Life Science Alliance

Rab40c Regulates Focal Adhesions and PP6 Activity by Controlling ANKRD28 Ubiquitylation

Ke-Jun Han, Valeryia Mikalayeva, Scott Gerber, Arminja Kettenbach, Vytenis Skeberdis, and Rytis Prekeris
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January 14, 2022

Re: Life Science Alliance manuscript #LSA-2021-01346-T

Prof. Rytis Prekeris
UC Denver
Cell and Developmental Biology
12801 E. 17th Ave.
Bldg. RC1, Room L18-12402
Aurora, CO 80045

Dear Dr. Prekeris,

Thank you for submitting your manuscript entitled "Rab40c Regulates Focal Adhesions and PP6 by Controlling ANKRD28 Ubiquitylation and Degradation" to Life Science Alliance. The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revised manuscript addressing the Reviewer comments.

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When submitting the revision, please include a letter addressing the reviewers' comments point by point.

We hope that the comments below will prove constructive as your work progresses.

Thank you for this interesting contribution to Life Science Alliance. We are looking forward to receiving your revised manuscript.

Sincerely,

Eric Sawey, PhD
Executive Editor
Life Science Alliance
http://www.lsajournal.org

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

In this study Han et al. show that the GTPase Rab40c interacts with the cullin5 complex to form an active E3 ubiquitin ligase. This complex leads to the ubiquitination and degradation of the PP6 complex subunit ANKRD28. Degradation of ANKRD28 affects the distribution of paxillin, which is part of focal adhesions that promote the migration of cells along the ECM. Together this illustrates that PP6 activity is required for proper focal adhesion distribution at the leading edge of lamellipodia on migrating cells. Migration is thus altered by the recruitment of the Rab40/CRL5 complex to focal adhesions. This is a very well executed and controlled set of experiments that support the conclusions. Importantly, this advances the field in a significant manner and will be of broad interest to the audience. That being said, there are some points that should be addressed prior to publication.

In Figure 4G-H the authors show that ANKRD28 is ubiquitinated in a Rab40c dependent manner. Interestingly, they also show that the Ub linkage is at K63. This is consistent with the lack of an effect by the proteosome inhibitor MG132 as well as the block in degradation by the H+-ATPase inhibitor Bafilomycin. Together this shows that ANKRD28 degradation occurs in the lysosome. K-63 Ub labeling has been shown in autophagy as well as ESCRT dependent MVB sorting. Can the authors address these pathways, and which is likely/known to occur in this instance? To verify that ANKRD28 is transported to lysosomes it would be beneficial to see that there is colocalization with lysosomal markers.

How is Rab40c activity/binding to the Cullin5 complex dependent on its GTP binding or GTPase activity?

Is the Rab40c associated with Cullin5 inserted into a membrane? Could it be that Golgi Rab40c is trafficked to the plasma membrane to target focal adhesions?

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

In this manuscript Han and co-workers have studied the role of the Rab40c isoform of the Rab40 subfamily in regulation of focal adhesion dynamics. They made the interesting discovery that Rab40c assembles with Cullin 5 into an E3 ubiquitin ligase complex that selectively binds and ubiquitinates targets for lysosomal degradation, such as several components of the PP6 protein phosphatase complex that are involved in FA regulation. Following selective Rab40c knock outs FAs are bigger, more numerous and lose their polarity towards the lamellipodia. Loss of Rab40c leads to stabilization of targets such as ANKRD28 (but possibly also others within the Rab40 interactome that they identified), which can be rescued by expression of Rab40c but not by a Rab40c variant that cannot interact with its substrates. They have characterized the binding requirements for Rab40c to ANKRD28 and PP6R1, and the data suggest that they can bind independently to Rab40c. From this the hypothesis arises that Rab40c regulation of FAs acts via degradation of (parts of) the PP6 complex, which alters phosporylation of various targets associated with FA dynamics. Indeed, loss of ANKRD28 in many respects has the opposite phenotype as Rab40c KO and can actually reverse the latter. This correlates with effects on phosphorylation status and stability of components of the Hippo signaling pathway and with changes in transcriptional regulation by the YAP/TAZ complex.
The paper is well written and presents a comprehensive body of work with interesting findings that futher our understanding of how Rab40c acts on the formation of FAs and on stability and function of the PP6 phosphatase complex.

