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Constitutive regulation of mitochondrial morphology by Aurora A kinase depends on a predicted cryptic targeting sequence at the N-terminus

Rhys Grant, Ahmed Abdelbaki, Alessia Bertoldi, Maria P. Gavilan, Jörg Mansfeld, David M. Glover and Catherine Lindon

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Original submission:	22 December 2017
1st revised submission:	8 April 2018
2nd revised submission:	17 May 2018
Final acceptance:	18 May 2018

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSOB-17-0272.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

Are each of the following suitable for general readers?

a) **Title**
Yes

b) **Summary**
Yes

c) **Introduction**
Yes

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Is it clear how to make all supporting data available?
No

Is the supplementary material necessary; and if so is it adequate and clear?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
Grant et al. investigate a novel role for Aurora-A kinase in the maintenance of mitochondrial morphology in interphase. For obvious reasons, the focus of research on AurA has so far been on its mitotic role and I think there will be wide interest in this work since it suggests that AurA has other functions. If this is correct it has implications for cancer since AurA is frequently over expressed in cancer cells and for its treatment since AurA inhibition as a cancer treatment is currently being trialed in the clinic.

I was happy to review this paper since I'd already read it on bioRxiv. Thanks to the authors for making it available ahead of publication. I found this paper to be very well written, the experimental data are of a high standard and the discussion of results is honest. My criticisms:

1. Results p.7: "Conversely, we found that tetracycline-induced overexpression of an AURKA-Venus transgene in RPE-1 cells resulted in increased fragmentation of the mitochondrial network (Figure 2D,E)." Looking at the figure it looks like there is no change. What do the stats say (see next point)? I see two reasons why the authors detect no fragmentation of mitochondria in RPE-1 with induced AurA OE: a) they just don't go any shorter in RPE-1 cells, b) in this series of experiments the cells were behaving differently (note that the distribution is shifted to longer mitochondria lengths when compared with other experiments in this figure. Looking at Fig S3 it seems like OE of AurA does fragment the mitochondria. It would be nice to show that the change can be induced the other way by OE, so it would be worth repeating this experiment to clarify this result

2. Some clarity over the stats and presentation would be good. The authors show the results of manual measurements (30 mitos/cell, 20 cells from 3 expts) as smoothed PDFs (using a kernel density estimation method). This is OK but makes me wonder why smoothing is applied at all. Second point is that a Mann-Whitney test is done and low p values are reported. As I understand it these tests are done on the outputs from MicroP and not the manual measurements. My guess is that the authors did not use the actual N value of 3 to calculate the p value from the MW test. The effects here are clear (even if not what the authors want, see point above) but the stats need to be valid and done properly. My suggestion here is that the authors should show the (raw) measurements as cumulative histograms and compare each pair of distributions using a Kolmogorov-Smirnov test.

3. The experiments in Fig 3 are really important to show a role for AurA at the mitochondria. It is

pretty convincing. However, there's a lot of AurA signal which is clearly not on the mitochondria. My worry is that the dots that are coincident with the mitochondrial network are not specifically targeted there. In a live cell movie, do the authors see the spots wobbling around stuck to the mitotracker-stained mitochondria? This would be good evidence that AurA is specifically targeted to mitochondria and might also reveal if it is indeed preferentially found at network branch points.

4. The authors say "cloning details are available on request". Really? Cloning details? This is important for anyone wanting to reproduce the work. Just add them to the paper and be done with it.

Review form: Reviewer 2

Recommendation
Major revision is needed (please make suggestions in comments)

Are each of the following suitable for general readers?

a) **Title**
Yes

b) **Summary**
Yes

c) **Introduction**
Yes

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
Yes

Is it clear how to make all supporting data available?
Yes

Is the supplementary material necessary; and if so is it adequate and clear?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
See attached file

Decision letter (RSOB-17-0272.R0)

18-Jan-2018

Dear Dr Lindon,

We are writing to inform you that the Editor has reached a decision on your manuscript RSOB-17-0272 entitled "Regulation of mitochondrial dynamics by Aurora A kinase", submitted to Open Biology.

As you will see from the reviewers' comments below, there are a number of criticisms that prevent us from accepting your manuscript at this stage. The reviewers suggest, however, that a revised version could be acceptable, if you are able to address their concerns. If you think that you can deal satisfactorily with the reviewer's suggestions, we would be pleased to consider a revised manuscript.

The revision will be re-reviewed, where possible, by the original referees. As such, please submit the revised version of your manuscript within six weeks. If you do not think you will be able to meet this date please let us know immediately.

To revise your manuscript, log into https://mc.manuscriptcentral.com/rsob and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, please revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, please respond to the comments made by the referee(s) and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referee(s).

