Nucleoplasmin is a limiting component in the scaling of nuclear size with cytoplasmic volume

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Re: JCB manuscript #201902124

Dr. Daniel L Levy
University of Wyoming
Molecular Biology
1000 E University Avenue
Laramie, Wyoming 82071

Dear Dan,

Thank you for submitting your manuscript entitled "Cytoplasmic volume and limiting components scale nuclear size during Xenopus laevis development". Three referees have now assessed your manuscript and are in agreement that the problem is an interesting one but the manuscript requires additional experimental work before it is suitable for publication in JCB.

In particular, all three reviewers are in agreement that more rigorous biochemical testing of the function of Npm2 in cell size control is necessary. The referees have suggested several lines of experimentation to solidify the function of Npm2 in nuclear size control. In addition, the referees have requested clarification of the data in Figures 1 and 2. Reviewer 1 and 3 echo the point that if the nuclei are at steady state with the extract then one might not expect additional size increase when added to the same stage extract. Both referees propose experiments to clarify this issue. Reviewer 3 points out that the concept of chromatin structure influencing nuclear size is not a new one and more detailed mechanistic data to understand the functions of Npm2 on nuclear size and chromatin structure would be required to distinguish this work from previous studies.

At this time, we cannot accept the manuscript in its current version. However, we invite you to address the referees concerns in a revised manuscript that incorporates new experimental data and text to address the technical and conceptual criticisms. Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed. I want to emphasize that a thorough revision that clearly addresses the issues raised by the referees is more important than resubmitting the manuscript within the standard 4-month window. Overall, the problem is an interesting and exciting one and we look forward to considering a revised manuscript.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

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Text limits: Character count for a Report is < 20,000; a full Research Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.
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Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures; a full Research Article may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Aaron Straight
Monitoring Editor

Andrea L. Marat
Scientific Editor

Journal of Cell Biology

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

In this manuscript Chen et al are interested in the factors that determine the nuclear size in the early Xenopus embryo after the cell cycle slows at the MBT. The authors use droplets of cytoplasmic extract of controlled size from various time points along with microinjection into live embryos and biochemical fractionation to identify and verify Npm2 as a regulator of nuclear size at this stage. They demonstrate that droplets of different sizes and cytoplasmic ages result in different size nuclei with smaller droplets and older extracts resulting in smaller nuclei. They then attempt to purify factor responsible for setting the nuclear size and identify Npm2. Next they show that Npm2 injection can cause a modest increase in nuclear size and that this effect is increased by co-injection of importin a. Finally they show that Npm2 affects histone concentrations in the
nucleus and link this to changes in chromatin state.

Overall I think that this is an interesting and important topic and that the approach and experiments presented are appropriate and reasonable. However, I do have several (I believe addressable) concerns about the interpretation and biological significance of these findings.

First, I am confused about the state of the stage 10-10.5 nuclei in vivo (before isolation). Are they still growing or do they reach steady state? If they have reached steady state then why do the same nuclei grow when placed into extract from the same stage as in Figure 1? I would assume that if the author's model is correct and a nuclear factor had become limiting that that factor would be removed from the extract with the nuclei and that no droplet size made from a stage 10+ embryo could result in a larger stag 10 nucleus. If they are not yet in steady state after a one hour G2 arrest then how am I to think about the rest of the in vivo results in this manuscript (especially the importin results)? In that case what is the arrest length required to reach steady state in the embryo and how do the resultant nuclear sizes compare to those obtained in the droplets? It would be very helpful to know the Npm2 concentration in these extracts when the nuclei are removed to see if the remaining cytoplasmic Npm2 can explain the observed growth in these extracts. A related question is if you remove the steady state nuclei and add fresh (smaller) nuclei do they not grow at all?

More broadly, the authors have shown that the total Npm2 is constant and that the nuclear concentration is falling. They propose (reasonably) that this is due to exhaustion of a cytoplasmic pool. This is a central point of their model. To actually show this it would be very helpful to see either the cytoplasmic (extract without nuclei) concentration falling and/or quantification of the total nuclear fraction (individual nuclear concentration multiplied by number of nuclei) increasing. I find their interpretation of their data plausible, but one more measurement is needed to fully support their claim.

I am most concerned about the interpretation of figure 5. This figure is critical because it brings in the biological significance of their findings and tries to tie the in vitro changes in nuclear size to actual developmental phenomena. However, I do not believe that chromatin relative area is an informative metric for chromatin state here because the denominator is the area of the nucleus and so it is necessarily true that if you divide the same amount of DNA (they are arrested in G2, which I assume means not replicating) by a larger area you will get a smaller chromatin relative area. The authors might choose another, less directly size sensitive metric for DNA homogeneity (perhaps even something as simple as pixel intensity distributions in the heochst channel scaled by nuclear size. Better would be to see some sort of additional experiments to demonstrate consequences for chromatin organization or downstream events. For example, staining for disproportionate changes to the area occupied by specific chromatin marks/features, MNase digests to look for changes in nucleosome occupany or spacing, or measurable differences in transcription. In some way, there needs to be more concrete evidence to tie their observations to larger biological consequences.

Finally, the sample sizes and statistical tests being used need to be made more clear in the text and figure legends. If all droplets are being treated as independent replicates is there a statistical justification for this (are droplets made from different extracts statistically distinguishable?) or are droplets only being compared within a given extract from the same day?

Minor points:
1C and D, the volumes are not the same, so hard to compare where reach the steady state. If this data is available it would be helpful to plot the same size droplets so that the curves were more
directly comparable.

This sentence is confusing "After an incubation period, we observed that nuclei grew larger in ~0.8 nL spherical droplets compared to ~0.1 nL spherical droplets (Fig. 1B, Movie 1), while droplet volume did not change (Fig. S1A)." It needs to be made clear that droplet volume did not change over time not between conditions.

In the presentation of figure 4 in the text it is important to note what stage the embryos were analyzed at.

In several figures the authors are plotting fold change and not absolute volume or delta volume. This choice makes it difficult to compare that data to the other data that is presented in absolute volume. Is there a reason that this data must be plotted as a fold change?

In figure 4F what are the statistical comparisons between? Actually, this could be clarified for all of the statistics in the figures.

I'd very much like to see more discussion of the effect of importin a on steady state nuclear size/Npm2 concentration. This result seems to imply that passive or active export of Npm2 has a large effect on final volume. Since Npm2 is in a fairly large complex, I wouldn't have expected passive nuclear export to be a major issue. Is Npm2 being actively shuttled? Truly answering these questions is beyond the scope of this paper, but some discussion of the implications of this result would be appropriate.

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

The manuscript by Chen et al. investigates the role of cytoplasmic volume in nuclear size scaling. As is explained by the authors, mechanisms that determine the scaling of cells and organelles remains an interesting and fundamental question in biology. Thus, this work will be of general interest. The authors leverage the Xenopus model and an innovative approach by encasing nuclei in different sized oil droplets containing Xenopus embryo cytosol. After an incubation period the nuclei grow and reach a steady-state size that mirrors the cytosolic volume (i.e. the larger the volume the larger the nuclei). In some of the more compelling experiments, the authors further demonstrate that specific contents of the cytosol derived from different stage embryos impacts the size scaling of the nuclei. These data clearly suggest that there are specific cytosolic factors that ultimately limit nuclear growth. By using a subcellular fractionation scheme and mass spectrometry protein identification the authors identify one such factor to be nucleoplasmin, a histone chaperone. They further establish that the size-scaling function of nucleoplasmin likely requires its nuclear import and its histone-binding capability. Thus, overall, this study introduces an innovative approach to exploring size-scaling and a new factor that contributes to nuclear size determination in at least Xenopus embryos. I do have some concerns regarding the quality of some of the biochemical data and the lack of depletion experiments, which detract from the manuscript’s clarity and impact.

Major Points:

1) Lack of detail/explanation of biochemical fractionation: While the rationale behind the biochemical fractionation experiments is clear, how proteins were identified in monoQ fractions is not. It would
greatly help the reader, for example, to have a better understanding of what the "proteomic analysis" consists of. The authors use terms like "highly enriched" but it is not clear what metric is used for this assessment (what is the rationale behind the order of the proteins listed in S1G?). Moreover, in the methods it is indicated that individual bands are cut out of SDS-PAGE gels suggesting there are a limited number of protein components in each fraction. This is great but it would be nice to see these gels of the fractions in supplemental data; a Western blot for anti-Npm2 would also be appropriate for every fraction not just number 2 to see how Npm2 segregates. These points are particularly relevant to explaining why Npm2 is found in at least two very different fractions. The authors suggest this could be due to its interaction with histones, but they need to explain why there aren't any histones identified in the MS data. I would also appreciate a clear rationale behind why Npm2 was chosen over anp32b, which is also found in both fractions. Ideally, this would also have been carried along as a specificity control in the scaling assays.

