EMBO reports

Post-mitotic expansion of cell nuclei requires nuclear actin filament bundling by alpha-actinin 4

Sylvia Krippner, Jannik Winkelmeier, Julian Knerr, David Virant, Dominique Brandt, Carsten Schwan, Ulrike Endesfelder, and Robert Grosse
DOI: 10.15252/embr.202050758

Corresponding author(s): Robert Grosse (robert.grosse@pharmakol.uni-freiburg.de) , Carsten Schwan (carsten.schwan@pharmakol.uni-freiburg.de), Ulrike Endesfelder (ulrike.endesfelder@synmikro.mpi-marburg.mpg.de)

Review Timeline:

Submission Date:	28th Apr 20
Editorial Decision:	20th May 20
Revision Received:	13th Jul 20
Editorial Decision:	14th Aug 20
Revision Received:	18th Aug 20
Accepted:	20th Aug 20

Editor: Deniz Senyilmaz Tiebe

Transaction Report:

(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 20th May 2020

Dear Robert,

Thank you for the submission of your research manuscript to our journal, which was now seen by two referees, whose reports are copied below.

We concur with the referees that the proposed role of ACTN4 in nuclear actin regulation and hence post-mitotic nuclear expansion. However, they the referees also raise important concerns that need to be addressed prior to publication in this journal. In particular,

1. Cell cycle phase dependence of ACTN4 nuclear localization should be reported (referee #2, 2nd paragraph).
2. All knock-down and reconstitution experiments (with wt or deletion mutants) require western blot controls (referee #1 point 1 and referee #2 points 4 and 8).
3. Currently there is not enough support for the following statements: 'ACTN4 sliding on actin bundles' ,'ACTN4 cross-links actin filaments' (referee #1 points 2 and 3) and ACTN4 mediated nuclear actin assembly (referee #2, point 6). If these cannot be addressed experimentally, the text should be re-phrased. Similarly, a different word should be used to define the nuclear actin structures (referee #2, point 5).
4. Better support into ACTN4 presence in the nucleus (not on the cytosolic face of the nuclear membrane) (referee #2, point 1). If this point cannot be addressed experimentally, the text should be re-phrased.
5. G1 cell size of ACTN4 depleted cells should be shown (referee #2, point 7)

I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

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 (where applicable).
2. Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter plots in these cases.

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

Please note that for all articles published beginning 1 July 2020, the EMBO Reports reference style will change to the Harvard style for all article types. Details and examples are provided at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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).

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

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

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

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

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

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

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

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

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

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

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

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

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

Data availability

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

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

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

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

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

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

Yours sincerely,

Deniz

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

Review of "Post-mitotic expansion of cell nuclei requires ACTN4-mediated nuclear actin filament bundling" by Krippner et al.

In recent years, it has become apparent that actin is not only a conserved component of the nucleus, but it also plays roles in several important processes. One of these roles, as show by previous work from this lab, is expansion of the nucleus following cell division. However, the details of this and other actin-dependent processes in the nucleus remain fuzzy, at least in part because the "parts list" for the nuclear actin cytoskeleton remains underdeveloped.

In the current study, the authors follow up on their previous identification of alpha-actinin4 (ACTN4) as a potential nuclear actin filament (F-actin) regulator. They first provide evidence that ACTN4 binds nuclear F-actin, then characterize the dynamics of ACTN4 and then assess the consequences of ACTN4 depletion. They find that loss of ACTN4 results in a striking deficit in post-division nuclear expansion. They also demonstrate, using super-resolution imaging, that the nuclear F-actin appears less "bundled" and less abundant in cells expressing a version of ACTN4 lacking one of its calponin homology domains. Based on their results, the authors conclude that ACTN4 is an important regulator of nuclear F-actin that contributes to nuclear expansion via

bundling of nuclear F-actin.

This is a fascinating study. It is also, by and large, well controlled and well-supported by the imaging results. However, as described below, the some key results are not included and at times the authors conclusions go beyond what they demonstrate.