Please see our detailed instructions for revision requirements
https://royalsociety.org/journals/authors/author-guidelines/

Once again, thank you for submitting your manuscript to Open Biology, we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,

The Open Biology Team
mailto: openbiology@royalsociety.org

Reviewer(s)' Comments to Author(s):

Referee: 1

Comments to the Author(s)
Grant et al. investigate a novel role for Aurora-A kinase in the maintenance of mitochondrial morphology in interphase. For obvious reasons, the focus of research on AurA has so far been on

its mitotic role and I think there will be wide interest in this work since it suggests that AurA has other functions. If this is correct it has implications for cancer since AurA is frequently over expressed in cancer cells and for its treatment since AurA inhibition as a cancer treatment is currently being trialed in the clinic.

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4. The authors say "cloning details are available on request". Really? Cloning details? This is important for anyone wanting to reproduce the work. Just add them to the paper and be done with it.

Referee: 2

Comments to the Author(s)
See attached file

Author's Response to Decision Letter for (RSOB-17-0272.R0)

See Appendix A.

RSOB-17-0272.R1 (Revision)

Review form: Reviewer 3

Recommendation
Accept with minor revision (please list in comments)

Are each of the following suitable for general readers?

a) Title
Yes

b) Summary
Yes

c) Introduction
Yes

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Is it clear how to make all supporting data available?
Yes

Is the supplementary material necessary; and if so is it adequate and clear?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This manuscript by Grant et al investigates the relationship between AURKA and the dynamics of mitochondrial network. The authors demonstrated that a fraction of cytoplasmic AURKA localizes to mitochondria and interacts with mitochondrial protein TOMM20, and they also identified a mitochondrial targeting sequence (MTS) in N-terminal domain of AURKA.
A better understanding of the control of AURKA activity beyond mitosis and the role of AURKA in interphase cells are important in optimizing the efficacy and interpreting potential downstream consequences of AURKA inhibitors in the clinic.
Current manuscript is a revised version after the first round of the review process. In my opinion the authors have addressed the questions raised by the reviewers.

Minor comments:
1) please check the reference to Figure 4C on page 4, it seems there is a mistake in this reference and Figure 4 can't be reference prior to Figure 3. "In further experiments we found that transient overexpression of AURKA-Venus in RPE-1 cells or in another
untransformed line, breast epithelial MCF10A cells, also led to increased fragmentation of themitochondrial network in cells overexpressing AURKA (Figure 4C, Supplementary Figure S2C)."

2) Please check the reference to Figure 3C on page 6, it seems to be a mistake and Figure 4C should be referenced instead. - "Moreover, AURKA Δ31 overexpression did not cause fragmentation of mitochondria (Figure 3C)".

Decision letter (RSOB-17-0272.R1)

15-May-2018

Dear Dr Lindon

We are pleased to inform you that your manuscript RSOB-17-0272.R1 entitled "Constitutive regulation of mitochondrial morphology by Aurora A kinase depends on a predicted cryptic targeting sequence at the N-terminus" has been accepted by the Editor for publication in Open Biology. The reviewer(s) have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, we invite you to respond to the reviewer(s)' comments and revise your manuscript.

Please submit the revised version of your manuscript within 14 days. If you do not think you will be able to meet this date please let us know immediately and we can extend this deadline for you.

To revise your manuscript, log into https://mc.manuscriptcentral.com/rsob and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, please revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referee(s) and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referee(s).

Please see our detailed instructions for revision requirements
https://royalsociety.org/journals/authors/author-guidelines/.

Before uploading your revised files please make sure that you have:

1) A text file of the manuscript (doc, txt, rtf or tex), including the references, tables (including

captions) and figure captions. Please remove any tracked changes from the text before submission. PDF files are not an accepted format for the "Main Document".

2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. Please note that PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file from the main text and meet our ESM criteria (see http://royalsocietypublishing.org/instructions-authors#question5). All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rsob.2016[last 4 digits of e.g. 10.1098/rsob.20160049].

4) A media summary: a short non-technical summary (up to 100 words) of the key findings/importance of your manuscript. Please try to write in simple English, avoid jargon, explain the importance of the topic, outline the main implications and describe why this topic is newsworthy.

Images
We require suitable relevant images to appear alongside published articles. Do you have an image we could use? Images should have a resolution of at least 300 dpi, if possible.

Data-Sharing
It is a condition of publication that data supporting your paper are made available. Data should be made available either in the electronic supplementary material or through an appropriate repository. Details of how to access data should be included in your paper. Please see http://royalsocietypublishing.org/site/authors/policy.xhtml#question6 for more details.