2) Lack of depletion experiments: To test the role of Npm2 in nuclear-size scaling, the authors rely on microinjection of excess Npm2 into embryos. This leads to variations in Npm2 staining in nuclei, which complicates the analysis and interpretation. It would greatly strengthen the work to deplete Npm2 from cytosolic extracts using the anti-Npm2 antibody in droplet experiments. This is particularly relevant as the major conclusion of the work is based on a limiting cytosolic factor(s) contributing to nuclear size.

Minor Points in need of clarification:

Figure 2D: The change in approach from the droplet to the egg extracts could be better explained for the non-expert. Also, in multiple figures, "NPC" is a bit confusing as a label. It should be "NPCs" or preferably denote that the staining is mab414. At a minimum, please indicate in figure legends that mAB414 was used here.

Figure 3A-B: The purpose and results presented in these figure panels need to be better explained. It is not obvious, particularly for the non-expert, what the IBB domain achieves, nor WGA as neither of these are even mentioned in the text.

Figure 4B: please clarify the metric for "high Npm2" staining.

Figure 4C. The connection between importin-alpha and Npm2 is not clear. Can the authors clarify whether Npm2 is actually imported by importin alpha? Also, were this the case, importin alpha alone cannot import cargoes without importin-beta. I would appreciate some rationale for the specificity of importin-alpha (even if this isn't explicitly tested). In the future, it might be useful to try an additional NLS not recognized by importin alpha to explicitly test whether import, or importin-alpha-mediated import, is what is required for nuclear size scaling.

Figure 4D: There is a bit of a disconnect between the 4.2 uM indicated in the text and the "relative [Npm2]" in the Figure.

The statement "Npm2 mutants defective for histone binding and/or histone chaperone activity, but still import-competent" need a reference or explicit experiments showing that these mutants are still imported into the nucleus.
Reviewer #3 (Comments to the Authors (Required)):

The authors have shown previously, using Xenopus embryos and cell-free extracts, that nuclear size can be controlled in this system by the levels of importin alpha and lamin B3, and also by protein kinase C. Here they further investigate this model, using a microfluidics-based approach for analyzing nuclei incubated in different volumes of cytoplasm. They have focused on cells at the onset of gastrulation, when cytoplasmic and nuclear size are diminished as compared to earlier stage embryos. They show that incubating nuclei from stage 10 embryos with cytoplasm from progressively earlier stages resulted in correspondingly greater increases in nuclear volume during in vitro incubation, suggesting a limiting factor. Based on biochemical fractionation of egg cytosol, they show that the abundant histone chaperone nucleoplasmin (Npm2) was one of several components of egg extract that results in increased nuclear growth of stage 10 nuclei; nuclear growth also was obtained with importin alpha and lamin B3. An increased size of stage 10 nuclei also occurred when Npm2 was injected into embryos at the onset of development. The authors provide evidence that the activity of Npm2 in these assays requires the histone-binding activity of Npm2, and not simply its acidic properties. Since nuclei arising from supplemented Npm2 showed increased areas with no DNA staining, they speculate that the increased nuclear volume arises from Npm2-induced changes in chromatin structure. This overall is a solid study, but would benefit from changes:

1) The authors should clarify (Figs. 1-2) why nuclear volume increased more when nuclei were incubated with increasing volumes of cytoplasm. Is the number of nuclei per unit volume smaller with increasing droplet size? This would be consistent with Fig. S1C. These two figures could be condensed, as they seem somewhat disconnected from the remainder of the manuscript.

2) Do nuclei assembled in vitro from sperm (vs stage 10 nuclei), when incubated with egg extract supplemented with recombinant Npm2, also grow larger than controls? This would indicate that the amount of Npm2 is limiting even with very large cytoplasmic/nuclear ratios, and would argue that Npm2 is not necessarily an important limiting determinant in development. In this regard, it would be valuable to see the effects of depleting Npm2 on in vitro growth of nuclei.

3) There clearly are two populations of stage 10 nuclei. What is different about those that grow more strongly and import more Npm2 and histone? Is there nucleus-to-nucleus heterogeneity in the number of nuclear pores from stage 10 embryos? This would alter the model somewhat and could suggest that the most proximal cause of the size effects results from differences in nuclear transport.

4) Does Npm2 have direct effects on chromatin, such as on nucleosome packing? Is it possible that high Npm2 has nonphysiological effects on chromatin by competition of other structural proteins?

Overall, this manuscript supports the notion that chromatin structure could be an important determinant of nuclear size. This point already is well-established in other systems: a number of papers have shown that depletion of proteins involved in chromatin compaction results in nuclear size increase in various experimental models, ranging from Tetrahymena micro/macro nuclei to mammalian neurons, lymphocytes and ESCs (reviewed by Bustin and Misteli, Science, 2016). Considering this, and the inconclusive mechanistic insight on the Npm2 effects, the level of new scientific advance obtained in this manuscript is relatively modest.
Point-by-point response to previous reviews

The comments of the editors and reviewers are copied here in italics. Our responses are just below each point raised by the editor or reviewer. Our major additions to the revised manuscript are in blue font here and highlighted in the manuscript. Other small changes were made throughout the text but are not explicitly mentioned here.

Editors

Thank you for submitting your manuscript entitled "Cytoplasmic volume and limiting components scale nuclear size during Xenopus laevis development". Three referees have now assessed your manuscript and are in agreement that the problem is an interesting one but the manuscript requires additional experimental work before it is suitable for publication in JCB.

We were happy to see that the referees were generally interested in our study.

In particular, all three reviewers are in agreement that more rigorous biochemical testing of the function of Npm2 in cell size control is necessary. The referees have suggested several lines of experimentation to solidify the function of Npm2 in nuclear size control.

We have performed the experimental work suggested by the reviewers. Major additions include: 1) demonstrating that the total nuclear Npm2 pool increases over development, 2) providing an alternate approach to quantifying chromatin distribution, 3) examining Npm2’s effect on nucleosome packing and euchromatin, 4) providing additional details and data relating to the biochemical fractionation, 5) performing Npm2 immunoinhibition, and 6) examining whether Npm2 affects NPC numbers. Below we detail the experiments performed, results, and how they have been incorporated into the revised manuscript. We have also addressed minor issues to clarify the presentation of some of our data and statistical analyses and provide additional discussion relating to the interpretation of some of our results.

In addition, the referees have requested clarification of the data in Figures 1 and 2. Reviewer 1 and 3 echo the point that if the nuclei are at steady state with the extract then one might not expect additional size increase when added to the same stage extract. Both referees propose experiments to clarify this issue.

We have performed the suggested experiments. In brief, we show that nuclei are at a steady-state size in stage 10 embryos and that there is a cytoplasmic pool of Npm2. We provide some additional discussion and rationalization of these results.
Reviewer 3 points out that the concept of chromatin structure influencing nuclear size is not a new one and more detailed mechanistic data to understand the functions of Npm2 on nuclear size and chromatin structure would be required to distinguish this work from previous studies.

We now include data showing that nucleoplasmin affects both nucleosome packing and the amount of euchromatin, more firmly establishing how nucleoplasmin impacts chromatin structure. With regard to the idea that chromatin structure is already known to influence nuclear size, our data suggest a distinctly different model. While previous studies have depleted major chromatin structural proteins and shown that massive chromatin decondensation is associated with larger nuclei, our data support a model whereby modest, and perhaps more physiological, chromatin condensation actually drives nuclear growth. So our results and model are quite different and novel compared to previous work. We now include some discussion of these previous studies, how our results differ, and some possible explanations for these differences.

At this time, we cannot accept the manuscript in its current version. However, we invite you to address the referees concerns in a revised manuscript that incorporates new experimental data and text to address the technical and conceptual criticisms. Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed. I want to emphasize that a thorough revision that clearly addresses the issues raised by the referees is more important than resubmitting the manuscript within the standard 4-month window. Overall, the problem is an interesting and exciting one and we look forward to considering a revised manuscript.

We believe our revised manuscript that includes new experimental data and text thoroughly addresses all concerns raised by the referees.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count for a Report is < 20,000; a full Research Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: A Report may include up to 5 main text figures; a full Research Article may have up to 10 main text figures. To avoid delays in production, figures must be prepared
according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Because our revised manuscript includes quite a bit of new experimental data as well as discussion and rationale requested by the referees, we have formatted our manuscript to be a Research Article. In particular, we have expanded the Discussion section to touch on many of the issues raised by the reviewers. If the editors feel our study is better suited as a Report, we can try to condense our manuscript back to the shorter format.