1. Unless I missed it, I could not find any immunoblots demonstrating knockdowns of the endogenous ACTN4 via siRNA. Likewise, there is no demonstration that the replacement experiments result in a level of expression comparable to endogenous expression.

2. The authors report that ACTN4 slides along actin filaments in the nucleus. However, in neither the movie nor the images provided to support this point, was any such sliding evident. The actin filaments move, and the ACTN4 clusters move, but movement of a cluster along filament (ie toward one end or the other while staying in association with the filament) cannot be seen. Indeed, in the absence of any kind of fiduciary marks on the actin filaments, it would be impossible to demonstrate such sliding at the long sampling intervals used for the live imaging experiments.

3. The authors allude to the localization of ACTN4 on nuclear actin filaments "that seemed to be crosslinking event". This is also not evident in the data provided. A crosslinking event would entail two or more filaments assuming a roughly parallel organization (if we assume that the authors bundling model is correct). There is one example of a cluster that is at the end of one filament and might be at the end of another filament (or filament bundle), but because the channel showing the actin alone is not presented it is hard to be sure. Regardless, because there is no before and after showing the filaments coming together, it is not really a crosslinking "event".

To be clear, while the authors should address point one above by providing the relevant data, they do not need to show actual crosslinking or sliding along filaments by ACTN4. But they should remove the assertion that it is occurring or provide convincing evidence that it is occurring.

Minor points:

Figure 2 has no micron bar for the time lapse although one is alluded to in the figure legend.

ACTN4 should be spelled out in the title and abstract-lots of people know what alpha actinin is but do not know what ACTN4 stands for.

What is meant by the phrase "The dashed white box is shown as magnification below"?

What is meant by the phrase "...after spontaneous cell division during early G1"? In particular, what is the "spontaneous" meant to imply and do we conclude that in this cell type, cell division occurs in G1 rather than M phase or are the authors referring specifically to telophase?

The distribution of ACTN4 along the nuclear actin filaments appears to be discontinuous (usually just a single, focal cluster) which is surprising if it is indeed responsible for bundling F-actin. Is it possible that ACTN4 does not play the same role in the nucleus that it does in the cytoplasm? The super resolution results would suggest otherwise but, strictly speaking, these do not reflect what happens in the absence of ACTN4, but rather the presence of an ACTN4 lacking one of its calponin homology domains.

It would be helpful to show what is currently figure 4B at a much higher magnification or at least

provide insets that allow the reader to compare the nuclear distribution of WT vs deltaCH1 ACTN4. One expects that the latter would be distributed diffusely, but this point cannot be assessed with the figures in their current form.

The authors may want to consider subjecting their live imaging results on ACTN4 to mean square displacement analysis to determine whether the movement displayed represents directed movement (although this might not be informative if the sampling interval is too long). If they cannot, they could try to determine whether the movement of ACTN4 varies as a function of its apparent association with nuclear F-actin.

Referee #2:

This manuscript shows that the actin bundling protein actinin4 (ACTN4) localizes to the nucleus in early G1 phase, and enriches on nuclear actin filaments during this time. Disruption of ACTN4 function causes reduced nuclear expansion during G1 and reduced numbers and thicknesses of nuclear actin structures. These effects are specific for ACTN4, and not ACTN1. These results build on previous results from this laboratory showing that nuclear actin polymerization during G1 is necessary for nuclear expansion.

The work in general is well done and interesting, adding to our growing knowledge of nuclear actin. However, it is a bit 'thin' in terms of mechanistic impact. Answering at least one additional question would make this work more suitable for EMBO Reports - is ACTN4 nuclear localization specific to G1 phase, and does it decrease in S and G2? The authors could answer this by either sub-cellular fractionation at specific time points, or microscopy (bearing in mind specific comment 1 just below this). It would be best to see overall ACTN4 protein levels as a function of cell cycle stage too, as part of this experiment. This question is especially important to answer, since previous work (Kumeta et al 2010 J. Cell Sci.) showed that nuclear ACTN4 is highest in G2 and lowest in G1.