Data accessibility section
To ensure archived data are available to readers, authors should include a ‘data accessibility’ section immediately after the acknowledgements section. This should list the database and accession number for all data from the article that has been made publicly available, for instance:
- DNA sequences: Genbank accessions F234391-F234402
- Phylogenetic data: TreeBASE accession number S9123
- Final DNA sequence assembly uploaded as online supplemental material
- Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311

Once again, thank you for submitting your manuscript to Open Biology, we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,

The Open Biology Team
mailto:openbiology@royalsociety.org

Reviewer(s)' Comments to Author:

Referee: 3

Comments to the Author(s)
This manuscript by Grant et al investigates the relationship between AURKA and the dynamics of mitochondrial network. The authors demonstrated that a fraction of cytoplasmic AURKA localizes to mitochondria and interacts with mitochondrial protein TOMM20, and they also identified a mitochondrial targeting sequence (MTS) in N-terminal domain of AURKA.
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Current manuscript is a revised version after the first round of the review process. In my opinion the authors have addressed the questions raised by the reviewers.

Minor comments:
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Author's Response to Decision Letter for (RSOB-17-0272.R1)

See Appendix B.

Decision letter (RSOB-17-0272.R2)

18-May-2018

Dear Dr Lindon

We are pleased to inform you that your manuscript entitled "Constitutive regulation of mitochondrial morphology by Aurora A kinase depends on a predicted cryptic targeting sequence at the N-terminus" has been accepted by the Editor for publication in Open Biology.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it within the next 10 working days. Please let us know if you are likely to be away from e-mail contact during this time.

Thank you for your fine contribution. On behalf of the Editors of Open Biology, we look forward to your continued contributions to the journal.

Sincerely,

The Open Biology Team
mailto: openbiology@royalsociety.org

Appendix A

Response to Referees.

Referee 1

1. We agree with the referee that this result – increased fragmentation of mitochondrial network upon induction of a stable AURKA-Venus transgene in RPE cells - did not look a strong result, however it is statistically significant (see point 2). We agree with the suggestion that RPE mitochondria are rather resistant to shortening. Higher level of overexpression of AURKA after transient transfection has a much clearer effect in shortening mitochondria (data now in Fig 3E and Fig S4A&B, with quantifications) and we have also tested the result in another untransformed cell line, MCF10A (Fig S2C), in response to Referee 2.
2. We acknowledge the commentary on suitable presentation and statistical tests for mitochondrial size data and thank Referee 1 for his suggestion of the K-S test, which we had not previously used. We have now reanalysed our data using the K-S test, with D- and p-values logged in all figure legends. The K-S test showed increased confidence in most of our results (compared to the MW test applied previously) EXCEPT for the result in Fig2E (previously Fig 2C), which Referee 1 had spotted as less convincing. However the *p*-value for this distribution still crosses the threshold for significance (see point 1).

   We chose kernel density plots to present our data because they are easier to look at than histograms of the same data, especially where comparing two conditions. We have added two new panels to Figure S2 to support our chosen methods: Fig S2A shows that the kernel density plot for the data in Fig 1A maps exactly onto a histogram plot of the same data. Fig S2B shows the cumulative density plots of the same data, to which the Kolmogorov-Smirnov (K-S) test is applied for statistical significance.
3. We agree that the large number of dots seen in AURKA IF are a worry in terms of establishing specific localization with mitochondria, and we spent a lot of time convincing ourselves of this result. Unfortunately it is not possible to assess colocalization through time as Referee 1 suggests, since our timelapse movies do not offer sufficient temporal resolution. However, we have included in FigS3 images that we obtained on an OMX, that show examples of AURKA-Venus dots localized to mitochondria at increased resolution. We also checked the statistical performance of random patterns of dots against our AURKA dots to confirm significant co-localization (see also response to Referee 2 point 3) – data not included in manuscript, but we would be happy to send for inspection to Referees.
4. We have added subcloning details in Materials & Methods.

Referee 2

1. We have included more information to validate use of PCNA as a marker for cell cycle phases. We have added a citation of the method [Zerjatke, T. et al. Quantitative Cell Cycle Analysis Based on an Endogenous All-in-One Reporter for Cell Tracking and Classification. Cell Rep 19, 1953–1966 (2017)] and have added to Fig 1B the cell cycle distribution stats which show that our scoring with PCNA gives the expected G1:S:G2 phases in the population. We chose this method in order to avoid

synchronisation protocols (suggested by Referee 2), since all methods we know of involve use of reagents eliciting cellular stress. We agree that Fig 1C was not clear and have reassembled it from raw image files to improve the quality of the figure panel as requested.