**Reviewer #1**

In this manuscript Chen et al are interested in the factors that determine the nuclear size in the early Xenopus embryo after the cell cycle slows at the MBT. The authors use droplets of cytoplasmic extract of controlled size from various time points along with microinjection into live embryos and biochemical fractionation to identify and verify Npm2 as a regulator of nuclear size at this stage. They demonstrate that droplets of different sizes and cytoplasmic ages result in different size nuclei with smaller droplets and older extracts resulting in smaller nuclei. They then attempt to purify factor responsible for setting the nuclear size and identify Npm2. Next they show that Npm2 injection can cause a modest increase in nuclear size and that this effect is increased by co-injection of importin a. Finally they show that Npm2 affects histone concentrations in the nucleus and link this to changes in chromatin state.

Overall I think that this is an interesting and important topic and that the approach and experiments presented are appropriate and reasonable. However, I do have several (I believe addressable) concerns about the interpretation and biological significance of these findings.

We appreciate the reviewer's support.
First, I am confused about the state of the stage 10-10.5 nuclei in vivo (before isolation). Are they still growing or do they reach steady state? If they have reached steady state then why do the same nuclei grow when placed into extract from the same stage as in Figure 1? I would assume that if the author’s model is correct and a nuclear factor had become limiting that that factor would be removed from the extract with the nuclei and that no droplet size made from a stage 10+ embryo could result in a larger stag 10 nucleus. If they are not yet in steady state after a one hour G2 arrest then how am I to think about the rest of the in vivo results in this manuscript (especially the importin results)? In that case what is the arrest length required to reach steady state in the embryo and how do the resultant nuclear sizes compare to those obtained in the droplets? It would be very helpful to know the Npm2 concentration in these extracts when the nuclei are removed to see if the remaining cytoplasmic Npm2 can explain the observed growth in these extracts. A related question is if you remove the steady state nuclei and add fresh (smaller) nuclei do they not grow at all?

We now include data showing that nuclei reach a steady-state size in stage 10 embryos arrested with cycloheximide. These data are described as follows:

Lines 102-105: To test this hypothesis, we isolated extract containing embryonic cytoplasm and endogenous embryonic nuclei from stage 10-10.5 embryos, which have average blastomere volumes of 0.07 nL and steady-state nuclear sizes (Fig. S1A).

We have also determined that stage 10 cytoplasm contains Npm2. When we form droplets that are similar in size to stage 10 blastomeres, the nuclei do not grow much, as expected. The nuclei only grow significantly when encapsulated in larger droplets of extract. These data are presented as follows:

Lines 186-191: We also determined that Npm2 is present in the cytoplasm of stage 10 extracts (Fig. S2D), potentially explaining why increasing droplet volume, and therefore the amount of available cytoplasmic Npm2, leads to nuclear growth. Although Npm2 lacks an obvious nuclear export signal, it likely still shuttles between the nucleus and cytoplasm to adopt a steady-state distribution (Kopito and Elbaum, 2007; Nguyen et al., 2019).

Why might Npm2 be found in the cytoplasm? While Npm2 lacks an obvious nuclear export signal, it has been shown that Npm2 actively shuttles between the cytoplasm and nucleus (PMID 17646647). It is also common for proteins with an NLS to be found in the cytoplasm such that dynamic, steady-state nucleocytoplasmic partitioning is achieved (PMID 26441354 and 26673895). In the Xenopus oocyte, most Npm2 is found in the nucleus, although a small fraction is also cytoplasmic. The likely reason for this is that oocytes exist for weeks or months, allowing a long period of time for Npm2 to partition to the nucleus. Stage 10 nuclei only persist for a few hours at most, potentially explaining why a significant fraction of the Npm2 is still cytoplasmic. Nonetheless, Npm2 can still become limiting for nuclear growth over development as the absolute amount of
Npm2 per cell decreases with cell size. Even though all Npm2 does not become nuclear-localized at a given time point, its absolute amount or shuttling capacity can still limit histone import and chromatin assembly.

With regard to the proposed experiment to remove nuclei from stage 10 extract and add-back fresh smaller nuclei, this is technically challenging. Stage 10 extracts contain hundreds of thousands of nuclei. We have experimented with different centrifugation conditions and can never completely remove the nuclei. Instead we have to dilute the extract prior to centrifugation, and extract dilution may compromise activities necessary for nuclear growth.

More broadly, the authors have shown that the total Npm2 is constant and that the nuclear concentration is falling. They propose (reasonably) that this is due to exhaustion of a cytoplasmic pool. This is a central point of their model. To actually show this it would be very helpful to see either the cytoplasmic (extract without nuclei) concentration falling and/or quantification of the total nuclear fraction (individual nuclear concentration multiplied by number of nuclei) increasing. I find their interpretation of their data plausible, but one more measurement is needed to fully support their claim.

We now show that the total nuclear fraction increases over development, and these data are presented in the manuscript as follows:

Lines 209-211: Interestingly, the total amount of nuclear Npm2 per embryo increases over development (Fig. S2G), indicating that Npm2 is progressively depleted from the cytoplasm as nucleus number increases.

I am most concerned about the interpretation of figure 5. This figure is critical because it brings in the biological significance of their findings and tries to tie the in vitro changes in nuclear size to actual developmental phenomena. However, I do not believe that chromatin relative area is an informative metric for chromatin state here because the denominator is the area of the nucleus and so it is necessarily true that if you divide the same amount of DNA (they are arrested in G2, which I assume means not replicating) by a larger area you will get a smaller chromatin relative area. The authors might choose another, less directly size sensitive metric for DNA homogeneity (perhaps even something as simple as pixel intensity distributions in the hoechst channel scaled by nuclear size.

We appreciate this suggestion and now include quantification of chromatin distribution as suggested in the main figures. The quantification approach and results are presented in the manuscript as follows:

Lines 259-267: To quantify chromatin distribution, we drew line scans through the middle of Hoechst-stained nuclei and measured the standard deviation of intensity values along these lines. We termed this parameter the "chromatin heterogeneity
index,” with larger values corresponding to more heterogeneous chromatin distributions. As nuclei grew in egg extract, the chromatin heterogeneity index increased (Fig. 5A). We also measured the area occupied by Hoechst-staining chromatin normalized to nuclear area and termed this parameter the “chromatin relative area” (Baarlink et al., 2017), finding that it decreased by more than 2-fold as nuclei expanded (Fig. S4B).

Lines 270-276: As per cell amounts of Npm2 decrease over development (Figs. 4D and S2F), we observed a reduction in both nuclear H2B staining intensity and the chromatin heterogeneity index, while chromatin relative area increased (Fig. 5B and S4C). In embryos microinjected to increase Npm2 levels, concomitant with an increase in nuclear size we observed more nuclear H2B staining, an increased chromatin heterogeneity index, and decreased chromatin relative area (Fig. 5C and S4D).

We have opted to retain the “chromatin relative area” measurements in Supplemental Information because we believe they are still informative. It is important to note that we measure the area occupied by the chromatin and not the Hoechst-staining intensity, so the chromatin area can differ depending on the level of compaction even if the total amount of DNA is the same. The “chromatin relative area” measurement is a well-established parameter that has been reported in a number of other studies (PMID 29131140, 25443297, 26541514, 28803781) so we feel it is worth including, especially in combination with our measurements of the “chromatin heterogeneity index.”

Better would be to see some sort of additional experiments to demonstrate consequences for chromatin organization or downstream events. For example, staining for disproportionate changes to the area occupied by specific chromatin marks/features, MNase digests to look for changes in nucleosome occupancy or spacing, or measurable differences in transcription. In some way, there needs to be more concrete evidence to tie their observations to larger biological consequences.

We have now performed MNase digestion assays and stained for euchromatin. These new data are described in the manuscript as follows:

Lines 277-284: To determine if these Npm2-induced changes in chromatin organization were due to altered nucleosome assembly, we performed micrococcal nuclease (MNase) digestion assays. Nucleosome occupancy was increased in embryos microinjected with either Npm2 protein or mRNA (Fig. 6A). Furthermore, this increase in chromatin compaction was associated with a reduction in histone H3 acetylation, indicating a reduction in euchromatin (Fig. 6B). Thus at the scale of both bulk chromatin and nucleosomes, Npm2 promotes chromatin compaction with concomitant increases in nuclear size.

The suggestion to examine differences in transcription is a good one but outside the scope of the current manuscript. At present, it would be difficult to dissect transcriptional effects resulting from altered nuclear size versus altered chromatin organization induced by Npm2. Our future work will focus on the downstream effects of altered nuclear size.
Finally, the sample sizes and statistical tests being used need to be made more clear in the text and figure legends. If all droplets are being treated as independent replicates is there a statistical justification for this (are droplets made from different extracts statistically distinguishable?) or are droplets only being compared within a given extract from the same day?

