Selective axonal translation of the mRNA isoform encoding prenylated Cdc42 supports axon growth

Seung Joon Lee, Matthew D. Zdradzinski, Pabitra K. Sahoo, Amar N. Kar, Priyanka Patel, Riki Kawaguchi, Byron J. Aguilar, Kelsey D. Lantz, Caylee R. McCain, Giovanni Coppola, Qun Lu and Jeffery L. Twiss
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First decision letter

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MS TITLE: Selective axonal translation of prenylated Cdc42 mRNA isoform supports axon growth

AUTHORS: Seung Joon Joon Lee, Amar N. Kar, Matthew D. Zdradzinski, Priyanka Patel, Pabitra K Sahoo, Riki Kawaguchi, Giovanni Coppola, Byron J. Aguilar, Kelsey D. Lantz, Caylee R. McCain, Qun Lu, and Jeffery L. Twiss
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We have now reached a decision on the above manuscript.

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

As you will see, the two reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. In particular, the underlying feeling is that the manuscript in its present form does not convey in a clear manner its mechanistic contribution and novelty to the field to fully merit publication in JCS.

However, I agree with one of the reviewers that a revised version addressing the points above and others listed in the reviewers' reports might prove acceptable. If you think that you can deal satisfactorily with these criticisms on revision, I would be pleased to see a revised manuscript. I am happy to discuss any aspects of the revision plan at your earliest convenience.

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

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

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

Reviewer 1

Advance summary and potential significance to field

In the present manuscript, Lee et al., present an extension to our knowledge of CDC42 signaling in neurons. They convincingly show that the two splice variants have are cleaved at different intra cellular locations and that this location is the main factor to introduce axonal growth in dorsal root ganglion cells of the rat.

Comments for the author

Mayor points:

Looking at the DRG neurons in culture I miss the characteristic Stem axon.
Does the interference with CDC42 stop the formation of this unique property seen in DRG neurons. Also the authors claim the palm-CDC42 is an active dendrite inducer. It would be important to see that in Multipolar neurons. Moreover, to claim the CaaX motive as a general translocator towards the axon one would need to show this in Multipolar neurons including the absents of CDC42 with this motive in dendrites.

As a second point, I would like to raise the siRNA unspecific knockdown of palm-CDC42 with the siPrenyl. Instead of overexpressing an artificial construct and knocking down the endogenous protein. To prove the point, it would be handy to use CRISPR-Cas13 REPAIR (Cox DB et al. Science 2017) this would give more specificity to the probes.

Minor points:

Statistics in Fig 1C.

The red channel in Fig 2A needs a boost Fig 2D is a little unclear it should for the bridge why DRG neurons are used in subsequent experiments.

Reviewer 2

Advance summary and potential significance to field

This study was aimed at investigating the functional importance of prenyl-Cdc42 during axon growth. It is already known since decades that Cdc42 is required for axon growth, and it is also known for many years that alternative splicing allows the formation of two Cdc42 isoforms that are subject of different posttranslational modifications. Of these, palm-Cdc42 has been linked to dendrite maturation and PSD formation, whereas former data suggested an axonal localization and function for prenyl-Cdc42. The current ms used state-of-the-art methodology by avoiding the employment of DN or activated small GTPase forms to provide compelling evidence for the axonal localization and localized translation of the Cdc42 mRNA. Their data quite clearly show that the 3Â’ UTR present in exon 7 is necessary and sufficient for axonal transport and local translation of the prenyl isoform. Whereas I appreciate these focused and carefully designed studies, I am not convinced that this analysis represents sufficient conceptual and/or mechanistic advances to merit publication in JCS in the present form.