Specific comments:
1) Figure 1 - the data shown here do not constitute proof of the authors conclusions:
a. From the micrographs in Figure 1C, the authors draw the conclusion that ACTN4 immunofluorescence staining shows some nuclear localization. However, it is impossible to make these conclusions from the micrographs supplied, as the ACTN4 could be on the cytoplasmic face of the nuclear membrane. High-quality 3D reconstructions would be necessary to make this conclusion.
b. Similar to comment a, in Figure 1B the authors use biotin-phalloidin pulldown to show endogenous ACTN4 in nuclear fractions and ACTN4 interacts with nuclear F-actin. This pulldown assay cannot rule out actin filaments accumulated on the outer nuclear membrane. Can the authors treat the nuclear extract with a large protease (that will not fit through the nuclear pore) to potentially degrade any ACTN4 on the ONM, then do the pulldown? Alternately, they could add a statement that these could in fact represent ONM ACTN4 and/or actin. The later experiments are pretty convincing that there is ACTN4 in the nucleus.
2) In Figure 2, the data shown could be much more informative:
a. 2A. The example of ACTN4 in filamentous structures in the nucleus is not very impressive, with few filamentous structures apparent. Can they show a better example?
b. 2B. This line scan does not provide any new information, and in fact argues against specific co-localization of ACTN4 and actin (ACTN4 is highest where actin is lowest).

c. 2C. This tracking of ACTN4 clusters adds nothing on its own. Much more informative would be to track both ACTN4 and actin, and show that they track in the same manner.
3)
4) For the dSTORM experiments in Figure 4, it is unclear why the authors chose to use cells expressing transfected ACTN4 constructs (WT and deltaCH1), instead of simply comparing cells knocked-down for ACTN4 versus control cells, which would have been a much cleaner comparison. The comparison of WT (without an extra NLS) versus deltaCH1 (with an added NLS) presents other problems as well. Is the deltaCH1 at much higher nuclear concentrations than WT? Is the deltaCH1 expressed at much higher overall levels than WT? Tests for these two parameters are important.
5) For the dSTORM experiments in Figure 4, it might be better to use a different wording than "filaments" to describe the structures quantified in panels C and D. "Filament" implies a single actin filament. What is quantified here appears to be any linear structure observed, regardless of width or intensity. I might use "fiber", or "linear structure", and define this. In the second part of this experiment, the authors attempt to resolve single filaments, and at that point the term filament is more appropriate.
6) In the Abstract, it is not correct to state "F-actin formation" and "nuclear actin assembly" as being processes affected by ACTN4. From the authors' results, it is unclear whether F-actin assembly is affected by ACTN4 functional disruption. It is possible that, in the dSTORM images shown in Figure 4, single filaments cannot be resolved. Actinins are not known to be involved in actin assembly in other systems, and the likelihood is that ACTN4 is just bundling filaments assembled by another mechanism here. It is possible that, without ACTN4, the filaments are subject to faster cofilin-mediated depolymerization. However, that still is not an assembly issue.
7) One possibility for altered nuclear expansion is that ACTN4 disruption perturbs overall cell health or cell size, indirectly changing nuclear expansion. Can the authors show that overall cell size in G1 is not affected?
8) All KD experiments should have western blots to confirm efficiency, especially for ACTN1 since that is a negative result.

Other comments:
1) For the methods on immunofluorescence fixing/staining, more detail must be given than "cells were fixed and stained by standard procedures". What was the fixative (PFA or other), where was it obtained (catalog #), in what buffer was it used, and how long at what temperature was fixation conducted? How were cells permeabilized?
2) In Figure 2A, it is unclear how the authors decide that the cell imaged is in early G1. Some mention of that in the text or methods is needed.
3) Figures 3E-G are confusing in labeling and it seems that they are not always correctly indicated in the Results. Clearer legends and descriptions would be useful. All the data seem to be there to justify the authors' conclusions.
4) For the dSTORM experiments in Figure 4, definitions of what constitutes an "interphase" cell must be given.

