Consistent with the Ribo-Seq data in vitro, mRNA levels quantified by RT-qPCR were unaffected by TAC surgery and Torin 1 treatment (Fig 1K).

5. **The authors perform Ribo-seq on hearts at different time points after TAC, but this should have been done on TAC hearts with and without Torin 1 to really define the in vivo mTOR targets during stress induced remodeling.**

This is valid suggestion and experiments are ongoing to define the mTOR translatome *in vivo* in different cell types in response to cardiac stress using cell type-specific Ribo-seq approaches. However, we feel that those experiments would be out of the scope of the current manuscript. Here, we have defined *in vitro* mTOR responsive target genes and have confirmed the increased translation of Cand2, a muscle specific protein, *in vivo*. We show that Torin 1 treatment blocks Cand2 protein expression after TAC and elucidated the function of Cand2 both *in vivo* and *in vitro*. We agree that performing Ribo-seq *in vivo* after mTOR inhibition with Torin1 likely identifies additional mTOR targets in adult diseased cardiomyocytes. Although we don't have formal evidence, most likely Cand2 will be one the identified mTOR targets *in vivo*, based on our existing follow up experiments in the current manuscript. We hope that we could convince the reviewer, that adding more sequencing data would not alter the overall result and impact of the current study.

6. **The authors take heart 2 days after pressure overload. However, at this point the hypertrophy is still known to be compensatory.**

We agree that hypertrophic growth 2 days after TAC surgery is compensatory. However, several pathological signaling events occur early after pressure overload, including mTOR signaling which is associated with changes in pathological gene expression as well as a clear increase in the heart weight to body weight ratio 2 days after pressure overload. In the revised manuscript, we added additional data about the role of Cand2 in early and late pathological cardiac remodeling. Cand2 deletion blocked the increase of both heart weight as well as the Mef2 target genes two days after pressure overload. Similarly, cardiac

function was improved two weeks after TAC which again was associated with decreased pathological gene expression in the Cand2 knockout mice compared to WT mice.
It would be very interesting to comprehensively map the mTOR dependent translatome later after TAC surgery or during cardiac failure and we will address these important questions in subsequent studies.

7. **In EV1C Torin 1 has no effect on Nppa expression. However, inhibition of Cand2 blocks Nppa expression and overexpression of Cand2 enhances Nppa expression. Also, Cand2 KO shows less Nppa 4 weeks after banding. The authors should explain this discrepancy**

True and we tried to address this important observation in the revised manuscript. We added additional experiments in neonatal rat cardiomyocytes to the revised manuscript and confirmed that Torin 1 does not block the induction of Nppa in response to PE treatment *in vitro*. Still, Cand2 depletion blocks Nppa both early as well as late after TAC surgery *in vivo*. However, Torin 1 treatment does also block Nppa induction *in vivo* after pressure overload. We speculate that on the one hand the difference in the cell types (neonatal vs adult) as well as the difference in the stress conditions (TAC vs PE) might explain this discrepancy.

We added this information into the revised discussion as follows:

Page 5, line 1
Molecular markers of pathological growth such as *Nppb* or *Xirp2* were blocked while *Nppa* was still induced (**EV1C**).

Page 5, line 13
In line with the *in vitro* data, mTOR inhibition with Torin 1, *in vivo*, blocked pathologic growth as well as induction of *Nppa* and *Nppb* induced by acute transverse constriction (TAC) surgery, a common model for cardiac hypertrophy and remodeling (**EV2**) (Doroudgar *et al*, 2019).

8. **While the authors show that Cand2 is induced 2 days after banding, it is unclear whether this is also the case 4 weeks after banding, when the authors perform the functional analysis. These data should be included.**

This is an important question and Cand2 is still induced. This information is presented in Figure 5D.

9. **How significant are the transcriptomic changes in Cand2 KO hearts versus wildtype hearts?**

A false-positive rate of α= 0.05 with FDR correction was taken as the level of significance.
The FDR for GRK5 was 6.87583^{-05}

Page 15, line 1
A false-positive rate of α= 0.05 with FDR correction was taken as the level of significance

10. **Why would Grk5 in vivo be regulated at the mRNA level under baseline conditions, but only under stress conditions at the protein level? And, in line with this, why would the regulation in vitro only be at the protein level?**

Again, a very important question. Many of the direct mTORC1 targets are predominantly regulated at the protein level, without changes at the mRNA level. Still, as the reviewer pointed out, we see a significant decrease of *Grk5* mRNA levels at baseline conditions in the knockout mice. Thus, we cannot conclude that Cand2 depletion regulates Grk5 only at the

protein level (see also your question 12). The mechanisms how *Grk5* mRNA levels might be regulated in the Cand2 KO mice are unknown. However, Grk5 levels are only regulated at the protein level *in vitro* after Cand 2 knockdown. We think that the shorter duration of the knockdown *in vitro* (72h) vs the global knockout model in which Cand2 is depleted for the entire cardiac development, might explain the differences of the regulation. Still, the induction in response to pathological growth either by PE *in vitro*, or in response to TAC *in vivo*, is regulated at the level of translation, which is in line with our sequencing data *in vitro* and the role of mTOR.