Major comments

Functional assays of FA dynamics are limited to image quantification of FA localization, number and morphology, but do not assess effects on actual adhesive and migratory phenotype. Experiments aimed at determining changes in migration and adhesive behavior dependent on the Rab40c - PP6 axis would further strengthen this paper.

Although a lot of data is presented that details the molecular composition of the Rab40c regulatory complex and the signaling hierarchy that it controls, the main weakness of this study is that it remains unclear exactly where all these regulatory events take place. Where does Rab40c label its targets for degradation ? In the lamellipodia, on the FA, somewhere in the cytosol, or on the Golgi? Figure 5 panels A and B show ectopically expressed FLAG-tagged ANKRD28 and PP6R1 somewhere in

lamellipodia. Is this representative for endogenous ANKRD28 and PP6R1? Are they part of the same structure and do they colocalize with (endogenous) Rab40c in lamellipodia subdomains? Figure 1 shows GFP-Rab40C also somewhere in lamellipodia, but it is not clear if this overlaps with its targets and again it is unclear to what extent this is representative of endogenous Rab40c. Endogenous localization of the players involved (Rab40c, PP6 components) would be very helpful for a better appreciation of what goes on, and how this changes in the absence of key components of this pathway as identified in this study.

Minor comments

In the proteomic survey of the Rab40c interactome a number of proteins were pulled down from the MDA-MB-231 cell lysates. Confirmation of these interactions using CO-IPs was performed in 293 cells, which seems less relevant and odd, considering MDA-MB-231 can also be used.

What's the level of ANKRD28 depletion throughout their experiments ? Can this be quantified?

I can't help to think that a lot of last minute rearrangement of panels and figures has taken place before submission, without updating the manuscript text accordingly. Some (but possibly not all) examples are: Figure 1B not mentioned in text, but referred to as 1C, 1D should be 1C, 1E-F should be 1D-E, Figure 4H incorrect (panel in figure, but no mention in text), Figure 5H incorrect (mentioned in text but no panel in H in the figure), Supplemental Figure 4 incorrect (mentioned in text, not actually present), two supplemental figures labeled Figure S3 in the supplement, mention of supplemental figure 2A in the IP of endogenous Rab40c from 293 cells makes little sense. The authors are advised to carefully proof read their article before submission, it would have saved me a lot of searching through the documents for figure panels that were mislabeled or non-existing.

We are very grateful to all reviewers for very constructive comments and suggestions. We incorporated vast majority of them, and I do believe that these changes significantly improved the manuscript. For the point-by-point changes see below.

Reviewer #1:

In this study Han et al. show that the GTPase Rab40c interacts with the cullin5 complex to form an active E3 ubiquitin ligase. This complex leads to the ubiquitination and degradation of the PP6 complex subunit ANKRD28. Degradation of ANKRD28 affects the distribution of paxillin, which is part of focal adhesions that promote the migration of cells along the ECM. Together this illustrates that PP6 activity is required for proper focal adhesion distribution at the leading edge of lamellipodia on migrating cells. Migration is thus altered by the recruitment of the Rab40/CRL5 complex to focal adhesions. This is a very well executed and controlled set of experiments that support the conclusions. Importantly, this advances the field in a significant manner and will be of broad interest to the audience. That being said, there are some points that should be addressed prior to publication.

1) In Figure 4G-H the authors show that ANKRD28 is ubiquitinated in a Rab40c dependent manner. Interestingly, they also show that the Ub linkage is at K63. This is consistent with the lack of an effect by the proteosome inhibitor MG132 as well as the block in degradation by the H+-ATPase inhibitor Bafilomycin. Together this shows that ANKRD28 degradation occurs in the lysosome. K-63 Ub labeling has been shown in autophagy as well as ESCRT dependent MVB sorting. Can the authors address these pathways, and which is likely/known to occur in this instance? To verify that ANKRD28 is transported to lysosomes it would be beneficial to see that there is colocalization with lysosomal markers.

As suggested, to confirm that ANKRD28 can be degraded by lysosomes, we tested whether ANKRD28 is present in CD63-positive (lysosomal marker) organelles. Since proteins targeted for lysosomal degradation are usually hard to detect we treated cells with Bafilomycin (inhibitor of lysosomal degradation). Our new data shows (see Figure 6C) FLAG-ANKRD28 puncta can be observed in lumen of CD63-positive organelles, which would be consistent with our model. It is more difficult to answer whether ANKRD28 is targeted to lysosomes via autophagy or via ESCRT-dependent MVB sorting since many inhibitors and lysosomal markers label both pathways. Since ABKRD28 is cytosolic protein, I suspect that autophagy is involved, but further studies will be needed to determine that. We added this discussion to the manuscript.