Point-by-point response (reviewer text in blue)

Referee #1:

Review of "Post-mitotic expansion of cell nuclei requires ACTN4-mediated
nuclear actin filament bundling" by Krippner et al.

In recent years, it has become apparent that actin is not only a conserved component of the nucleus, but it also plays roles in several important processes. One of these roles, as show by previous work from this lab, is expansion of the nucleus following cell division. However, the details of this and other actin-dependent processes in the nucleus remain fuzzy, at least in part because the "parts list" for the nuclear actin cytoskeleton remains underdeveloped.

In the current study, the authors follow up on their previous identification of alpha-actinin4 (ACTN4) as a potential nuclear actin filament (F-actin) regulator. They first provide evidence that ACTN4 binds nuclear F-actin, then characterize the dynamics of ACTN4 and then assess the consequences of ACTN4 depletion. They find that loss of ACTN4 results in a striking deficit in post-division nuclear expansion. They also demonstrate, using super-resolution imaging, that the nuclear F-actin appears less "bundled" and less abundant in cells expressing a version of ACTN4 lacking one of its calponin homology domains. Based on their results, the authors conclude that ACTN4 is an important regulator of nuclear F-actin that contributes to nuclear expansion via bundling of nuclear F-actin.

This is a fascinating study. It is also, by and large, well controlled and well-supported by the imaging results. However, as described below, the some key results are not included and at times the authors conclusions go beyond what they demonstrate.

1. Unless I missed it, I could not find any immunoblots demonstrating knockdowns of the endogenous ACTN4 via siRNA. Likewise, there is no demonstration that the replacement experiments result in a level of expression comparable to endogenous expression.

We agree that this is an important control that we missed to provide. We now provide Western and RT-PCR knockdown controls as well as Western blot controls for our replacement experiments in EV2 also showing comparable expression levels to endogenous ACTN4. Since we have no access to a functional ACTN1-specific antibody we had performed and now show RT-PCR quantifications for efficient ACTN1 knockdown. An additional knockdown Western blot for ACTN4 is shown in the new figure EV3B. We usually achieve almost complete silencing of ACTN4 to undetectable protein levels.

2. The authors report that ACTN4 slides along actin filaments in the nucleus. However, in neither the movie nor the images provided to support this point, was any such sliding evident. The actin filaments move, and the ACTN4 clusters move, but movement of a cluster along filament (ie toward one end or the other while staying in association with the filament) cannot be seen. Indeed, in the absence of any kind of fiduciary marks on the actin filaments, it would be impossible to demonstrate such sliding at the long sampling intervals used for the live imaging experiments.

We agree with the reviewer. We now performed tracking analysis for both, actin and ACTN4 from 3 independent experiments. These data are now shown in the new Figure 2C. A representative movie (Movie 2) was added as well. Our data show that ACTN4 displayed a synchronized movement with actin.

3. The authors allude to the localization of ACTN4 on nuclear actin filaments "that seemed to be crosslinking event". This is also not evident in the data provided. A crosslinking event would entail two or more filaments assuming a roughly parallel organization (if we assume that the authors bundling model is correct). There is one example of a cluster that is at the end of one filament and might be at the end of another filament (or filament bundle), but because the channel showing the actin alone is not presented it is hard to be sure. Regardless, because there is no before and after showing the filaments coming together, it is not really a crosslinking "event".

To be clear, while the authors should address point one above by providing the relevant data, they do not need to show actual crosslinking or sliding along filaments by ACTN4. But they should remove the

assertion that it is occurring or provide convincing evidence that it is occurring.

We agree with the reviewer and rephrased the text accordingly and removed interpretations of “crosslinking” events (page 5, first paragraph).

Minor points:

Figure 2 has no micron bar for the time lapse although one is alluded to in the figure legend.

We could not find a missing scale bar in the Figure 2A. The legend refers to two scale bars. One in the overview and one in the time series, both are present. In the time series, first color image (mitotic exit) a vertical scale bar is on the right. An additional scale bar was now added to the magnifications lower line of images.