We added this information into the revised discussion as follows:

Page 12, line 30
We found that in response to stress such as pressure overload, Cand2 regulates expression of Grk5 predominantly independently of the transcript levels. However, Grk5 mRNA levels are decreased in Cand2 KO mice, suggesting that additional mechanisms involving regulation of Grk5 transcription exist in the Cand2 KO mice.

11. What is the effect of Torin 1 on NRCM under baseline conditions and does it regulate Cand2?

We analyzed the effect of Torin1 in NRCM under baseline conditions in the revised manuscript. Torin 1 decreased Cand2 protein levels in a time dependent manner.
We added this information into the revised manuscript as follows:

Page 6, line 5
Torin 1 inhibited Cand2 expression also without pharmacological stimulation of NRCMs (**EV2F).**

12. Why do the authors say Cand2 regulates Grk5 at the protein level if they found Grk5 to be regulated by Cand2 by mRNA sequencing?

Agreed and also see our response to your point #10. We revised the manuscript to clarify this point as follows:

Page 9, line 7
Our findings showed that Cand2 depletion *in vitro* results in decreased Grk5 protein levels mostly independent from changes in transcript levels (**Fig 5B**), suggesting that post-transcriptional mechanisms might regulate Grk5 expression by Cand2. *In vivo*, both *Grk5* mRNA levels and protein levels are regulated by Cand2.

Page 12, line 30
We found that in response to stress such as pressure overload, Cand2 regulates expression of Grk5 predominantly independently of the transcript levels. However, *Grk5* mRNA levels are decreased in Cand2 KO mice, suggesting that additional mechanisms involving regulation of Grk5 transcription exist in the Cand2 KO mice.

13. Can the effects on MEF2 targets and the change in Cul1 neddylation be confirmed in vivo in the Cand2 KO mice?

We analyzed the expression levels of MEF2 target genes in the Cand2 KO mice at baseline and after TAC surgery at the two different timepoints. Indeed, we do observe a reduction in MEF2 target gene expression *in vivo* in the Cand2 KO mice. We also quantified the change in Cul1 neddylation *in vivo*. In line with the in vitro data, Cand2 KO mice showed higher levels of Cul1 neddylation in *vivo*. This was associated with decreased Grk5 protein levels early after TAC.

We added this new information into the new Figure 8 into the revised manuscript:

Page 10, line 35
Since Cand2 expression is induced early after TAC, we examined early pathological remodeling 2 days after TAC surgery in Cand2 KO mice compared to WT mice. Cand2 KO mice along WT littermates were subjected to TAC or sham surgery and phenotypic consequences were analyzed 2 days after surgery. TAC-induced *Nppa* and *Nppb* levels as well as induction of MEF2 target genes *Xirp1* and *Nr4a1* in WT mice were significantly reduced in Cand2 KO mice (**Fig 8A**). TAC surgery resulted in an increased LV/BW ratio in WT mice (**Fig 8B**). In contrast, Cand2 deficient mice showed a significantly decreased LV/BW ratio compared to TAC-operated WT mice. Early cardiac remodeling was associated with increased Grk5 protein levels in WT mice, which was predominantly regulated at the protein levels since Grk5 mRNA levels were not significantly increased in TAC-operated mice compared to sham-operated animals. Induction of Grk5 was blocked in Cand2 KO mice (**Fig 8C)**. In line with our observation *in vitro*, Cand2 depletion resulted in higher levels of neddylated Cul1 (**Fig 8D).**

Minor issues:

1. **The manuscript and figure legends would benefit from a spelling check as there are currently many typos.**

We apologize and carefully revised the manuscript.

2. **Legend Figure 6 Raptor is misspelled**

Corrected.

3. **The legend of Figure 3C unclear and proteins should indicated in capitals**

Corrected.

We are thankful for the constructive critique of Reviewer #1, and hopeful that the articulated concerns have been satisfactorily redressed.

Referee #2:

This paper explores the mechanism through which mTORC1 enhances the translation of specific, not general, mRNAs to promote cardiomyocyte hypertrophy. Combined 2 Ribo-seq analyses using 1) rat neonatal cardiomyocytes in the presence or absence of Torin1, a mTOR inhibitor, under phenylephrine (PE) stimulus and 2) hearts in response to TAC or sham identified 17 mRNAs as mTOR targets in response to hypertrophic stimuli. Among them, the authors focused on Cand2, Cullin-associated NEDD8-associated protein 2, muscle-specifically expressed gene. Cand2 knockout (KO) attenuated TAC-induced hypertrophy and dysfunction, whereas AAV-mediated Cand2 overexpression increased PE-induced cardiomyocyte hypertrophy. Mechanistically, they showed that mTOR translationally, not transcriptionally, upregulates Cand2, which interacts with and inhibits Cullin 1, resulting in an increase in Grk5. Grk5 stimulates the MEF2 transcriptional activity and promotes hypertrophy. Ribo-seq combined with RNA-seq analyses unbiasedly identified Cand2 as a target of mTOR signaling for cardiac hypertrophy. The authors' finding of mTOR specific translational targets under hypertrophic stimuli is interesting. Furthermore, RNA-seq

analysis using Cand2 KO mouse hearts identified Grk5 as a potential downstream of Cand2. These findings are novel and strengths of this paper. However, there are some weaknesses with regard to mechanistic studies.

Although the authors' findings that Cand2 upregulation mediates pathological hypertrophy is novel and interesting, the underlying mechanism is investigated in a superficial manner. The involvement of GRK5 is not very convincing. My suggestion is that the authors should show that GRK5 is critically mediated the effect of Cand2 upon cardiac hypertrophy in vivo by conducting rescue experiments (downregulated GRK5 in the presence of Cand2 overexpression and restore the level of GRK5 in the presence of Cand2KO). In vivo analyses of cardiac hypertrophy and heart failure are insufficient. For example, although Cand2 is upregulated 2 days after pressure overload, the analysis of cardiac hypertrophy and heart failure was conducted 2weeks later when Cand2 is no longer upregulated. In addition, the authors used systemic Cand 2 KO mice. It is unclear whether the cardiac phenotype observed in Cand 2 KO mice was mediated through the effect in cardiomyocytes.

Thank you for your very positive comments about our manuscript. Below are our specific responses to your comments. Based on our discussion with the Editor after the initial decision about the manuscript, we decided to perform the suggested rescue experiments *in vitro*. Those data are now included in the revised manuscript, and we now show that downregulation of Grk5 blocks the hypertrophic growth induced by Cand2 overexpression. In addition, overexpression of Grk5 partly rescues the decreased cell size of Cand2 depleted myocytes.

Major issues

1. **Although the authors propose that stabilization of GRK5 plays a major role in mediating the effect of Cand 2, considering the fact that Cul1 may degrade many other substrates, it is unclear whether the proposed mechanism really mediates the effect of mTORC1 stimulation. Only data shown in Figure 6 is very shaky and hard to prove the critical involvement of GRK5 in vivo.**

The reviewer raises an important point. We agree that Cul1 very likely degrades many other substrates, some of which might be dependent on the Cand2-Cul1 interaction. Moreover, Cand2 could affect the activity or stability of several other proteins in addition to Cul1. In order to answer this question *in vitro*, we performed several rescue experiments as suggested by this reviewer (and reviewer #3). We agree that this cannot clarify the consequence of mTOR dependent stimulation of Cand2 expression on Grk5 expression *in vivo*. However, generating and characterization of several genetic models including GRK5 knockout mice with Cand2 overexpression, or Cand2 knockout with GRK5 overexpression would require an amount of mice work that would be beyond the scope of the current manuscript.

We could show in the revised manuscript that Grk5 knockdown in Cand2 overexpressing myocytes blocked induction of cell size and the induction of the MEF2 targets (revised Fig. 7). Conversely, Grk5 overexpression in Cand2 depleted myocytes resulted in increased expression of Mef2 targets but did not fully restore the decreased cell size upon Cand2 depletion. Again, this fits very well with your argument that Cand2 has additional targets that mediate the antihypertrophic effect of Cand2. We carefully revised the manuscript and added this information in as follows:

Page 10, line 17
To confirm that Cand2 affects cell size through Grk5, we examined whether Grk5 re-expression is sufficient to rescue the decreased cell size phenotype of Cand2-deficient cardiomyocytes. Grk5 overexpression and Cand2 depletion was confirmed by immunoblots

(Fig 7G). While Cand2 knockdown reduced cellular size, Grk5 overexpression significantly increased cell size. Grk5 overexpression partly rescued the phenotype in Cand2 depleted NRCMs. In parallel, RT-qPCR analysis revealed upregulation of direct MEF2 targets such as *Nr4a1* and *Xirp2* after overexpression of Grk5 in Cand2 depleted cells (**Fig 7H**). Conversely, Grk5 knockdown in the presence of Cand2 overexpression blocked the hypertrophic cell growth induced by Cand2 (**Fig 7I).** RT-qPCR analysis revealed upregulation of direct MEF2 targets such as *Nr4a1* and *Xirp2* after overexpression of Cand2 which was reversible after Grk5 knockdown (**Fig 7J**), suggesting that Grk5 signaling is downstream of Cand2 *in vitro*.