2. Indeed we agree with these comments from Referee 2. We have written up our findings as a 'short report' precisely because we felt the claims we could associate to this work are rather modest, and we carefully avoid making the 'global' claim about differential expression of AURKA and mitochondrial morphologies in cancers. The amount of work involved in a systematic study (to support a global claim) would warrant a much bigger publication. However, in response to this Referee's concerns we have repeated our core finding – that raising AURKA levels leads to shortening of mitochondria and inhibition of AURKA activity has the opposite effect – in another pair of cell lines showing opposite mitochondrial morphology (non-transformed MCF10A and HCC1143 breast cancer cells). These additional data are in Fig S2C,D.

   We are pursuing the 'global' question as a two year collaboration with other labs. This future work will include figuring out an assay sensitive enough to answer Referee 2's question about activity: Given it is the activity of AURKA at the mitochondria that is responsible for the effect on morphology, we would indeed want to detect activity of this subpopulation of the kinase. Existing tools are not sensitive enough, and we plan to use new FRET-based tools in development.

3. We have looked carefully at Fig S3A (previously Fig S1C), and although we understand the Referee's concern that there could be mutually exclusive staining of the AURKA 'dots' (in green) with the mitochondrial staining (purple), we are confident that our interpretation is safe. We have added arrows to indicate colocalization. Notably, in the knockdown image, where cytoplasmic staining is much reduced, the remaining signal appears more likely to colocalise to mitochondria, which we think argues against the interpretation of 'mutually exclusive staining' in the control image. We have modified the figure legend to clarify our interpretation.

   In a study to compare the colocalization of mitochondria with AURKA 'dots', versus random fields of 'dots' generated with 100nm fluoresent beads, we found highly significant differences in Pearson's Correlation Coefficients, in favour of the idea that the AURKA staining pattern contains a component specific to mitochondria.

   Finally, we generated higher resolution images on an OMX system that appear to confirm colocalization of AURKA dots with mitochondria, and have included this data in Fig 3.

4. Figure S3F (previously S3D) demonstrates the successful endogenous tagging of AURKA (ie it behaves as predicted through the cell cycle). We have reduced the number of images in this panel and explained in more detail in the figure legend.

5. To address Referee 2 comments on Fig3: Aurora A overexpression in our hands rarely leads to mitotic abnormalities, and certainly never to arrest in cytokinesis. If anything, it promotes cytokinesis (as might be expected from the recent work of Vernos and Prigent labs showing AURKA-dependent phosphorylation of p150 is required for anaphase). We have removed the previous Fig 3C quantification of AURKA-Venus overexpression, which was not an important result, to make way for new data in this figure (now Fig 3D). The previous Fig 3D showed fixed cells from the endogenously tagged AURKA-Venus line shown in Fig S3. We can't explain the bright dots, but they are not

centrosomal abnormalities. We had chosen this image because it showed a particularly nice example of colocalization of the endogenous-tagged AURKA with mitochondrial staining. We have chosen a different image (now Fig 3C) that we hope is more acceptable.

6. Thanks to Referee 2 for pointing out the error in this panel. We had intended to remove lanes 4 and 8, which show cells transfected with a mutated version of AURKA (a non-degradable version that we were testing for altered localization to the mitochondria). In the interests of telling a simple story we had decided not to add this negative result to the story. We have now removed these lanes in the revised version of Figure 3.
7. We based our study on experiments carried out in two different cell lines. This was a necessary choice: For experiments to examine the consequences of reducing AURKA levels, we looked at U2OS cells, where AURKA levels are higher than RPE. For experiments to analyse the effect of different versions on AURKA on mitochondrial morphology, we chose RPE cells, since the mitochondria are already rather fragmented in U2OS cells. In the same experiments we analysed the mitochondrial morphology and examined the localization. The result of this choice does mean therefore that the colocalization data are mostly from RPE cells. To address this criticism we have carried out a new experiment to show enhanced colocalization of AURKA-Δ8-Venus with mitochondria in U2OS cells (Fig S4D).
8. We have re-done all of our statistical analyses in line with the suggestions of Referee 1, and have added them to all figure legends.
9. We have modified the title of our study as recommended.

Appendix B

Response to Referees.

The referee points out a couple of errors with figure citations in the main text of our revised manuscript. We are uploading a final version of the manuscript in which these errors have been corrected as follows:

1) The reference to Figure 4C has been removed from p4
2) The reference to Figure 3C on p6 has been altered to read Figure 4C.

We are delighted that our manuscript is ready for publication, and thank the referee for his time and effort.

Cath Lindon

16/6/2018