We have added some additional clarification to the Figure Legends regarding sample sizes and statistical tests applied. Generally we used a different extract for each droplet volume tested because it is technically difficult to test more than two droplet volumes using the same extract on the same day. Filling and imaging of each device takes several hours, and extract viability decreases over time, even on ice. However, we have now compared initial nuclear volumes among different extracts used in our experiments and they are not statistically different:

![Initial nuclear volumes for all spherical and flattened droplet experiments presented in Figure 1E.](image)

Two-tailed Student’s t-tests assuming equal variances: ns, not significant. Error bars represent SD.

For droplet experiments where we supplemented extract with recombinant Npm2, we did use the same extract supplemented with buffer for the control comparison, and this is noted in the Figure Legend for Fig. S3A.

**Minor points:**
1C and D, the volumes are not the same, so hard to compare where reach the steady state. If this data is available it would be helpful to plot the same size droplets so that the curves were more directly comparable.

Because of the difference in droplet shape, it is difficult to generate droplets with exactly the same volume. Also, for some droplet volumes, we do not have full time courses and only have data for intermittent time points until a final steady-state size is reached, allowing us to calculate the fold change in nuclear volume, which is the parameter we felt was most informative. The data we present for several representative droplet volumes in Fig. 1C-D span a similar range of sizes. We do not have the specific data requested, but Fig. 1E shows data for a wide range of droplet volumes that can be
directly compared, and Fig. S1C shows how nuclear growth speed varies as a function of droplet volume.

This sentence is confusing "After an incubation period, we observed that nuclei grew larger in ~0.8 nL spherical droplets compared to ~0.1 nL spherical droplets (Fig. 1B, Movie 1), while droplet volume did not change (Fig. S1A)." It needs to be made clear that droplet volume did not change over time not between conditions.

We clarified the text as follows:

Lines 108-110: After an incubation period, we observed that nuclei grew larger in ~0.8 nL spherical droplets compared to ~0.1 nL spherical droplets (Fig. 1B, Movie 1), while droplet volume did not change over time (Fig. S1B).

In the presentation of figure 4 in the text it is important to note what stage the embryos were analyzed at.

We clarified the text as follows:

Lines 192-193: To test if Npm2 levels affect nuclear size in vivo, we microinjected one-cell X. laevis embryos and analyzed nuclei at stage 10-10.5.

In several figures the authors are plotting fold change and not absolute volume or delta volume. This choice makes it difficult to compare that data to the other data that is presented in absolute volume. Is there a reason that this data must be plotted as a fold change?

While initial nuclear sizes are generally similar across different stage 10 embryos and extracts, there can be some slight variability. For this reason, when comparing many different conditions and combining multiple data sets, we believe it is more accurate to plot fold change so as to normalize for this inherent variability. We include some representative plots of absolute nuclear volume to give the reader a sense of the actual nuclear sizes. In fact, plotting delta volume versus fold change does not make a substantial difference in the trends and appearance of the data as shown by this representative delta volume plot for Fig. 1E:
In figure 4F what are the statistical comparisons between? Actually, this could be clarified for all of the statistics in the figures.

This has been clarified in the legends for Fig. 4F, S1F-G, S3G, S4A.

I'd very much like to see more discussion of the effect of importin a on steady state nuclear size/Npm2 concentration. This result seems to imply that passive or active export of Npm2 has a large effect on final volume. Since Npm2 is in a fairly large complex, I wouldn't have expected passive nuclear export to be a major issue. Is Npm2 being actively shuttled? Truly answering these questions is beyond the scope of this paper, but some discussion of the implications of this result would be appropriate.

It has been shown that Npm2 exhibits dynamic nucleocytoplasmic shuttling (PMID 17646647) and this issue was discussed above in response to the first Major Point. One possibility is that importin α positively regulates the import of other cargos, in addition to Npm2, that are important for nuclear size control. Alternatively, importin α directly increases Npm2 import to drive nuclear growth. These issues are now discussed in some detail as follows:

Lines 340-356: We observed additive effects when Npm2 levels were manipulated along with importin α. It is worth noting that increasing importin α levels alone enhances nuclear growth in pre-MBT embryos (Jevtic and Levy, 2015; Levy and Heald, 2010) but not in post-MBT embryos where overexpression of both importin α and Npm2 was required. Perhaps import limits nuclear growth in early embryos while the availability of nuclear-sizing cargos becomes limiting later. Npm2 does not possess an obvious nuclear export signal and stage 10 nuclear size is not sensitive to leptomycin B treatment (Edens and Levy, 2014), suggesting active export is not a major determinant of nuclear size at this developmental stage. However, Npm2 has been demonstrated to shuttle between the cytoplasm and nucleus (Kopito and Elbaum, 2007), as have many histone chaperones (Keck and Pemberton, 2013), and it is common for proteins with an NLS to exhibit dynamic nucleocytoplasmic partitioning even at steady-state (Kirli et al., 2015; Nguyen et al., 2019; Wuhr et al., 2015). Given these observations, increased importin α may drive nuclear growth by directly increasing Npm2 nuclear import, although importin α likely also promotes import of other nuclear-sizing cargos secondarily to Npm2, such as lamin B3.

Reviewer #2

The manuscript by Chen et al. investigates the role of cytoplasmic volume in nuclear size scaling. As is explained by the authors, mechanisms that determine the scaling of cells and organelles remains an interesting and fundamental question in biology. Thus, this work will be of general interest. The authors leverage the Xenopus model and an innovative approach by encasing nuclei in different sized oil droplets containing Xenopus embryo cytosol. After an incubation period the nuclei grow and reach a
steady-state size that mirrors the cytosolic volume (i.e. the larger the volume the larger the nuclei). In some of the more compelling experiments, the authors further demonstrate that specific contents of the cytosol derived from different stage embryos impacts the size scaling of the nuclei. These data clearly suggest that there are specific cytosolic factors that ultimately limit nuclear growth. By using a subcellular fractionation scheme and mass spectrometry protein identification the authors identify one such factor to be nucleoplasmin, a histone chaperone. They further establish that the size-scaling function of nucleoplasmin likely requires its nuclear import and its histone-binding capability. Thus, overall, this study introduces an innovative approach to exploring size-scaling and a new factor that contributes to nuclear size determination in at least Xenopus embryos. I do have some concerns regarding the quality of some of the biochemical data and the lack of depletion experiments, which detract from the manuscript's clarity and impact.

We appreciate the reviewer's support.

Major Points:

1) Lack of detail/explanation of biochemical fractionation: While the rationale behind the biochemical fractionation experiments is clear, how proteins were identified in monoQ fractions is not. It would greatly help the reader, for example, to have a better understanding of what the "proteomic analysis" consists of. The authors use terms like "highly enriched" but it is not clear what metric is used for this assessment (what is the rationale behind the order of the proteins listed in S1G?).

We have now provided additional details about how proteins were identified after fractionation as follows:

Lines 176-181: We ultimately identified two active fractions (Fig. 3D), and we determined the protein composition of these fractions by mass spectrometry (Fig. S2A-B). Because the nuclear growth activity was dependent on nuclear import (Fig. 3A-B), we focused on the most abundant proteins with nuclear localization signals. We were struck by the fact that nucleoplasmin (Npm2) was identified in both fractions (Fig. 3D, S2B-C).

Lines 1167-1175: Proteins in fractions 2 and 9 from the biochemical fractionation shown in Figure 3 were identified by mass spectrometry. The mass spectrometry analysis provided a rough approximation of the relative abundance of each protein in each fraction, based on the sum intensity of the peptides for a given protein. Three and two independent fractionation samples were analyzed for fractions 2 and 9, respectively. For a given fraction, we identified the most abundant NLS-containing proteins present in all samples. We list these proteins in order of abundance. Proteins present in trace amounts with peptide sum intensities less than 4x10^5 were excluded from the list.
Moreover, in the methods it is indicated that individual bands are cut out of SDS-PAGE gels suggesting there are a limited number of protein components in each fraction. This is great but it would be nice to see these gels of the fractions in supplemental data; a Western blot for anti-Npm2 would also be appropriate for every fraction not just number 2 to see how Npm2 segregates.

We now include images of the Coomassie-stained gels corresponding to representative protein fractions subjected to mass spectrometry analysis in Fig. S2A.

Lines 1163-1167: Representative fractions 2 and 9 from the biochemical fractionation shown in Figure 3 were separated on 10% SDS-PAGE gels, and the gels were stained with Coomassie. Gel slices 2A, 2B, and 9A were subjected to mass spectrometry analysis for protein identification. Proteins were also identified from two other fraction 2 samples and one other fraction 9 sample.

We also include an anti-Npm2 western blot for every fraction from one representative fractionation experiment in Fig. S2C showing that Npm2 elutes in two distinct complexes.

Lines 1178-1185: Six µl of each Mono Q fraction after dialysis and concentration were separated on an SDS-PAGE gel and probed for Npm2 by western blot. Mass spectrometry identified all four core histones in fraction 2 but not fraction 9 (data not shown). Note that the MonoQ fractions with peak activity varied slightly between the three different fractionation experiments. Generally, peak activity was identified in fractions 2 and 9, while in the representative western blot the highest amounts of Npm2 were found in fractions 2 and 10.

These points are particularly relevant to explaining why Npm2 is found in at least two very different fractions. The authors suggest this could be due to its interaction with histones, but they need to explain why there aren't any histones identified in the MS data.

In fact, the mass spectrometry data did identify all four core histones in the lower salt elution, however the abundance was relatively low so we did not include them on the list (see above). We thank the reviewer for drawing our addition to this omission. We have revised the text as follows to clarify this issue:

Lines 183-186: We suspect that it eluted in two different ion exchange chromatography fractions due to its association with different protein complexes. Consistent with this idea, mass spectrometry identified core histones in only the lower salt Npm2 elution (data not shown).

Lines 1180-1181: Mass spectrometry identified all four core histones in fraction 2 but not fraction 9 (data not shown).
I would also appreciate a clear rationale behind why Npm2 was chosen over anp32b, which is also found in both fractions. Ideally, this would also have been carried along as a specificity control in the scaling assays.

We focused on Npm2 for a number of reasons. First, Npm2 is a well studied histone chaperone. The structure has been solved and mutants have been identified that are deficient for histone binding and nuclear import. This greatly facilitated our functional studies of how Npm2 influences nuclear size. Second, Anp32b protein levels increase over development, which is generally inconsistent with a limiting component model of developmental nuclear size scaling (PMID 26555057). This rationale is briefly mentioned in the manuscript as follows:

Lines 1175-1178: We focused on Npm2 over Anp32b because Npm2 is well studied and because Anp32b protein levels increase over development, which is generally inconsistent with a limiting component model of developmental nuclear size scaling (Peshkin et al., 2015).

Third, the amount of experimental work required to test Anp32b is beyond the scope of the current study. We would have to essentially repeat all in vitro and in vivo experiments in the paper with Anp32b, which could require a year or more of work. Ultimately, in our opinion, these data would not substantially improve the paper. If Anp32b influences nuclear size, that would suggest that histone chaperones are general nuclear size effectors. If Anp32b does not affect nuclear size, then that would suggest the effect may be more specific to Npm2, although it would be necessary to test additional histone chaperones. Either result would not substantially change the overall message of the paper. As discussed in the manuscript, there are likely many limiting factors and this paper provides detailed insight into one. That being said, we agree that it will be important in the future to establish whether histone chaperones generally influence nuclear size and we now mention this idea as follows:

Lines 429-437: Because Npm2 belongs to a large family of nucleophosmin/nucleoplasmin histone chaperones that are conserved throughout metazoans (Frehlick et al., 2007), it will be important to determine if other histone chaperones impact nuclear size and whether such mechanisms are conserved in other systems. Of note, 8-cell stage Npm2-null mouse embryos exhibited smaller nuclei compared to wild-type as well as a loss of heterochromatin and altered chromatin organization (Burns et al., 2003). Interestingly, our biochemical fractionation identified a number of proteins with histone chaperone activity.

2) Lack of depletion experiments: To test the role of Npm2 in nuclear-size scaling, the authors rely on microinjection of excess Npm2 into embryos. This leads to variations in Npm2 staining in nuclei, which complicates the analysis and interpretation. It would greatly strengthen the work to deplete Npm2 from cytosolic extracts using the anti-Npm2 antibody in droplet experiments. This is particularly relevant as the major
conclusion of the work is based on a limiting cytosolic factor(s) contributing to nuclear size.

We have now performed immunoinhibition experiments in extract using two different neutralizing Npm2 antibodies. Both antibodies reduced nuclear growth. These new data are described in the manuscript as follows:

Lines 237-240: To address potential caveats of increasing Npm2 amounts, we inhibited Npm2 by supplementing extract with Npm2 neutralizing antibodies, which resulted in a modest reduction in nuclear growth and size (Fig. S3H).

Minor Points in need of clarification:

Figure 2D: The change in approach from the droplet to the egg extracts could be better explained for the non-expert.

The following explanations have been added:

Lines 157-165: Because our data suggested that one or more cytoplasmic components are limiting for nuclear growth, we undertook a biochemical fractionation approach to identify putative limiting components. We reasoned that X. laevis egg extract would be a good starting material rather than stage 10 embryo extract because: 1) fractionation necessitates large extract volumes and it is straightforward to obtain sufficiently large volumes of egg extract but not embryo extract, 2) egg extract contains large amounts of stockpiled maternal proteins, and 3) unencapsulated egg extract induces significant growth of stage 10 nuclei (Fig. 2D), providing a relatively facile assay for nuclear sizing activities without time-consuming microfluidic encapsulation.

Also, in multiple figures, "NPC" is a bit confusing as a label. It should be "NPCs" or preferably denote that the staining is mab414. At a minimum, please indicate in figure legends that mAB414 was used here.

The labels have been changed to: NPCs. The NPC antibody mAb414 is now mentioned in the relevant figure legends.

Figure 3A-B: The purpose and results presented in these figure panels needs to be better explained. It is not obvious, particularly for the non-expert, what the IBB domain achieves, nor WGA as neither of these are even mentioned in the text.

We have added the following additional explanations:

Lines 165-172: We first performed high-speed fractionation of egg extract and determined that the nuclear growth inducing activity was present in cytosol but not in the
heavy or light membrane fractions (Fig. 3A). We also determined that nuclear growth was dependent on importin α/β mediated import because cytosol treated with the importin β binding domain of importin α (IBB) failed to induce growth of stage 10 nuclei (Fig. 3A). Consistent with this finding, nuclei also failed to grow in stage 10 extract droplets when import was blocked with IBB or wheat germ agglutinin (Fig. 3B).

Lines 1012-1014: IBB, added at ~30 µM, is the importin β binding domain of importin α and inhibits nuclear import (Weis et al., 1996).

Lines 1016-1018: Experiments were performed as in Figure 1C-E except that extracts were supplemented with IBB or wheat germ agglutinin (WGA) to block nuclear import (Cox, 1992).

Figure 4B: please clarify the metric for “high Npm2” staining.

The metric for “high Npm2” staining is described in the figure legend as follows:

Lines 1047-1049: Orange bars represent sizes for nuclei with Npm2 staining intensity values greater than one standard deviation above the XB control (see also Figure 4C).

Lines 1051-1061: Nuclei from microinjections described in (B) were stained with an antibody against Npm2. Nuclear volumes and total nuclear Npm2 staining intensities were measured for at least 330 nuclei per condition per experiment. 25 embryos on average were microinjected per condition, and two independent experiments were performed. For the Npm2+importin α condition, individual nuclear volume was plotted as a function of nuclear Npm2 staining intensity. For XB-microinjected embryos, nuclear Npm2 staining intensity was 2.05 ± 1.35 (average ± SD). In Figure 4B and S4D, the orange bars represent data for nuclei with Npm2 staining intensity values greater than one standard deviation above the XB control, therefore nuclei with Npm2 staining intensities greater than 2.05 + 1.35 = 3.4 (indicated by the open bracket on the x-axis in Figure 4C).

Figure 4C. The connection between importin-alpha and Npm2 is not clear. Can the authors clarify whether Npm2 is actually imported by importin alpha? Also, were this the case, importin alpha alone cannot import cargoes without importin-beta. I would appreciate some rationale for the specificity of importin-alpha (even if this isn't explicitly tested). In the future, it might be useful to try an additional NLS not recognized by importin alpha to explicitly test whether import, or importin-alpha-mediated import, is what is required for nuclear size scaling.

Npm2 is imported by importin α. We now mention this and cite a relevant review, to avoid citing the large number of primary literature references necessary to support this statement.
Npm2 is a known importin α/β cargo (Kim et al., 2017), and nuclear size was unaffected in embryos microinjected with Npm-core lacking the NLS (Fig. 4A), demonstrating that nuclear import of Npm2 is required to have an effect on nuclear size.