ACTN4 should be spelled out in the title and abstract-lots of people know what alpha actinin is but do not know what ACTN4 stands for.

-We now spell out ACTN4 in the title and abstract as suggested.

What is meant by the phrase "The dashed white box is shown as magnification below"?

We apologize if this was unclear. The Figure 2 and accompanying figure legend was changed entirely.

What is meant by the phrase "...after spontaneous cell division during early G1"? In particular, what is the "spontaneous" meant to imply and do we conclude that in this cell type, cell division occurs in G1 rather than M phase or are the authors referring specifically to telophase?

We apologize if this was unclear. We now write in the Method section on page 12: Cells at mitotic exit (early G1) were identified by following live cells through mitotic cell division under the microscope until cytokinesis when new daughter cell nuclei are formed.

The distribution of ACTN4 along the nuclear actin filaments appears to

be discontinuous (usually just a single, focal cluster) which is surprising if it is indeed responsible for bundling F-actin. Is it possible that ACTN4 does not play the same role in the nucleus that it does in the cytoplasm? The super resolution results would suggest otherwise but, strictly speaking, these do not reflect what happens in the absence of ACTN4, but rather the presence of an ACTN4 lacking one of its calponin homology domains.

We thank the reviewer for this comment. We have reanalyzed our data from several experiments. We now performed tracking analysis for both, actin and ACTN4 from 3 independent experiments. These data are now shown in the new Figure 2C. A representative movie (Movie 2) was added as well. Our data show that ACTN4 displayed a synchronized movement with actin, which would be in principle compatible with a bundling function. Of course, further studies are needed to understand nuclear ACTN4.

It would be helpful to show what is currently figure 4B at a much higher magnification or at least provide insets that allow the reader to compare the nuclear distribution of WT vs deltaCH1 ACTN4. One expects that the latter would be distributed diffusely, but this point cannot be assessed with the figures in their current form.

The purpose of the microscopy images in the upper panel of 4B was the identification of ACTN4 transfected cells for subsequent STORM analysis. As mentioned in the legend the images are just regular confocal images (that were acquired with rather low magnification and resolution compared to the STORM images next to it). The used STORM setup unfortunately did not allow for dual color imaging or 3D imaging. Therefore, we cannot provide the requested information at this time.

The authors may want to consider subjecting their live imaging results on ACTN4 to mean square displacement analysis to determine whether the movement displayed represents directed movement (although this might not be informative if the sampling interval is too long). If they cannot, they could try to determine whether the movement of ACTN4 varies as a function of its apparent association with nuclear F-actin.

Please see above and our new Figure 2.

Referee #2:

This manuscript shows that the actin bundling protein actinin4 (ACTN4) localizes to the nucleus in early G1 phase, and enriches on nuclear actin filaments during this time. Disruption of ACTN4 function causes reduced nuclear expansion during G1 and reduced numbers and thicknesses of nuclear actin structures. These effects are specific for ACTN4, and not ACTN1. These results build on previous results from this laboratory showing that nuclear actin polymerization during G1 is necessary for nuclear expansion.

The work in general is well done and interesting, adding to our growing knowledge of nuclear actin. However, it is a bit 'thin' in terms of mechanistic impact. Answering at least one additional question would make this work more suitable for EMBO Reports - is ACTN4 nuclear localization specific to G1 phase, and does it decrease in S and G2? The authors could answer this by either sub-cellular fractionation at specific time points, or microscopy (bearing in mind specific comment 1 just below this). It would be best to see overall ACTN4 protein levels as a function of cell cycle stage too, as part of this experiment. This question is especially important to answer, since previous work (Kumeta et al 2010 J. Cell Sci.) showed that nuclear ACTN4 is highest in G2 and lowest in G1.