2. **The authors show that Cand2 is transiently activated at 2days after TAC but not at 2weeks. The effect TAC upon hypertrophy and LV function was investigated only after 2weeks. The authors should show how pressure overload affects the function of the heart in Cand2 KO mice.**

This is an important comment. Cand2 protein levels are elevated two weeks after TAC surgery (**Fig. 5D**). We performed additional TAC experiments in the Cand2 KO early after TAC surgery to analyze the induction of MEF2 targets *in vivo*. In addition, we included echocardiographic data of Cand2 KOs compared to WT mice both early and late after TAC surgery.

We included this information into the revised manuscript as follows:

Page 10, line 35
Since Cand2 expression is induced early after TAC, we examined early pathological remodeling 2 days after TAC surgery in Cand2 KO mice compared to WT mice. Cand2 KO mice along WT littermates were subjected to TAC or sham surgery and phenotypic consequences were analyzed 2 days after surgery. TAC-induced *Nppa* and *Nppb* levels as well as induction of MEF2 target genes *Xirp1* and *Nr4a1* in WT mice were significantly reduced in Cand2 KO mice (**Fig 8A**). TAC surgery resulted in an increased LV/BW ratio in WT mice (**Fig 8B**). In contrast, Cand2 deficient mice showed a significantly decreased LV/BW ratio compared to TAC-operated WT mice. Early cardiac remodeling was associated with increased Grk5 protein levels in WT mice, which was predominantly regulated at the protein levels since Grk5 mRNA levels were not significantly increased in TAC-operated mice compared to sham-operated animals.

Page 7, line 21
Serial echocardiographic analysis revealed improved function of Cand2 KO mice four weeks after surgery compared to WT mice (**Fig. EV3)**.

3. **The authors used systemic Cand2 KO mice. This raises the question of whether the effect of Cand2 KO is mediated through the effect in cardiomyocytes.**

True, and we cannot completely exclude the possibility that the effect of Cand2 is mediated by non-myocytes. However, Cand2 expression is predominantly expressed in myocytes in the heart, and we added additional data to support this statement. Immunofluorescence showed expression of Cand2 in myocytes but not in interstitial cells (**Fig. 2C**). We isolated cells from mouse hearts and analyzed the expression of Cand2 in cardiomyocytes, fibroblast as well as endothelial cells. These data again indicate that Cand2 is predominantly expressed in the myocyte fraction.

We added this information in the revised manuscript as follows:

Page 6, line 27

Immunofluorescent staining confirmed specific Cand2 expression only in cardiomyocytes (**Fig 3C**). In addition, immunoblots from isolated cardiac cells showed Cand2 expression only in cardiomyocytes but not in endothelial cells or fibroblasts (**Fig 3D**), suggesting that Cand2 protein expression is restricted to muscle cells in the heart.

4. **Considering that Cand2 KO mice had no apparent phenotype at baseline but protected against TAC, transcriptome analysis should be done in the hearts of mice subjected to pressure overload, not baseline hearts, in Figure 5A. The effect of Cand2 knock-down upon gene expression in vitro was also evaluated at baseline in Figure 6. The analysis should be conducted in the presence of stress.**

We agree that performing transcriptome analysis in hearts of mice subjected to TAC would be a very interesting and important dataset. Considering the molecular function of Cand2, transcriptome analysis might not identify all targets of Cand2, and additional experiments would be needed including Ribo-Seq or protein stability assays. Given the complexity of those methods, we believe that this should be addressed in future experiment, together with the search of additional direct interacting partner of Cand2 by mass-spectrometry.
We added the effect of Cand2 knock-down upon gene expression *in vitro* in the presence of stress in the revised manuscript. Again, we show that Cand2 depletion inhibits the induction of MEF2 target genes in response to PE compared to control myocytes.
We added this information in the revised manuscript as follows:

Page 8, line 20
Moreover, the expression of MEF2-dependent mRNAs such as *Nr4a1* and *Xirp2* (Lehmann *et al*, 2018)'(Huang *et al*, 2006) was downregulated when Cand2 was depleted both at baseline and after stimulation with PE (**Fig 6B**).

Minor issues:

1. **In Figure 5E, the results require quantification and statistical analyses.**

Agreed, and we quantified and analyzed the results of figure 5E in the revised manuscript.