Previous studies have implicated importin α, but not importin β, as a developmental regulator of nuclear size scaling. Importin β has been explicitly tested in *Xenopus* extract and shown to not induce nuclear growth (PMID 20946986), possibly because importin β is in excess of importin α (PMID 26555057). We now briefly mention this as follows:

We focused on importin α because Npm2 is an importin α/β cargo (Kim et al., 2017) and reductions in the cytoplasmic levels of importin α, but not importin β, contribute to developmental nuclear size scaling (Brownlee and Heald, 2019; Levy and Heald, 2010; Wilbur and Heald, 2013).

With regard to other cargos, it was shown that addition of transportin to *Xenopus* extract did not lead to increased nuclear growth (PMID 20946986). Furthermore, import of M9, a transportin cargo, did not scale with nuclear size in *Xenopus* extracts (PMID 20946986), consistent with nuclear size scaling acting predominantly through importin α/β-mediated import. In support of this idea, we show that IBB blocks nuclear growth (Fig. 3A-B), and IBB specifically inhibits importin α-mediated nuclear import (PMID 8617227).

*Figure 4D: There is a bit of a disconnect between the 4.2 uM indicated in the text and the "relative [Npm2]" in the Figure.*

We changed the relative concentrations to absolute concentrations in this figure panel.

The statement "Npm2 mutants defective for histone binding and/or histone chaperone activity, but still import-competent" need a reference or explicit experiments showing that these mutants are still imported into the nucleus.

For mRNA microinjections, the wild-type and mutant Npm2 constructs are 6xHis-tagged. We isolated nuclei from microinjected embryos and performed immunofluorescence using an anti-6xHis antibody. Nuclei from embryos microinjected with wild-type and mutant Npm2 stained similarly for 6xHis, as shown in the following representative images:
We now mention this result in the manuscript as follows:

Lines 1067-1070: Mutants 1 and 2 have mutations in the histone-binding region of Npm2 (Warren et al., 2017), and mutant 3 is defective in histone chaperone activity (Salvany et al., 2004). We verified that these mutants are nuclear import-competent (data not shown).

Reviewer #3

The authors have shown previously, using Xenopus embryos and cell-free extracts, that nuclear size can be controlled in this system by the levels of importin alpha and lamin B3, and also by protein kinase C. Here they further investigate this model, using a microfluidics-based approach for analyzing nuclei incubated in different volumes of cytoplasm. They have focused on cells at the onset of gastrulation, when cytoplasmic and nuclear size are diminished as compared to earlier stage embryos. They show that incubating nuclei from stage 10 embryos with cytoplasm from progressively earlier stages resulted in correspondingly greater increases in nuclear volume during in vitro incubation, suggesting a limiting factor. Based on biochemical fractionation of egg cytosol, they show that the abundant histone chaperone nucleoplasmin (Npm2) was one of several components of egg extract that results in increased nuclear growth of stage 10 nuclei; nuclear growth also was obtained with importin alpha and lamin B3. An increased size of stage 10 nuclei also occurred when Npm2 was injected into embryos at the onset of development. The authors provide evidence that the activity of Npm2 in these assays requires the histone-binding activity of Npm2, and not simply its acidic properties. Since nuclei arising from supplemented Npm2 showed increased areas with no DNA staining, they speculate that the increased nuclear volume arises from Npm2-induced changes in chromatin structure. This overall is a solid study, but would benefit from changes:
We appreciate the reviewer's support.

1) The authors should clarify (Figs. 1-2) why nuclear volume increased more when nuclei were incubated with increasing volumes of cytoplasm. Is the number of nuclei per unit volume smaller with increasing droplet size? This would be consistent with Fig. S1C. These two figures could be condensed, as they seem somewhat disconnected from the remainder of the manuscript.

Unless otherwise noted as in Fig. S1D, all droplets contained only one nucleus. We vary the cytoplasmic volume, and presumably the amounts of Npm2 and other limiting components, not the number of nuclei. We have clarified this in the text as follows:

Lines 107-108: Unless otherwise noted, we only analyzed droplets containing one nucleus.

As to the question of why nuclear volume increased more in larger cytoplasmic volumes, we now show that stage 10 cytoplasm contains Npm2, and these data are presented as follows:

Lines 186-191: We also determined that Npm2 is present in the cytoplasm of stage 10 extracts (Fig. S2D), potentially explaining why increasing droplet volume, and therefore the amount of available cytoplasmic Npm2, leads to nuclear growth. Although Npm2 lacks an obvious nuclear export signal, it likely still shuttles between the nucleus and cytoplasm to adopt a steady-state distribution (Kopito and Elbaum, 2007; Nguyen et al., 2019).

We appreciate the recommendation to combine Figures 1 and 2, however each figure contains a lot of data and combining them would likely render some of the text and images illegible.

2) Do nuclei assembled in vitro from sperm (vs stage 10 nuclei), when incubated with egg extract supplemented with recombinant Npm2, also grow larger than controls? This would indicate that the amount of Npm2 is limiting even with very large cytoplasmic/nuclear ratios, and would argue that Npm2 is not necessarily an important limiting determinant in development. In this regard, it would be valuable to see the effects of depleting Npm2 on in vitro growth of nuclei.

The proposed experiment is a good one that has already been published. Adding a range of Npm2 concentrations (0.14 – 19 µM) to nuclei assembled in unencapsulated Xenopus egg extract had no effect on nuclear size (PMID 20946986). The likely explanation is that Npm2 is in large excess in the egg and early embryo, so adding more Npm2 has little effect. We agree that this indicates that Npm2 is not limiting early in development but only becomes limiting later. We now discuss this issue as follows:
Why might Npm2 become physiologically limiting in vivo in post-MBT embryos? In the egg, Npm2 binds to and sequesters maternal core histone stores (Onikubo et al., 2015). In pre-MBT embryos, Npm2 and histones are presumably in large excess to the number of nuclei, which explains why supplementing Xenopus egg extract with recombinant Npm2 did not increase nuclear size (Levy and Heald, 2010). At the MBT, while the Npm2 concentration remains constant, core histone levels increase (Peshkin et al., 2015; Sun et al., 2014), meaning the Npm2:histone ratio decreases in post-MBT embryos. Furthermore, bulk nuclear import kinetics decrease over development (Levy and Heald, 2010). As a result, limiting amounts of Npm2 are imported more slowly, correlating with reductions in nuclear size and consistent with limiting Npm2 and import both contributing to nuclear size-scaling over development.

The Npm2 depletion experiment is also a good one. We have now performed immunoinhibition experiments in extract using two different neutralizing Npm2 antibodies. Both antibodies reduced nuclear growth. These new data are described in the manuscript as follows:

To address potential caveats of increasing Npm2 amounts, we inhibited Npm2 by supplementing extract with Npm2 neutralizing antibodies, which resulted in a modest reduction in nuclear growth and size (Fig. S3H).

3) There clearly are two populations of stage 10 nuclei. What is different about those that grow more strongly and import more Npm2 and histone? Is there nucleus-to-nucleus heterogeneity in the number of nuclear pores from stage 10 embryos? This would alter the model somewhat and could suggest that the most proximal cause of the size effects results from differences in nuclear transport.

We primarily noted these two populations of nuclei with differential Npm2 staining only after microinjecting embryos with recombinant Npm2 protein. We suspect this is due to incomplete diffusion of microinjected Npm2 protein within the embryo, such that some blastomeres received more Npm2 protein than others. We mention this in the text as follows:

For embryos microinjected with Npm2 protein, Npm2 immunofluorescence revealed two populations of nuclei: one group with control level Npm2 staining and one group with on average ~4-fold more intense Npm2 staining (Fig. 4A), suggesting that microinjected Npm2 protein was not evenly distributed across the one-cell embryo.

To test if Npm2 and NPC staining intensity were positively correlated, we co-stained nuclei isolated from Npm2-microinjected embryos for Npm2 and NPCs using mAb414. We found that nuclei with stronger Npm2 staining in fact exhibited somewhat reduced NPC staining, as shown below:
This argues against the idea that increased levels of nuclear Npm2 result from more nuclear pores that accommodate increased Npm2 import. We now discuss this model as follows:

Lines 361-369: While Npm2 influences nuclear size independently of transcription, open questions remain about precisely how Npm2 promotes nuclear growth. NPC assembly is known to require nucleosomes (Inoue and Zhang, 2014; Zierhut et al., 2014), so one possibility is that increased nucleosome assembly by Npm2 leads to larger numbers of NPCs and increased nuclear import capacity. We disfavor this model because growth of stage 10 nuclei with egg cytosol did not lead to an increase in NPC density, and NPC staining intensity was not significantly altered in Npm2-microinjected embryos and was not greater for nuclei with high Npm2 staining (data not shown).