We thank the reviewer for the comments on ACTN4 nuclear localization as a potential function of cell cycle stage to which we would like to respond as follows. Reviewer #2 refers to the study that by Kumeta et al (2010 J. Cell Sci) that we also cite in our manuscript and to which we previously already stated on page 4, second paragraph :”Consistent with this, we observed endogenous localization of ACTN4 to the nucleus of NIH3T3 or RPE-1 cells by immunostaining at mitotic exit but also in interphase cells confirming previous results showing nucleocytoplasmic shuttling of ACTN4 (Kumeta et al., 2010). No obvious difference could be observed in nuclear localization of ACTN4 at early G1 or in

interphase cells, indicating continuous nuclear distribution or shuttling and no specific nuclear accumulation after mitosis."

We think that there are several points worth noticing from that study by Kumeta et al. in 2010. Firstly, they used HeLa cells, which are significantly different from the RPe-1 and 3T3 cells used in our study. Secondly, a home-made antibody (#92B) was used to which we have no access and that apparently differs remarkably to an anti-actinin-4 control antibody they used in terms of endogenous cellular distribution. Thus, for example, the 92B did not stain any F-actin structures like the anti-actinin-4 control antibody did that are typical of actinin-4 localisation and function. Instead it stained nuclei very strongly (see Figure 1D, Kumeta et al).

Thus, the experiments performed for cell cycle analysis by Kumeta et al. were all done in HeLa cells with that 92B antibody. These images in Figure 5A in Kumeta et al show what appears to be a rather comparable nuclear distribution between G2 (9h) and G1 (12h), while in G2 (7h) and S phase nuclear actinin-4 is much lower than in G1 (12h). Importantly however, a control experiment with the anti-actinin-4 commercial antibody they used in Figure 1 is not provided. Furthermore, the quantifications in 5B do not show data from independently performed analysis and hence do not demonstrate any statistical significance!

We think it is fair to say, also given cell type differences as well as commonly known HeLa cell problems with respect to phenotypic differences that these data from that paper do not justify a general claim that actinin-4 nuclear localisation really differs between G2 or G1 or interphase for that matter. We write in our paper: "we observed endogenous localization of ACTN4 to the nucleus of NIH3T3 or RPE-1 cells by immunostaining at mitotic exit but also in interphase cells".
We further do show ACTN4 in nuclear fractions as well as from nuclear Phalloidin pulldowns (Figure 1) - so it is clearly well detectable, plus, our study investigates daughter cell nuclear expansion and thus does not deal with S phase or G2 but with a nuclear actinin-4 function at mitotic exit. Hence, we would kindly like to point out that this issue of potentially differing or changing levels of nuclear ACTN4 over the entire cell cycle might be a future task beyond our current study. However, we now provide another fractionation experiment from postmitotic cells in 1B showing endogenous nuclear ACTN4.

Specific comments:

1) Figure 1 - the data shown here do not constitute proof of the authors conclusions:
a. From the micrographs in Figure 1C, the authors draw the conclusion that ACTN4 immunofluorescence staining shows some nuclear localization. However, it is impossible to make these conclusions from the micrographs supplied, as the ACTN4 could be on the cytoplasmic face of the nuclear membrane. High-quality 3D reconstructions would be necessary to make this conclusion.

-We agree that it would be desirable to have high resolution 3D reconstructions of endogenous nuclear ACTN4. We have tried to address this with several novel stainings in the past weeks. Unfortunately, the antibodies available to us are at present not sensitive enough to perform such analysis. We have therefore rephrased our text and now refer to “nuclear area” (page 4, second paragraph). However, we now provide another fractionation experiment from postmitotic cells in 1B showing endogenous nuclear ACTN4.

b. Similar to comment a, in Figure 1B the authors use biotin-phalloidin pulldown to show endogenous ACTN4 in nuclear fractions and ACTN4 interacts with nuclear F-actin. This pulldown assay cannot rule out actin filaments accumulated on the outer nuclear membrane. Can the authors treat the nuclear extract with a large protease (that will not fit through the nuclear pore) to potentially degrade any ACTN4 on the ONM, then do the pulldown? Alternately, they could add a statement that these could in fact represent ONM ACTN4 and/or actin. The later experiments are pretty convincing that there is ACTN4 in the nucleus.