2) How is Rab40c activity/binding to the Cullin5 complex dependent on its GTP binding or GTPase activity?

As suggested we completed new binding experiments and now show that GTP-Rab40c binding to ANKRD28 is enhanced by GTP. This new data is now included in Supplemental Figure 2D.

3) Is the Rab40c associated with Cullin5 inserted into a membrane? Could it be that Golgi Rab40c is trafficked to the plasma membrane to target focal adhesions?

Since all Rab40-subfamily proteins binds to Cullin5 in GTP independent fashion (this can be associated with GDP-Rab40c and GTP-Rab40c) it is likely that it can associate with membrane bound Rab40c. It is also quite possible that Golgi Rab40c is trafficked to FAs from Golgi. That is actually how Rab18 (closes paralogue of Rab40 subfamily) functions. However, there is no easy way to answer these questions. We tried to address that by co-staining with Cullin5, but majority of Cullin 5 is cytosolic (since it binds to many other proteins in addition to Rab40c). Our previous work (PMID: 33999101) have shown that mutation of Cullin5 bidnign site within Rab40 proteins does not have any effect on Rab40b or Rab40c localization. That all suggest that Rab40c cycling and Rab40c binding to Cullin5 are regulated differentially. We are very interested expoling this, but that is outside the scope of this mansucipt.

Reviewer #2:

In this manuscript Han and co-workers have studied the role of the Rab40c isoform of the Rab40 subfamily in regulation of focal adhesion dynamics. They made the interesting discovery that Rab40c assembles with Cullin 5 into an E3 ubiquitin ligase complex that selectively binds and ubiquitinates targets for lysosomal degradation, such as several components of the PP6 protein phosphatase complex that are involved in FA regulation. Following selective Rab40c knock outs FAs are bigger, more numerous and lose their polarity towards the lamellipodia. Loss of Rab40c leads to stabilization of targets such as ANKRD28 (but possibly also others within the Rab40 interactome that they identified), which can be rescued by expression of Rab40c but not by a Rab40c variant that cannot interact with its substrates. They have characterized the binding requirements for Rab40c to ANKRD28 and PP6R1, and the data suggest that they can bind independently to Rab40c. From this the hypothesis arises that Rab40c regulation of FAs acts via degradation of (parts of) the PP6 complex, which alters phosporylation of various targets associated with FA dynamics. Indeed, loss of ANKRD28 in many respects has the opposite phenotype as Rab40c KO and can actually reverse the latter. This correlates with effects on phosphorylation status and stability of components of the Hippo signaling pathway and with changes in transcriptional regulation by the YAP/TAZ complex. The paper is well written and presents a comprehensive body of work with interesting findings that futher our understanding of how Rab40c acts on the formation of FAs and on stability and function of the PP6 phosphatase complex.

Major comments

1) Functional assays of FA dynamics are limited to image quantification of FA localization, number and morphology, but do not assess effects on actual adhesive and migratory phenotype. Experiments aimed at determining changes in migration and adhesive behavior dependent on the Rab40c - PP6 axis would further strengthen this paper.

As suggested, we added cell adhesion analysis (now shown in new Figure 2). New data is fully consistent with our model and shows that Rab40c-KO has enhanced substrate adhesion.

2) Although a lot of data is presented that details the molecular composition of the Rab40c regulatory complex and the signaling hierarchy that it controls, the main weakness of this study is that it remains unclear exactly where all these regulatory events take place. Where does Rab40c label its targets for degradation? In the lamellipodia, on the FA, somewhere in the cytosol, or on the Golgi?

Figure 5 panels A and B show ectopically expressed FLAG-tagged ANKRD28 and PP6R1 somewhere in lamellipodia. Is this representative for endogenous ANKRD28 and PP6R1? Are they part of the same structure and do they colocalize with (endogenous) Rab40c in lamellipodia subdomains? Figure 1 shows GFP-Rab40C also somewhere in lamellipodia, but it is not clear if this overlaps with its targets and again it is unclear to what extent this is representative of endogenous Rab40c. Endogenous localization of the players involved (Rab40c, PP6 components) would be very helpful for a better appreciation of what goes on, and how this changes in the absence of key components of this pathway as identified in this study.