4) Does Npm2 have direct effects on chromatin, such as on nucleosome packing? Is it possible that high Npm2 has nonphysiological effects on chromatin by competition of other structural proteins?

We have now performed MNase digestion assays and stained for euchromatin. These new data are described in the manuscript as follows:

Lines 277-284: To determine if these Npm2-induced changes in chromatin organization were due to altered nucleosome assembly, we performed micrococcal nuclease (MNase) digestion assays. Nucleosome occupancy was increased in embryos microinjected with either Npm2 protein or mRNA (Fig. 6A). Furthermore, this increase in chromatin compaction was associated with a reduction in histone H3 acetylation, indicating a reduction in euchromatin (Fig. 6B). Thus at the scale of both bulk chromatin and nucleosomes, Npm2 promotes chromatin compaction with concomitant increases in nuclear size.

We would argue that the Npm2 concentrations tested in our studies are physiological. In general, we increased the Npm2 concentration by only ~2-fold. In terms of absolute amounts of Npm2 per cell, this is much less than is found at earlier normal stages of
development. Concerns about nonphysiological effects of Npm2 overexpression are further alleviated by the inclusion of Npm2 immunoinhibition data showing a reduction in nuclear size (Point 2 above). Nonetheless, we agree that competition of other structural proteins is a potential caveat of our Npm2 overexpression experiments, and we briefly mention this idea as follows:

Lines 391-395: Why might increased nucleosome assembly and chromatin compaction promote nuclear growth? While changes in nucleosome assembly might affect chromatin-NE interactions and/or chromatin-association of linker histones and chromatin binding/remodeling proteins, it is also well-established that more condensed chromatin is stiffer (Chalut et al., 2012; Stephens et al., 2017; Stephens et al., 2018).

Overall, this manuscript supports the notion that chromatin structure could be an important determinant of nuclear size. This point already is well-established in other systems: a number of papers have shown that depletion of proteins involved in chromatin compaction results in nuclear size increase in various experimental models, ranging from Tetrahymena micro/macro nuclei to mammalian neurons, lymphocytes and ESCs (reviewed by Bustin and Misteli, Science, 2016). Considering this, and the inconclusive mechanistic insight on the Npm2 effects, the level of new scientific advance obtained in this manuscript is relatively modest.

First, we hope the reviewer agrees that our new data showing how Npm2 affects nucleosome packing and euchromatin (Point 4 above) provides additional mechanistic insights into how Npm2 regulates nuclear size.

With regard to previous studies implicating chromatin structure in nuclear size determination, we will first summarize the major findings in the papers referenced by the reviewer.

Shen et al. (1995) show in Tetrahymena that deletion of micronuclear or macronuclear histone H1 leads to nuclear enlargement of only the micronucleus or macronucleus, respectively. They did not quantify changes in chromatin structure. Presumably chromatin decondensation induced by loss of H1 led to nuclear enlargement, which is the opposite of our result where Npm2 led to increased chromatin compaction and nuclear size.

Rawlings et al. (2011) show that T-cell activation leads to a more open chromatin structure, and that chromatin condensation during thymocyte development is abrogated in a condensin II mutant. They did not quantify nuclear size under these various conditions.

Fazzio et al. (2010) show that knockdown of condensin subunits leads to nuclear enlargement and chromatin decompaction in ES cells. Again opposite to our observations, they correlate chromatin decompaction with increased nuclear size.
George et al. (2014) show that knockdown of condensin subunits in cultured *Drosophila* and HeLa cells disrupts nuclear architecture and increases nuclear size.

Furusawa et al. (2015) show that HMGN5 overexpression leads to chromatin decompaction and nuclear enlargement in the heart, likely by counteracting the compacting activity of histone H1.

Yazdani et al. (2012) show that neuronal progenitor nuclei do not grow as much in the absence of MeCP2. They did not explicitly examine chromatin structure.

A key difference between our manuscript and these other studies is that we find that Npm2-induced chromatin compaction drives nuclear growth while these other studies describe a correlation between chromatin decompaction and increased nuclear size. There are a number of possible explanations for these differences. 1) Differences in the species, cell type, or developmental stage. 2) Differences due to manipulating a histone chaperone as opposed to chromatin structural and binding proteins like histone H1, condensin, and MeCP2. 3) These other studies delete or knockdown major chromatin structural and binding proteins that might be expected to induce cell cycle delays, which can artificially increase nuclear size. So it is possible that delayed cell cycle progression indirectly leads to increased nuclear size, rather than chromatin decompaction directly driving nuclear growth. Our in vitro experiments can exclude these potential indirect cell cycle effects. 4) Manipulating the levels of chromatin structural and binding proteins might be expected to affect gene expression, that could also indirectly impact nuclear size. For instance, Yazdani et al. found that transcription was reduced in the absence of MeCP2. In our study, we can alleviate concerns about indirect transcriptional effects on nuclear size because we show that Npm2 still increases nuclear size in the presence of α-amanitin and because Npm2 inhibition decreases nuclear size in *Xenopus* egg extracts that are transcriptionally inert. 5) Because these other studies delete or knockdown major chromatin structural and binding proteins, this likely leads to extensive chromatin decompaction. While this large increase in DNA volume might drive nuclear growth, this situation is perhaps artificial because such a large-scale chromatin decompaction might not occur under normal physiological conditions. In our study, we vary Npm2 levels within a normal physiological range and induce more subtle changes in chromatin compaction. Under these conditions, we find that a subtle increase in chromatin compaction also drives increased nuclear growth. As we are studying this process during *Xenopus* development, the underlying mechanism we describe may be more relevant to the regulation of nuclear size in an unperturbed cell or organism. Thus there might be two distinct regimes of chromatin packing that both lead to increased nuclear size: extreme decompaction and more subtle compaction might both drive nuclear growth, with the latter dominating under normal physiological conditions.

To summarize all of these issues, we have added the following text to the Discussion:

Lines 375-390: *We favor a model in which Npm2 induces nuclear growth by increasing nuclear histone localization, nucleosome assembly, and chromatin compaction. Previous studies have shown that large-scale chromatin decompaction induced by*
knockdown of histone H1, condensins, or MeCP2 can lead to nuclear enlargement (Bustin and Misteli, 2016), while we find that Npm2-induced chromatin compaction drives nuclear growth. Potential explanations for these differing results include: 1) differences in cell type or developmental stage, 2) differing effects of a histone chaperone as opposed to bulk chromatin structural and binding proteins, or 3) indirect effects of protein knockdown on nuclear size resulting from delayed cell cycle progression or altered gene expression. It is also possible that there are two distinct regimes of chromatin packing that both lead to increased nuclear size. Extreme decompaction induced by knockdown of chromatin structural and binding proteins might increase nuclear size because the DNA occupies a much larger volume. On the other hand, subtle chromatin compaction by Npm2 under more normal physiological conditions might also drive nuclear growth. Regardless of the explanation, we have identified Npm2 as a novel chromatin-based regulator of nuclear size.

Lines 413-420: Our model that Npm2 is a nuclear size-scaling factor over development is consistent with known developmental changes in histone occupancy and transcription. As embryos approach the MBT and total DNA content in the embryo rapidly increases, histone titration is one mechanism that contributes to the upregulation of zygotic transcription (Amodeo et al., 2015; Onikubo and Shechter, 2016). Reduced nucleosome assembly and increased transcription at the MBT correlate with less condensed chromatin (Bogdanovic et al., 2012; Hontelez et al., 2015), which by our model is predictive of reduced nuclear size during developmental progression.
Dear Dr. Levy,

Thank you for submitting your revised manuscript entitled "Cytoplasmic volume and limiting components scale nuclear size during Xenopus laevis development". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that two of reviewers are positive but Reviewer #1 has some remaining concerns that should be addressed, such as the sample size and normalization queries. We also agree with the reviewer comments recommending revisions to the text and that the discussion should be more clear, terse and focused, but we feel the paper fits with remaining formatted as a short Article.

- Please provide a short eTOC blurb
- Provide main and supplementary texts as .doc or .docx files
- Provide main and supplementary figures as separate, editable files according to the instructions for authors on JCB's website *paying particular attention to the guidelines for preparing mages and blots at sufficient resolution for screening and production*
- Suggested alternative title to make the advance clear to as broad an audience as possible? "nucleophosmin is a limiting component in the scaling of nucleus size with cytoplasmic volume"
- Add conflict of interest statement to "Acknowledgements" section

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

In the revised manuscript Chen et al. have added several additional experiments and discussion to address the issues of the state of the stage 10 nuclei and the biological consequences of the size changes observed in response to alterations in Npm2. In particular they measure the amount of Npm2 in the nuclei at different stages, add a better metric for nuclear inhomogeneity, and assess the effects of Npm2 over expression on both nucleosome occupancy and histone acetylation. They have also added greater detail about the biochemical purification as well as clarified their statistical comparisons and added significant discussion of both the caveats and potential interpretations of their findings.

Overall, I the authors have addressed the majority of my concerns, though the new experiments and discussion have a raised a few new issues that I think should be addressed.

The additional measurements and discussion of import and export have convinced me that their model is reasonable for how the nuclei can be at steady state in a given volume and yet still change size when volume is changed in stage 10. I also appreciate their new metric for chromatin homogeneity and find it compelling that changes in Npm2 result in changes in the chromatin environment within the cell.

The MNase results are very exciting and add a lot to my understanding of potential mechanisms of how this process might work. However, it looks like these experiments were only done once each (?) and the results are fairly subtle in the quantifications. I would like to see a few more replicates and statistics.

Also in Figure 6B, it looks like they are plotting average pixel intensity, not total integrated signal (either way they need to be more clear what "Euchromatin staining intensity" means). If this is average pixel intensity in just the H3Ac stained channel (not normalized to DNA), then this has the same problem as the chromatin occupied fraction in that the same amount of staining in a smaller volume will give a greater pixel intensity by definition. To say that the acetylation state of H3 is actually being changed they either need to normalize to a DNA stain or take the total integrated staining intensity for an entire nucleus. If this is what they have done, then the data are compelling, and the figure legend simply needs to be clarified. If not, at least one of these two normalizations must be done. If this is impossible due to the way that the data was collected, I think that the work stands without this particular experiment. So, while I do think that this is interesting and important, I think that the paper would be stronger with H3Ac experiment removed entirely than presented without proper normalization.

Finally, the discussion has become overwhelming and unfocused. I appreciate the speculation about
possible mechanisms, however it would be much more readable in a more concise, condensed form that focuses more narrowly on a few key points.

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

The authors were able to address the majority of my criticisms. I support publication of this interesting work.

Reviewer #3 (Comments to the Authors (Required)):

The authors have addressed the points I raised in my earlier review to my satisfaction, and the present manuscript is clearly improved. The discussion about chromatin stiffness related to increased nuclear volume is particularly interesting, as a more compact chromatin state might be predicted to decrease nuclear volume (opposite from the results seen). That said, the current Discussion section is lengthy, and would be improved by shortening.
Dear Aaron and Marie,

We are submitting our revised manuscript "Nucleoplasmin is a limiting component in the scaling of nuclear size with cytoplasmic volume" by Pan Chen, Miroslav Tomschik, Katherine Nelson, John Oakey, Jay Gatlin, and myself for consideration as a JCB Research Article. This manuscript is an updated version of JCB manuscript #201902124R.

We were happy to see that all reviewers are generally supportive of publishing our work. We have now addressed the remaining concerns raised by Reviewer #1 and shortened the discussion. Our point-by-point responses follow. We hope you agree that our manuscript has now reached a level appropriate for publication in JCB. Please let me know if you have any additional questions or concerns.

Sincerely,

Daniel Levy

Daniel Levy
Point-by-point response to reviews

The comments of the editors and reviewers are copied here in italics. Our responses are just below each point raised by the editor or reviewer.

Editors

Thank you for submitting your revised manuscript entitled "Cytoplasmic volume and limiting components scale nuclear size during Xenopus laevis development". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that two of reviewers are positive but Reviewer #1 has some remaining concerns that should be addressed, such as the sample size and normalization queries. We also agree with the reviewer comments recommending revisions to the text and that the discussion should be more clear, terse and focused, but we feel the paper fits with remaining formatted as a short Article.

We appreciate the continued support for our work. We have addressed the remaining concerns raised by Reviewer #1 and shortened the discussion by >30% (see below).

- Please provide a short eTOC blurb

How is nuclear size regulated relative to cell size? Microfluidic encapsulation of Xenopus embryo extracts, biochemical fractionation, and in vivo experiments demonstrate that reductions in cytoplasmic volume and limiting components, including the histone chaperone nucleoplasmin, contribute to developmental nuclear-size scaling.

- Provide main and supplementary texts as .doc or .docx files

Uploaded.

- Provide main and supplementary figures as separate, editable files according to the instructions for authors on JCB's website *paying particular attention to the guidelines for preparing mages and blots at sufficient resolution for screening and production*

Uploaded.

- Suggested alternative title to make the advance clear to as broad an audience as possible? "nucleophosmin is a limiting component in the scaling of nucleus size with cytoplasmic volume"

We have changed the title as suggested. Note that our study deals with nucleoplasmin rather than nucleophosmin.
- Add conflict of interest statement to “Acknowledgements” section

Added.

Reviewers

Reviewer #1:

In the revised manuscript Chen et al. have added several additional experiments and discussion to address the issues of the state of the stage 10 nuclei and the biological consequences of the size changes observed in response to alterations in Npm2. In particular they measure the amount of Npm2 in the nuclei at different stages, add a better metric for nuclear inhomogeneity, and assess the effects of Npm2 over expression on both nucleosome occupancy and histone acetylation. They have also added greater detail about the biochemical purification as well as clarified their statistical comparisons and added significant discussion of both the caveats and potential interpretations of their findings.

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The additional measurements and discussion of import and export have convinced me that their model is reasonable for how the nuclei can be at steady state in a given volume and yet still change size when volume is changed in stage 10. I also appreciate their new metric for chromatin homogeneity and find it compelling that changes in Npm2 result in changes in the chromatin environment within the cell.

We appreciate the continued support for our work.

The MNase results are very exciting and add a lot to my understanding of potential mechanisms of how this process might work. However, it looks like these experiments were only done once each (?) and the results are fairly subtle in the quantifications. I would like to see a few more replicates and statistics.

We actually performed MNase digestion assays three times for Npm2 protein microinjection and three times for Npm2 mRNA microinjection, displaying results from one representative experiment for each type of microinjection in Figure 6A. We now clarify this in the figure legend for Figure 6B. We were initially unsure of the best way to combine results from multiple experiments because the degree of MNase digestion is extremely sensitive to MNase amount and digestion time. As such, the observed amount of MNase digestion varied significantly between experiments. It is for this reason that we chose to only show representative experiments. To address statistical significance, we have now calculated the fold change in nucleosome occupancy between control- and Npm2-microinjected embryos for a given experiment. We then compared these values across multiple experiments, revealing a statistically significant difference. This new analysis is now presented in Figure 6B.
Also in Figure 6B, it looks like they are plotting average pixel intensity, not total integrated signal (either way they need to be more clear what “Euchromatin staining intensity” means). If this is average pixel intensity in just the H3Ac stained channel (not normalized to DNA), then this has the same problem as the chromatin occupied fraction in that the same amount of staining in a smaller volume will give a greater pixel intensity by definition. To say that the acetylation state of H3 is actually being changed they either need to normalize to a DNA stain or take the total integrated staining intensity for an entire nucleus. If this is what they have done, then the data are compelling, and the figure legend simply needs to be clarified. If not, at least one of these two normalizations must be done. If this is impossible due to the way that the data was collected, I think that the work stands without this particular experiment. So, while I do think that this is interesting and important, I think that the paper would be stronger with H3Ac experiment removed entirely than presented without proper normalization.

To clarify, we have replaced “Euchromatin staining intensity” with “Total nuclear acetyl-histone H3 staining intensity” in Figure 6C and in the figure legend. We have repeated this quantification as suggested, measuring total integrated nuclear acetyl-histone H3 staining intensity. This analysis still shows a reduction in histone H3 acetylation upon Npm2 protein microinjection, so we feel it is appropriate to retain this result in our manuscript. We have replaced our previous quantification with this new analysis and clarified our measurement metric in the figure legend.

Finally, the discussion has become overwhelming and unfocused. I appreciate the speculation about possible mechanisms, however it would be much more readable in a more concise, condensed form that focuses more narrowly of a few key points.

We understand this criticism and have now significantly shortened our discussion by >30%. Nevertheless, the previous round of reviews raised a large number of diverse issues that required discussion. To insure that these issues are still adequately addressed in our manuscript, we have retained text that specifically addresses concerns raised in the first round of review.

Reviewer #2:

The authors were able to address the majority of my criticisms. I support publication of this interesting work.

Reviewer #3:

The authors have addressed the points I raised in my earlier review to my satisfaction, and the present manuscript is clearly improved. The discussion about chromatin stiffness related to increased nuclear volume is particularly interesting, as a more compact chromatin state might be predicted to decrease nuclear volume (opposite from the results seen). That said, the current Discussion section is lengthy, and would be improved by shortening.

The discussion has been shortened as described above in response to Reviewer #1.