Comments for the author

Major concerns:
1. As outlined above, previous research revealed nearly everything this paper was dealing with, except for the experimental demonstration of the role of the 3' UTR of the prenyl-Cdc42 encoding mRNA. Although this major finding is backed up by a consistent data set, this finding is anything but a surprise or a major breakthrough in the field. If this result is somewhat followed up to gain mechanistic or other insights, I would be far more convinced that this paper deserves publication in JCS.
2. My understanding of current literature on protein lipidation is that mono-lipidation, such as prenylation of Cdc42 in the CaaX motif alone, is not sufficient for stable membrane association. As compared to this prenylation followed by palmitoylation highly increases the membrane bound fraction of the proteins. In light of that knowledge I find it surprising that, according to Figure 6D-F, prenyl-Cdc42 exhibits a much stronger membrane association than palm-Cdc42 (targeted to the axon through 3' prenyl UTR). This seems to suggest that the strong peripheral growth cone membrane association of the prenyl-Cdc42 is not exclusively dependent on prenylation instead other factors also play a role, because otherwise palm-Cdc42 (that is also prenylated, in theory) should display an even stronger and more stable membrane association. This issue should be better discussed in the paper, both in page 11 where CCaX lipidation is described, and also in the Discussion of the results.

Minor comments:
1. page 5: DRG is not resolved in the text, neither here nor elsewhere
.
2. page 5: Actb is not resolved.
3. page 6: in Figure S1B the neurites are barely visible and it is impossible to see where are the dendrites and axons on these images.
4. page 7: "synthesized G" is presumably meant to be "synthesized GFP"
5. page 9: the authors report that their siPrenyl construct partly depletes the palm-Cdc42 mRNA as well. Would it be possible to design and try another construct with higher specificity for Prenyl-Cdc42 mRNA?
6. In Figure 4 legend, panel E is not correctly referred.
7. In case of Figure S3B, description and label of the statistical significance levels is quite confusing, clarity should be improved. It would be interesting to see if the effect of siPrenyl is significantly stronger than the one of pan-siCdc42 when cells are treated with ZCL278? According to the figure, this is the case, but the explanation for that remains elusive.
8. Figures 1,2,3: The method used for pixel intensity measurements does not appear entirely clear to me. In my understanding, pixel/mikrometer2
(indicated on the panels) is not equal to mean pixel intensity mentioned in the legends.
9. It is unclear from the M&M section whether normality tests were done on the data sets before the statistical analysis.

First revision

Author response to reviewers' comments

Response to Reviewers' comments: We sincerely thank the reviewers for their careful assessment of our previous submission and their constructive comments. We have made several changes to the revised manuscript that we hope the reviewers and editor will agree fully addresses the critiques and strengthen the manuscript. We outline these changes with response to each of the reviewers' comments below.

Reviewer 1:

Advance summary and potential significance to field In the present manuscript, Lee et al., present an extension to our knowledge of CDC42 signaling in neurons. They convincingly show that the two

splice variants have are cleaved at different intra cellular locations and that this location is the main factor to introduce axonal growth in dorsal root ganglion cells of the rat.

We appreciate the reviewer's comments and hope that they will find the revised manuscript makes a stronger case for this splicing mechanism as differentially affecting axonal and dendritic growth.

Major points:

1. Looking at the DRG neurons in culture I miss the characteristic Stem axon. Does the interference with CDC42 stop the formation of this unique property seen in DRG neurons?

We presume that the reviewer is referring to the 'stem axon' or pseudounipolar nature that DRG neurons exhibit in vivo. In culture the DRG neurons extend multiple axons from each cell body and it is exceptionally rare to see this type of stem axon, with only a few neurons out of thousands showing that morphology in culture. In our experience, even if a process does show that stem axon type of appearance, the soma will still have multiple other axonal processes. Please note that we and others have previously showed that all of the DRG processes in culture are axonal in nature based on microtubule polarity (Vuppalanchi et al., 2011, J Biol Chem 285:18025-38) and exclusion of MAP2 protein and mRNA from these axonal processes (Zheng et al., 2001, J Neurosci 21:9291-303). This brings advantages for isolation of axons as we have used in Figure 1B-C of the revised manuscript. We have added a sentence to the results to emphasize the axonal nature of the DRG neurites and that culture does not typically recapitulate the classic pseudo-unipolar appearance seen for these neurons in vivo.

Disrupting CDC42 has no effect on axon numbers or axon branching. It seems to only affect axon extension. We have added a sentence to the results to indicate that numbers of axons on each DRG neuron is not affected by the manipulations.

Please note that the DIV9 cortical neuron cultures show true axonal and dendritic polarity based on MAP2 and SMI312 staining. We outline effects of prenyl vs. palm-CDC42 in these neurons below. Notably pan-Cdc42 knockdown, pan-Cdc42 knockdown + rescue by Prenyl- or Palm-CDC42, or overexpression of prenyl- or palm-CDC42 had no effect on axonal and dendritic polarity. But these manipulations affect axon (prenyl-CDC42) and dendrite (palm-CDC42) length, and dendrite branching and the numbers of dendrites per cell body (prenyl-CDC42). Supranumery axons were not seen in the cortical cultures with these conditions.

2. Also the authors claim the palm-CDC42 is an active dendrite inducer. It would be important to see that in Multipolar neurons. Moreover, to claim the CaaX motive as a general translocator towards the axon one would need to show this in Multipolar neurons including the absents of CDC42 with this motive in dendrites.

We provide new data showing that the UTRs of the palm-Cdc42 mRNA are sufficient for dendritic localization, but does not localize a heterologous reporter mRNA into axons of cortical neurons. In contrast, the UTRs of the prenyl-Cdc42 mRNA are sufficient for both axonal and dendritic localization in these multipolar neurons (revised Figure 5C-D). These data are consistent with localization shown for the endogenous palm- and prenyl-Cdc42 mRNAs in these neurons (revised Figure 5A-B).

We address the functional significance of this differential localization of prenyl-Cdc42 and palm-Cdc42 in axonal and dendritic growth by depleting both Cdc42 mRNA isoforms (with 'siCdc42') from polarized cortical neuron cultures and performing rescue experiments with the GFP-prenyl-Cdc42 vs. GFP-palm-Cdc42 constructs that contained the axonally localizing 3'UTRs and unique C-termini (revised Figure 5E-F and Supplemental Figure S4B-E). These new data show that the palm-CDC42 isoform increases length of dendrites and not dendrite branching, and consistent with the studies in the DRGs, the Cdc42-prenyl isoform increases axon length but not axon branching.

Surprisingly the prenyl-CDC42 isoform increased dendrite branching and numbers of dendrites on each cell body. We are not sure what to make of this, but include these data as Supplemental Figures S4D-E. We hope that the reviewers and editor will allow us to leave resolving this issue for a future manuscript. We have added a sentence to indicate that prenyl-CDC42 isoform may have a

role in initiation of neurite growth, since these cultures are initially nucleofected with the plasmid DNA at DIV0 and then transfected with siRNAs at DIV5 (in our hands ,it typically takes 3-4 days for plasmid expression in the cortical neurons).

3. As a second point, I would like to raise the siRNA unspecific knockdown of palm-CDC42 with the siPrenyl. Instead of overexpressing an artificial construct and knocking down the endogenous protein. To prove the point, it would be handy to use CRISPR-Cas13 REPAIR (Cox DB et al. Science 2017) this would give more specificity to the probes.

We fully appreciate the reviewer's suggestion here and would love to move in this direction. We are moving that direction for some of our work, but it has not been straightforward, and efficiency is an issue. We fully agree with the reviewer's point on siPrenyl depleting palm-Cdc42 mRNA. We were frustrated with the siPrenyl and tried multiple siRNAs for the 3'UTR . After several months, we moved to the pan-siCdc42 + rescue constructs. Together with the non-specific siRNA knockdowns and new data in the cortical neurons, we hope that the reviewer and editor will agree that our data indicate axonally localized prenyl-Cdc42 mRNA supports axon growth.

Minor points:

Statistics in Fig 1C.

We have added the statistics in Figure 1C.

The red channel in Fig 2A needs a boost Fig 2D is a little unclear it should for the bridge why DRG neurons are used in subsequent experiments.

We have revised the Fig 2D (now revised Figure 1G and Supplemental Figure S1C). Additionally, we have reorganized the manuscript and hope that the use of DRGs for the bulk of the experiments is now clear.

Reviewer 2

Advance summary and potential significance to field This study was aimed at investigating the functional importance of prenyl-Cdc42 during axon growth. It is already known since decades that Cdc42 is required for axon growth, and it is also known for many years that alternative splicing allows the formation of two Cdc42 isoforms that are subject of different posttranslational modifications. Of these, palm-Cdc42 has been linked to dendrite maturation and PSD formation, whereas former data suggested an axonal localization and function for prenyl-Cdc42. The ms used state-of-the-art methodology by avoiding the employment of DN or activated small GTPase forms to provide compelling evidence for the axonal localization and localized translation of the Cdc42 mRNA. Their data quite clearly show that the 3' UTR present in exon 7 is necessary and sufficient for axonal transport and local translation of the prenyl isoform. Whereas I appreciate these focused and carefully designed studies, I am not convinced that this analysis represents sufficient conceptual and/or mechanistic advances to merit publication in JCS in the present form.

We hope that the revised manuscript will change the reviewer's mind on the lack of conceptual and mechanistic advances in this work. We fully agree that there are decades of information linking CDC42 functions to axon growth. However, the overwhelming majority of these studies did not distinguish CDC42 isoforms and only a few of the recent publications focused on the role of RNA localization. We reference these recent publications showing localization of Cdc42 mRNAs in neurites of neural cell lines and hippocampal neurons. These publications did not fully address differential functions of the localized mRNAs as we have here. Nor did they consider the roles of the post-translational modification motifs in the localized mRNAs as we have here. So, we think that our revised manuscript presents an appropriate conceptual and mechanistic advance to merit publication in JCS.

Major concerns:

1. As outlined above, previous research revealed nearly everything this paper was dealing with, except for the experimental demonstration of the role of the 3' UTR of the prenyl-Cdc42 encoding

mRNA. Although this major finding is backed up by a consistent data set, this finding is anything but a surprise or a major breakthrough in the field. If this result is somewhat followed up to gain mechanistic or other insights, I would be far more convinced that this paper deserves publication in JCS.

The prenyl-Cdc42 mRNA localizes into both axons and dendrites (shown in revised Figure 5). We now include data on CDC42 isoform function for axon vs. dendrite growth as outlined above for reviewer # 1, point # 2. Also, please do note that many of the studies on fully polarized neurons focused on much later stages of neuronal polarization and maturation than we have done here, a point that we emphasize in the Discussion.

2. My understanding of current literature on protein lipidation is that mono-lipidation, such as prenylation of Cdc42 in the CaaX motif alone, is not sufficient for stable membrane association. As compared to this, prenylation followed by palmitoylation highly increases the membrane bound fraction of the proteins. In light of that knowledge I find it surprising that, according to Figure 6D-F, prenyl-Cdc42 exhibits a much stronger membrane association than palm-Cdc42 (targeted to the axon through 3' prenyl UTR). This seems to suggest that the strong peripheral growth cone membrane association of the prenyl-Cdc42 is not exclusively dependent on prenylation, instead other factors also play a role, because otherwise palm-Cdc42 (that is also prenylated, in theory) should display an even stronger and more stable membrane association. This issue should be better discussed in the paper, both in page 11 where CCaX lipidation is described, and also in the Discussion of the results.

The reviewer is correct that additional sequences in the hypervariable regions of Rho GTPase family members and isoforms contribute to membrane localization. We now emphasize this point in the Discussion. However, also pertinent to this point is that the only difference between proteins encoded by the axonally targeted prenyl-Cdc42 and palm-Cdc42 + prenyl 3'UTR used here is in the C-terminal 10 amino acids. We remind the reader of this in the revised manuscript where effects of the mutations in only the CaaX in prenyl-Cdc42 and CCaX motifs in the coding sequence of these constructs on the subcellular localization shown in Figure 6 are discussed.

We also found it extremely surprising that when we target palm-Cdc42 mRNA into axons for local translation, the encoded protein shows much less localization to the growth cone's peripheral membrane. This was not predicted, but it is the data that derived from those experiments. We have added more discussion on this differential localization. This includes the following points: 1) palmitoylated proteins localize to cholesterol rich 'lipid raft' domains in membrane while prenylated and farnesylated proteins are excluded from these regions; 2) even for a single protein, the similarly unsaturated fatty acyl modifications of farnesylation and prenylation can result in differential subcellular localization; 3) we refer to previous work where palm-CDC42 with mutation CCaX mutated to CAaX still shows membrane motility significantly higher than prenly-CDC42 (Wirth et al., 2013); and, 4) we emphasize throughout the mansucript that we have examined effects of the motifs needed for prenylation and palmitoylation in CDC42 protein rather than the post-translational modifications.

Minor comments:

1. page 5: DRG is not resolved in the text, neither here nor elsewhere

This has been fixed.

2. page 5: Actb is not resolved.

We now refer to this as the mRNA encoding β-actin protein (Actb) – we use similar nomenclature for γ-actin's mRNA (Actg). Based on reviewer and editor recommendations from other manuscripts, this nomenclature helps to distinguish the use of the mRNA from the protein for the readers.

3. page 6: in Figure S1B the neurites are barely visible and it is impossible to see where are the dendrites and axons on these images.

We have revised these images.

4. page 7: “synthesized G” is presumably meant to be “synthesized GFP”

Thank you for catching this. This has been fixed.

5. page 9: the authors report that their siPrenyl construct partly depletes the palm-Cdc42 mRNA as well. Would it be possible to design and try another construct with higher specificity for Prenyl-Cdc42 mRNA?

We have gone through many siRNAs to get specific depletion. Note that we are strictly limited to the 3’UTR for this, and unfortunately we have not found one that behaved better. After many months ordering new siRNAs (and funds depleted), we moved to the pan-siCdc42 approach with rescue constructs. We hope the reviewer will agree with this approach and consider that the sum of our results with the knockdown, knockdown + rescue constructs, and pharmacological manipulations are conclusive.

6. In Figure 4 legend, panel E is not correctly referred.

We have fixed this.

7. In case of Figure S3B, description and label of the statistical significance levels is quite confusing, clarity should be improved. It would be interesting to see if the effect of siPrenyl is significantly stronger than the one of pan-siCdc42 when cells are treated with ZCL278? According to the figure, this is the case, but the explanation for that remains elusive.

With the different conditions used here, we were trying to balance a readable/clear figure while representing the most pertinent statistical comparisons. Nonetheless, we have attempted to clarify the statistics better in the figure as suggested by the reviewer.

The siPrenyl + ZCL278 is not significantly different from the pan-siCdc42 + ZCL278.

8. Figures 1,2,3: The method used for pixel intensity measurements does not appear entirely clear to me. In my understanding, pixel/mikrometer2 (indicated on the panels) is not equal to mean pixel intensity mentioned in the legends.

We have restricted the terms to pixels/µm2 to avoid confusion.

9. It is unclear from the M&M section whether normality tests were done on the data sets before the statistical analysis.

Thank you for the suggestion. We have run normality tests on all of the data presented. As I think anticipated by the reviewer, the growth measurements do not show a normal distribution. This is not surprising as we have a mix of different neuron types (nociceptive, mechanoceptive, and proprioceptive neurons at a nominal level) and perform the analyses across multiple culture preparations. We have revised our statistical tests to take this into account and noted this in the figure legends and methods.

Second decision letter

MS ID#: JOCES/2020/251967

MS TITLE: Selective axonal translation of prenylated Cdc42 mRNA isoform supports axon growth

AUTHORS: Seung Joon Lee, Matthew D Zdradzinski, Pabitra K Sahoo, Amar N Kar, Priyanka Patel, Riki Kawaguchi, Byron J Aguilar, Kelsey D Lantz, Caylee R McCain, Giovanni Coppola, Qun Lu, and Jeffery L Twiss
ARTICLE TYPE: Research Article

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