We fully agree with the reviewer that those are very interesting questions that we want to explore in the future but is outside the scope of this manuscript. Part of the issue is the fact that there are no good antibodies for ANKRD28, PP6R1 and Rab40c that work for microscopy. Furthermore, Rab40a, Rab40b and Rab40c are very closely related and we could not generate (despite numerous tries) antibodies that specifically recognize Rab40c by microscopy. At this point the only way to investigate dynamics of endogenous Rab40c is to generate knock-in GFP tags. That, however, is significant undertaking and outside the scope of this study.

Minor comments

1) In the proteomic survey of the Rab40c interactome a number of proteins were pulled down from the MDA-MB-231 cell lysates. Confirmation of these interactions using CO-IPs was performed in 293 cells, which seems less relevant and odd, considering MDA-MB-231 can also be used.

Our apologies for a mix up but immunoprecipitation experiments in Figure 4B and C were actually done using MDA-MB-231 cells. Experiments in Figure 4D-E were indeed done using 293T cells. The main reason for this is that MDA-MB-231 are difficult to transiently transfect. Consequently, to map PP6 interaction with rab40c we have used 293T cells.

2) What's the level of ANKRD28 depletion throughout their experiments? Can this be quantified?

As suggested, we added quantification of the level of ANKRD28 depletion (see Figure S3C).

3) I can't help to think that a lot of last minute rearrangement of panels and figures has taken place before submission, without updating the manuscript text accordingly. Some (but possibly not all) examples are: Figure 1B not mentioned in text, but referred to as 1C, 1D should be 1C, 1E-F should be 1D-E, Figure 4H incorrect (panel in figure, but no mention in text), Figure 5H incorrect (mentioned in text but no panel in H in the figure), Supplemental Figure 4 incorrect (mentioned in text, not actually present), two supplemental figures labeled Figure S3 in the supplement, mention of supplemental figure 2A in the IP of endogenous Rab40c from 293 cells makes little sense. The authors are advised to carefully proof read their article before submission, it would have saved me a lot of searching through the documents for figure panels that were mislabeled or non-existing.

Our apologies for the mistakes. We have proof-read the manuscript very carefully to fix all the miss-labelled figures.

April 22, 2022

RE: Life Science Alliance Manuscript #LSA-2021-01346-TR

Prof. Rytis Prekeris
University of Colorado Anschutz Medical Campus
Cell and Developmental Biology
12801 E. 17th Ave.
Bldg. RC1, Room L18-12402
Aurora, CO 80045

Dear Dr. Prekeris,

Thank you for submitting your revised manuscript entitled "Rab40c Regulates Protein Phosphatase 6 Activity by Controlling ANKRD28 Ubiquitylation". We would be happy to publish your paper in Life Science Alliance pending final revisions necessary to meet our formatting guidelines.

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Sincerely,

Eric Sawey, PhD
Executive Editor
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Reviewer #1 (Comments to the Authors (Required)):

This is a resubmitted paper and the authors have satisfactorily addressed my critiques. As such, I believe that this paper is ready for publication.

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

In their revised submission, Han and colleagues have added new experiments to address some of the points that I have raised.The authors have adequately responded to my comments.

April 26, 2022

RE: Life Science Alliance Manuscript #LSA-2021-01346-TRR

Prof. Rytis Prekeris
University of Colorado Anschutz Medical Campus
Cell and Developmental Biology
12801 E. 17th Ave.
Bldg. RC1, Room L18-12402
Aurora, CO 80045

Dear Dr. Prekeris,

Thank you for submitting your Research Article entitled "Rab40c Regulates Focal Adhesions and PP6 Activity by Controlling ANKRD28 Ubiquitylation". It is a pleasure to let you know that your manuscript is now accepted for publication in Life Science Alliance. Congratulations on this interesting work.

The final published version of your manuscript will be deposited by us to PubMed Central upon online publication.

Your manuscript will now progress through copyediting and proofing. It is journal policy that authors provide original data upon request.

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Again, congratulations on a very nice paper. I hope you found the review process to be constructive and are pleased with how the manuscript was handled editorially. We look forward to future exciting submissions from your lab.

Sincerely,

Eric Sawey, PhD
Executive Editor
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