To our best of knowledge we have no indication from our fractionations that the nuclear fractions might contain detectable ONM material. We conclude this from the fact that no tubulin was detected in our nuclear fractions. Microtubules as well as actin filaments are connected to the ONM via nesprin interactions. Hence, if ACTN4 would indeed be present on the ONM (to our knowledge no literature exists on that) it would likely be contaminating our nuclear fractions as much as tubulin would. We therefore think that our tubulin control is valid to exclude significant contaminations from ONM material.

2) In Figure 2, the data shown could be much more informative:
a. 2A. The example of ACTN4 in filamentous structures in the nucleus is not very impressive, with few filamentous structures apparent. Can they show a better example?
b. 2B. This line scan does not provide any new information, and in fact argues against specific co-localization of ACTN4 and actin (ACTN4 is highest where actin is lowest).
c. 2C. This tracking of ACTN4 clusters adds nothing on its own. Much more informative would be to track both ACTN4 and actin, and show that they track in the same manner.

We agree with the reviewer. We now performed tracking analysis for both, actin and ACTN4 from 3 independent experiments. These data are now shown in the new Figure 2C. A representative movie (Movie 2) was added as well. Our data show that ACTN4 displayed a synchronized movement with actin.
We also have added another example showing nuclear F-actin now as new Figure EV1.
The line scan was replaced (Figure 2B).

3)
4) For the dSTORM experiments in Figure 4, it is unclear why the authors chose to use cells expressing transfected ACTN4 constructs (WT and deltaCH1), instead of simply comparing cells knocked-down for ACTN4 versus control cells, which would have been a much cleaner comparison. The comparison of WT (without an extra NLS) versus deltaCH1 (with an added NLS) presents other problems as well. Is the deltaCH1 at much higher nuclear concentrations than WT? Is the deltaCH1 expressed at much higher overall levels than WT? Tests for these two parameters are important.
5) For the dSTORM experiments in Figure 4, it might be better to use a different wording than "filaments" to describe the structures quantified in panels C and D. "Filament" implies a single actin filament. What is quantified here appears to be any linear structure observed, regardless of width or intensity. I might use "fiber", or "linear structure", and define this. In the second part of this experiment, the authors attempt to resolve single filaments, and at that point the term filament is more appropriate.

We used the deltaCH1 (NLS) interfering approach to specifically act in the nucleus in the presence of endogenous ACTN4 and to clearly identify the transfected cells for further STORM analysis. Due to the current pandemic situation we cannot perform additional STORM experiments using siRNAs for example. Our collaborator Ulrike Endesfelder was supposed to start a new position and laboratory in January in Pittsburgh (USA). Due to the travel ban they cannot unpack the already shipped STORM equipment, while at the same time being “trapped” in Germany.
We would like to adhere to the term “filaments”. The term “fiber” could also refer to a single filament per definition. We did explain on Page 7 in detail how we interpret our findings and when we refer to single versus bundled filaments.

6) In the Abstract, it is not correct to state "F-actin formation" and "nuclear actin assembly" as being processes affected by ACTN4. From the authors' results, it is unclear whether F-actin assembly is affected by ACTN4 functional disruption. It is possible that, in the dSTORM images shown in Figure 4, single filaments cannot be resolved. Actinins are not known to be involved in actin assembly in other systems, and the likelihood is that ACTN4 is just bundling filaments assembled by another mechanism here. It is possible that, without ACTN4, the filaments are subject to faster cofilin-mediated depolymerization. However, that still is not an assembly issue.

We follow the reviewer’s argumentation and have rephrased these sentences in the Abstract accordingly.

7) One possibility for altered nuclear expansion is that ACTN4 disruption perturbs overall cell health or cell size, indirectly changing nuclear expansion. Can the authors show that overall cell size in G1 is not affected?