Page 8, line 12
Since Grk5 has been shown to translocate to the nucleus of cardiomyocytes following pressure overload *via* its NLS (Johnson *et al*, 2004)'(Martini *et al*, 2008)'(Gold *et al*, 2013), we examined Grk5 levels in the cytoplasm and nucleus *in vivo* in Cand2 KO mice (**Fig 5F)**. Neither TAC nor lack of Cand2 affected Grk5 levels in the cytoplasmic fraction, but Grk5 levels significantly increased in the nucleus in WT mice in response to TAC, which was blocked in Cand2 KO mice.

2. **In Figure 6H, why does Raptor downregulation downregulate Nppa, which is different from other results where Nppa expression is not affected.**

True and we tried to address this important observation in the revised manuscript. We added additional experiments in neonatal rat cardiomyocytes to the revised manuscript and confirmed that Torin 1 does not block the induction of *Nppa* in response to PE treatment. Still, Cand2 depletion as well as Raptor knockdown blocks *Nppa*. However, Torin 1 treatment does also block *Nppa* induction *in vivo* after pressure overload. We speculate that on the one hand the difference in the cell types (neonatal vs adult) as well as the difference in the stress conditions (TAC vs PE) might explain this discrepancy.
We added this information into the revised discussion as follows:

Page 5, line 1

Molecular markers of pathological growth such as *Nppb* or *Xirp2* were blocked while *Nppa* was still induced (**EV1C**).

Page 5, line 13
In line with the *in vitro* data, mTOR inhibition with Torin 1, *in vivo*, blocked pathologic growth as well as induction of *Nppa* and *Nppb* induced by acute transverse constriction (TAC) surgery, a common model for cardiac hypertrophy and remodeling (**EV2**) (Doroudgar *et al*, 2019).

3. **In Figure 7B, why is Cand2 upregulated in the presence of MLN? In C, why wasn't siCand2-induced decrease in cell size rescued by MLN?**

Again, an important point. We have performed RT-PCR analysis to analyze whether Cand2 is induced on the transcriptional level by MLN. This data now indicate that Cand2 expression is post-transcriptionally induced by treatment with MLN. The exact mechanism is unknown; however, we speculate based on the molecular action of MLN that Cand2 protein stability might be affected by MLN induced changes of overall neddylation. Since MLN induced Cand2, and increased Cand2 expression is associated with enlarged cells, depletion of Cand2 blocked the effect of MLN on cell size, suggesting that Cand2 is required for the cellular response induced by MLN treatment.

We added this information into the revised discussion as follows:

Page 9, line 34
MLN4924 induced Cand2 protein levels independent of transcripts levels, suggesting that post-transcriptional mechanisms are responsible for increasing Cand2 protein levels (**EV5**). The MLN4924-induced cell growth was blocked by Cand2 knockdown, suggesting that Cand2 is required for the increased cell size induced by MLN4924 **(Fig 7C)**.

We are thankful for the very constructive critique of reviewer #2 and hopeful that the articulated concerns have been satisfactorily redressed.

Referee #3:

In this study, Gorska et al. focused on the role of Cand2 in cardiac pathological remodeling in the heart under pressure overload. The authors explored the possibility that Cand2 is critically mediating the action of mTORC1. Importantly, the authors identified the downstream effectors of Cand2, GRK5 and MEF2, in cardiac remodeling. The main hypothesis is Cand2 is upregulated by mTORC1 signaling and stabilizes GRP5, and the latter migrates to nucleus and enhances MEF2. Additionally, the authors showed that Cand2 suppresses Cullin 1 neddylation and this is a potential mechanism underlying GRP5 stabilization. Overall, the study was thoughtfully designed and elegantly executed. A few issues remain before the final conclusions may be firmly drawn.

We appreciate the reviewer's enthusiasm toward our work!

1. **mTORC1 is a master regulator of cardiac cell growth under pressure overload. mTORC1 exerts its roles largely via general actions on protein translation with certain transcriptional regulations. While it is interesting and also important to identify one target of mTORC1 and explore its relevance, Cand2 is likely one of many mechanisms for mTORC1. Nevertheless, this study is comprehensive and convincing. I would however recommend adjusting the tone in the manuscript, stating Cand2 is one of mediators of complex actions of mTORC1.**

Current version sounds like Cand2 is the most important mediator, if not the only one, in this process.

We completely agree, and a similar concern was also raised by reviewer #2 (point 1). mTORC1 has several targets, and Cand2 is only one of them. We have carefully revised the manuscript and adjusted the tone in the manuscript and added this information into the revised discussion as follows

Page 11, line 28

We identified several mTOR-dependent genes *in vitro* and characterized Cand2 in follow-up experiments. Given the complexity of mTORC1 dependent signaling in response to stress, Cand2 upregulation is definitely only one of many effectors downstream of mTOR that regulate cardiac remodeling.

2. **The authors did great work in linking mTORC1, Cand2, GRP5, and MEF2 in pathological remodeling. However, the authors have spent more efforts on correlations, rather mechanistic explorations. A few questions remain: Does overexpression of MEF2 rescue the effect of Cand2 silencing? Does knockdown of GRK5 reverse the effects of Cand2 overexpression? What about overexpression GRK5 under the condition of Raptor silencing?**

Those are all great suggestions and we performed additional experiments in the revised manuscripts. MEF2 resulted in increased expression of target genes but did not rescue the decreased cell size in Cand2 depleted cells, suggesting that additional mechanisms mediate the effect of Cand2 on cardiomyocyte growth. However, Grk5 knockdown reversed the effects of Cand overexpression on gene expression and cell size *in vitro*. However, overexpression of GRK5 under the condition of Raptor silencing does not result in increased cell size, suggesting that mTORC1 activity itself is needed for the pro-hypertrophic effect of Grk5.
Those data are now included into the revised manuscript as follows:

Page 10, line 17

To confirm that Cand2 affects cell size through Grk5, we examined whether Grk5 re-expression is sufficient to rescue the decreased cell size phenotype of Cand2-deficient cardiomyocytes. Grk5 overexpression and Cand2 depletion were confirmed by immunoblots **(Fig 7G)**. While Cand2 knockdown reduced cellular size, Grk5 overexpression significantly increased cell size. Grk5 overexpression partly rescued the phenotype in Cand2-depleted NRCMs. In parallel, RT-qPCR analysis revealed upregulation of MEF2 targets such as *Nr4a1* and *Xirp2* after overexpression of Grk5 in Cand2 depleted cells (**Fig 7H**). Conversely, Grk5 knockdown in the presence of Cand2 overexpression blocked the hypertrophic cell growth induced by Cand2 (**Fig 7I).** RT-qPCR analysis revealed upregulation of direct MEF2 targets such as *Nr4a1* and *Xirp2* after overexpression of Cand2 which was reversible after Grk5 knockdown (**Fig 7J**), suggesting that Grk5 signaling is downstream of Cand2 *in vitro*. Furthermore, we tested whether overexpression of MEF2 could rescue the effect of Cand2 silencing (**EV5**). Adenoviral overexpression of MEF2c in NRCMs resulted in increased expression of MEF2 target genes, as well as increased cell size. MEF2c overexpression also induced target genes in Cand2-depleted cells but was not sufficient to increase cell size in Cand2 depleted cells, suggesting that Cand2 regulates cellular growth also via additional pathways.

Page 8, line 33

We also tested whether adenoviral overexpression GRK5 under the condition of Raptor silencing reverses the phenotype of Raptor depletion in NRCMs (**Fig 6J**). Grk5

overexpression alone increased cell size as well as MEF2 targets (**Fig 6K-L**), but Grk5 overexpression in Raptor depleted NRCMs only partially rescued both cellular size as well as MEF2 target gene expression, suggesting that mTORC1 activity is needed for the pro-hypertrophic effect of Grk5.

3. **Does Cand1 change by TAC or PE treatment in vitro?**

This is an important question and Cand1, in contrast to Cand2, does not change by TAC *in vivo* or PE *in vitro*.
Those data are now included into the revised manuscript as follows:

Page 6, line 6
In contrast, protein expression of Cand1 did not change after TAC surgery *in vivo* or after PE treatment of NRCMs *in vitro*.

4. **In Figure 4, the authors determined cardiomyocyte cross-sectional area 2 weeks after TAC. Have the authors tried different time points, such as 2 days and longer time (e.g. 4 weeks) after TAC?**

Great suggestions, and we added additional time points into the revised manuscript. We performed additional TAC experiments and analyzed the early response to TAC surgery in the Cand2 KO mice. In addition, we included echocardiographic data of Cand2 KOs compared to WT mice both early and late after TAC surgery. Cand2 KO have decreased HW/BW ratio as well as MEF2 target gene expression two days after TAC compared to WT mice. We also evaluated cardiac function four weeks after TAC. Cardiac function in both WT and KO mice deteriorated compared to Sham operated animals, but Cand2 KO mice still had a trend to better cardiac function compared to WT animals.

Those data are now included into the revised manuscript as follows:

Page 10, line 35
Since Cand2 expression is induced early after TAC, we examined early pathological remodeling 2 days after TAC surgery in Cand2 KO mice compared to WT mice. Cand2 KO mice along WT littermates were subjected to TAC or sham surgery and phenotypic consequences were analyzed 2 days after surgery. TAC-induced *Nppa* and *Nppb* levels as well as induction of MEF2 target genes *Xirp1* and *Nr4a1* in WT mice were significantly reduced in Cand2 KO mice (**Fig 8A**). TAC surgery resulted in an increased LV/BW ratio in WT mice (**Fig 8B**). In contrast, Cand2 deficient mice showed a significantly decreased LV/BW ratio compared to TAC-operated WT mice. Early cardiac remodeling was associated with increased Grk5 protein levels in WT mice, which was predominantly regulated at the protein levels since Grk5 mRNA levels were not significantly increased in TAC-operated mice compared to sham-operated animals. Induction of Grk5 was blocked in Cand2 KO mice (**Fig 8C)**.

Page 7, line 21
Serial echocardiographic analysis revealed improved function of Cand2 KO mice four weeks after surgery compared to WT mice (**Fig. EV3)**.

5. **In Figure 5, the authors showed GRK5 is increased by TAC at 4 weeks after the surgery. Since the induction of Cand2 is early, it would be interesting to know the time course of change for GRK5.**

Again, a very important point. We analyzed and quantified Grk5 levels early after TAC. Grk5 is induced already two days after TAC and this increase is blunted in the Cand2 KO mice.

Those data are now included into the revised manuscript as follows:

Page 11, line 6
Early cardiac remodeling was associated with increased Grk5 protein levels in WT mice, which was predominantly regulated at the protein levels since Grk5 mRNA levels were not significantly increased in TAC-operated mice compared to sham-operated animals. Induction of Grk5 was blocked in Cand2 KO mice (**Fig 8C)**.

6. **Figure 7E, the WB is not representative, for time 0 of Cand2 OE.**

We agree, repeated the experiment and updated the WB in Figure 7E.

7. **Figure 7F, GRK5 is heterogenous in the siCul1 group.**

Again, we agree, repeated the experiment and updated the WB in Figure 7F.

8. **Although PE is a classical drug to induce mTORC1, more specific activation of mTORC1 would be preferred, such as silencing TSC2. Under this condition, what about the changes for Cand2, GRK5, and MEF2.**

We followed this excellent suggestion of the reviewer and silenced TSC2. This resulted in increased mTORC1 activity, which was associated with increased Cand2 and Grk5 protein levels. We also measured MEF2 activity using the luciferase assay. TSC2 silencing increased Mef2 activity compared to control cells.
Those data are now included into the revised manuscript as follows:

Page 8, line 27
Consistent with this, genetic activation of mTORC1 after knockdown of TSC2 resulted in increased MEF2 luciferase activity and increased Cand2 and Grk5 expression (**Fig 6F**).

We are thankful for the constructive critique of Reviewer #3, and hopeful that the articulated concerns have been satisfactorily redressed.

References:

Doroudgar S, Hofmann C, Boileau E, Malone B, Riechert E, Gorska AA, Jakobi T, Sandmann C, Jürgensen L, Kmietczyk V, *et al* (2019) Monitoring cell-Type-specific gene expression using ribosome profiling in vivo during cardiac hemodynamic stress. *Circ Res* 125: 431–448

Gold JI, Martini JS, Hullmann J, Gao E, Chuprun JK, Lee L, Tilley DG, Rabinowitz JE, Bossuyt J, Bers DM, *et al* (2013) Nuclear Translocation of Cardiac G Protein-Coupled Receptor Kinase 5 Downstream of Select Gq-Activating Hypertrophic Ligands Is a Calmodulin-Dependent Process. *PLoS One* 8

Huang HT, Brand OM, Mathew M, Ignatiou C, Ewen EP, McCalmon SA & Naya FJ (2006) Myomaxin Is a novel transcriptional target of MEF2A that encodes a Xin-related α-

actinin-interacting protein. *J Biol Chem* 281: 39370–39379

Johnson LR, Scott MGH & Pitcher JA (2004) G Protein-Coupled Receptor Kinase 5 Contains a DNA-Binding Nuclear Localization Sequence. *Mol Cell Biol* 24: 10169–10179

Lehmann LH, Jebessa ZH, Kreusser MM, Horsch A, He T, Kronlage M, Dewenter M, Sramek V, Oehl U, Krebs-Haupenthal J, *et al* (2018) A proteolytic fragment of histone deacetylase 4 protects the heart from failure by regulating the hexosamine biosynthetic pathway. *Nat Med* 24: 62–72

Martini JS, Raake P, Vinge LE, DeGeorge BR, Chuprun JK, Harris DM, Gao E, Eckhart AD, Pitcher JA & Koch WJ (2008) Uncovering G protein-coupled receptor kinase-5 as a histone deacetylase kinase in the nucleus of cardiomyocytes (Proceedings of the National Academy of Sciences of the United States of America (2008) 105, (12457-12462) DOI: 10.1073/pnas.0803153105). *Proc Natl Acad Sci U S A* 105: 17206

Thoreen CC, Chantranupong L, Keys HR, Wang T, Gray NS & Sabatini DM (2012) A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature* 485: 109–113

1st Revision - Editorial Decision 16th Aug 2021

Dear Dr. Völkers,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees that were asked to assess it, and I am happy to say that both support the publication of your study now.

Some more minor editorial changes will be required before we can proceed with the official acceptance of your manuscript:

- Most bands in the source data are not labeled, or only the band size is labeled. Please add all labels (protein names) so that the bands in the source data can be related to the ones in the figure panels.

- Please upload 1 source data file per figure (the file can have several pages).

- "Disclosures" needs to be changed to "Conflict of Interest"

- Please add the author contributions to the manuscript file.

- All funding info also needs to be entered into our online manuscript tracking system when you upload the final manuscript.

- Fig. EV2B-E callouts are missing. Fig EV3-EV5 panel callouts are missing.
The dataset tables are not called out. There is a callout to supplementary Table 1 which should be corrected/removed. Please correct.

- The 2 Dataset files need titles/legends that can be added to the first tab of the excel file.

- Please check that all figure legends mention the number of replicates.

I attach to this email a relate manuscript file with comments by our data editors. Please address all comments in the final manuscript.

I would like to suggest some minor changes to the title and abstract that needs to be written in present tense. Please let me know whether you agree with the following:

Muscle-specific Cand2 is translationally upregulated by mTORC1 and promotes adverse cardiac remodeling

The mechanistic target of rapamycin (mTOR) promotes pathological remodeling in the heart by activating ribosomal biogenesis and mRNA translation. Inhibition of mTOR in cardiomyocytes is protective; however, a detailed role of mTOR in translational regulation of specific mRNA networks in the diseased heart is unknown. We performed cardiomyocyte genome-wide sequencing to define mTOR-dependent gene expression control at the level of mRNA translation. We identify the muscle-specific protein Cullin-associated NEDD8-dissociated protein 2 (Cand2) as a translationally upregulated gene, dependent on the activity of mTOR. Deletion of Cand2 protects the myocardium against pathological remodeling. Mechanistically, we show that Cand2 links mTOR signaling to pathological cell growth by increasing Grk5 protein expression. Our data suggest that cell-type-

specific targeting of mTOR might have therapeutic value against pathological cardiac remodeling.

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Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #2:

The authors are very responsive to my criticisms and the paper is improved significantly. I recommend acceptance of the work.

Referee #3:

The authors have addressed my previous concerns. I do not have other questions.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 10th Sep 2021

Dr. Mirko Völkers
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Dear Dr. Völkers,

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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- 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
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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.
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- 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 $\chi 2$ 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?	All experiments were repeated at least three independent times. Sample size was chosen to ensure reproducibility and allow stringent statistical analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	All experiments were repeated at least in five animals. We described sample size in each individual experiment .
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from our studies and to conduct statistical analysis.
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4.b. For animal studies, include a statement about blinding even if no blinding was done	All of the experiments were conducted in a blinded manner. Therefore the handling and experiments on the animal was blinded to experimental conditions and groups
5. For every figure, are statistical tests justified as appropriate?	Yes, we used appropiate statistical test. Data were analyzed using analysis of variance (ANOVA) or unpaired t-test as appropriate.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used statistical methods as described in the method section. Data were analyzed using analysis of variance (ANOVA) or unpaired t-test as appropriate and an alpha of 0.05. Analysis were performed using Graphpad software. P < 0.05 was considered significant .
Is there an estimate of variation within each group of data?	Data are expressed as mean ± SEM(Standard Error of the Mean)

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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
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	For the unpaired t-test, a statistical test of variance was apllied

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).	Information on antibodies is given in "Reagents table".
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Primary cells were used in this study

* 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.	Cand2 KO and wild-type littermates bred on a C57BL/6 background were generated from EUCOMM and housed according to institutional guidelines. Mice werehoused in a 12 hours light/dark cycle, in a temperature (22°C)-controlled environment with ad libitum access to food and water. All experiments were performed on 8-12 week-old male mice.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were approved by the Regierungspräsidium Karlsruhe
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.	Yes we followed the ARRIVE Guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n.a
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.	n.a
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n.a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n.a
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n.a
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.	n.a
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.	n.a

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	Raw sequencing data have been made publicly available and can be accessed. RNA-Seq: Raw data have been uploaded to GEO (accession ID: GSE153364) Ribo-Seq: Raw data have been uploaded to SRA (accession ID:SRP156230)
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).	Raw sequencing data have been made publicly available and can be accessed. RNA-Seq: Raw data have been uploaded to GEO (accession ID: GSE153364) Ribo-Seq: Raw data have been uploaded to SRA (accession ID:SRP156230)
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).	n.a
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.	n.a

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