We agree that this is an important point. We now provide a quantification of cytoplasmic volumes of ACTN4 knock-downs showing that inhibition of nuclear ACTN4 does not impact on general cell size in G1 (new EV3).

8) All KD experiments should have western blots to confirm efficiency, especially for ACTN1 since that is a negative result.

We agree that this is an important control that we missed to provide. We now provide Western and RT-PCR knockdown controls as well as Western blot controls for our replacement experiments in new Figure EV2, also showing comparable expression levels to endogenous ACTN4. Since we have no access to a functional ACTN1-specific antibody we had performed and now show RT-PCR quantifications for efficient ACTN1 knockdown. An additional knockdown Western blot for ACTN4 is shown in the new figure EV3B. We usually achieve almost complete silencing of ACTN4 to undetectable protein levels.

Other comments:

1) For the methods on immunofluorescence fixing/staining, more detail must be given than "cells were fixed and stained by standard procedures". What was the fixative (PFA or other), where was it obtained (catalog #), in what buffer was it used, and how long at what temperature was fixation conducted? How were cells permeabilized?

We apologize that this information was missing. We now include this information on page 11, last paragraph).

2) In Figure 2A, it is unclear how the authors decide that the cell imaged is in early G1. Some mention of that in the text or methods is needed.

We apologize if this was unclear. We now write in the Method section on page 12: Cells at mitotic exit (early G1) were identified by following live cells through mitotic cell division under the microscope until cytokinesis, when new daughter cell nuclei are formed.

3) Figures 3E-G are confusing in labeling and it seems that they are not always correctly indicated in the Results. Clearer legends and descriptions would be useful. All the data seem to be there to justify the authors' conclusions.

We included a subheader for 3F to make this more consistent with 3E and G. We apologize for the figure indication errors and we corrected these in the text accordingly. We reread the figure legend and found it to be correct and understandable.

4) For the dSTORM experiments in Figure 4, definitions of what constitutes an "interphase" cell must be given.

We agree. We now label these images as “post-mitotic” and “non-dividing”.

1st Revision - Editorial Decision 14th Aug 2020

Dear Robert,

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

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

• Please provide 3-5 keywords for your study. These will be visible in the html version of the paper and on PubMed and will help increase the discoverability of your work.
• As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database.
• Please add Conflict of Interest and Author Contributions sections to the manuscript.
• We note that the funding information in the manuscript submission system is incomplete.
• We realize that Figures EV2 and EV3 are currently not called out in the text and the movie callouts are missing EV. The nomenclature should be 'Movie EV#'. The movies need to be ZIPped with their legends.
• In the legends of EV Fig 3C and D you mention that n=2, however, in the dot plots there are more data points. Please clarify.
• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

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

Kind regards,

Deniz

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors have thoroughly addressed all of my points.

Referee #2:

The authors have addressed my comments satisfactorily.

Authors made the requested changes.

2nd Revision - Editorial Decision 20th Aug 2020

Dear Robert,

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

Congratulations on a nice work!

When I was performing the final checks, I noted that the labels of the synopsis image are a bit small, and it becomes hard to read when resized to the online publication format (please see attached). Could you please increase the size of the letters? You can send me the file per email.

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

--
Please note that under the DEAL agreement of German scientific institutions with our publisher Wiley, you could be eligible for free publication of your article in the open access format. Please contact either the administration at your institution or our publishers at Wiley (embojournal@wiley.com) for further questions.
--

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2020-50758V3 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Prof. Dr. Robert Grosse, Prof. Dr. Ulrike Endesfelder, Dr. Carsten Schwan
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-50758V2

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ 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?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	see Materials and Methods
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	see Materials and Methods

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-reporting	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumour	REMARK Reporting Guidelines (n
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

Is there an estimate of variation within each group of data?	see Materials and Methods
Is the variance similar between the groups that are being statistically compared?	see Materials and Methods

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).	see Materials section in Manuscript
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	all cell lines used are tested monthly for mycoplasma contamination by PCR --> no contamination detected

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA