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Review COMMONS

Transcription inhibition suppresses nuclear blebbing and rupture independently of nuclear rigidity

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Reviewer 1

Evidence, reproducibility and clarity

Summary:

In this manuscript by Berg et al the authors demonstrate that RNA polymerase activity is important for the formation of nuclear blebs. This is an interesting and significant finding because prior work has suggested nuclear bleb formation is a result of changes in nuclear rigidity (lamins) or chromatin (via histone modifications). Overall I thought the manuscript was quite interesting and the data well presented. I think the inclusion of multiple mechanisms of blebbing (VPA treatment, as well as lamin B KO) helps to further support the importance of RNA polymerase/transcription activity in the blebbing process. However, I do have some concerns regarding the conclusions of the data that I think should be addressed as a revision.

Major Comments:

1. One concern I have is that the alpha-amanitin inhibitor has been shown to also inhibit RNA polymerase III. In an old study (1974 Weinmann PNAS) it appears that the inhibitor starting at 1 to 10 ug/ml. In this study the authors are using 10 uM alpha-amanitin, which is ~ 9 ug/ml and within the range of inhibiting some RNA polymerase III. Additionally, the other drug (actinomycin D) is even less specific for RNA polymerase II. I would suggest that the authors consider one of the following approaches 1) acknowledge in the manuscript the potential for RNA polymerase III to be important in the blebbing process 2) try a 10-fold lower dose of alpha-amanitin and see if that also inhibits blebbing, 3) try to find a way to demonstrate that RNA polymerase III activity is not inhibited at the 10 uM alpha-amanitin dosage, or 4) consider an alternate method to perturb RNA polymerase II activity (see Zhang Science Advances 2021 for an auxin-based approach to downregulate RNA polymerase II).
2. A second concern I have is that the inhibition of RNA polymerase is global. Thus it is difficult to know for sure the biophysical function of the polymerase occurs immediately at the bleb, or instead is somehow affecting the overall chromatin state throughout the entire nucleus. I agree that figure 3 does provide some evidence that major mechanical and biophysical properties of the nuclei are not changed in response to the inhibition of the polymerase. However, micromanipulation experiments are done with isolated nuclei, which may be somehow mechanically altered already by isolation from cells. I feel that there still must be given some consideration in the discussion of the possibility that RNA polymerase activity outside of the bleb may be having some role in the stabilization of the chromatin and blebbing propensity.

While I lack expertise to evaluate the basis of the model, I appreciate the model can show that motor activity can influence bulge. But it is not clear in the manuscript that RNA polymerase can generate these kinds of forces. The Liu citation is a model, and does not provide direct evidence that the RNA polymerase can generate force, or forces large enough to be meaningful. To me the model in this paper (Figure 7) felt as if it was only a possible hypothesis of why the RNA polymerase has an effect on blebbing, but I imagine there could be other hypotheses that would cause the same effect. The authors state (in the abstract) that RNA pol II can generate active forces, but I am concerned this is not sufficiently established. Since this motor/force activity of RNA polymerase is not not experimentally demonstrated in this paper the authors should either do a better job of including evidence of this from the literature or consider removing this part of the manuscript.

Minor Comments:

1. Did the authors do any analysis to see if the increased RNA transcription with VPA treatment (Figure 1B) has any spatial relationship to where the bleb occurs? Could an analysis of this be done similar to Figure 6 (with a bleb/body ratio)?
2. Is there anything known about lamin B1 KO cells as to whether or not they have increased transcription? Or could the authors do an analysis like they did with VPA treatment to check this? If they were to have increased transcription this would further support the authors' proposed mechanism of transcription itself (or RNA polymerase activity) driving blebbing).
3. Figure 1D, the VPA ser2 image appears much brighter than the untreated image. Yet the graph shows they are similar. Perhaps a more representative image should be used?
4. Can the authors comment if there is less DNA at the bleb site? In Figure 6 A this appears to be the case (based on the VPA image). If true, is the alpha-amanitin treatment rescuing this such that there is more DNA at the bleb (maybe causing the bleb to be smaller?).
5. What is the significance of bleb vs non-bleb nuclear rupture? Is there anything known in the literature as to how these ruptures may be different in terms of biophysics, impact to DNA, repair? It would be helpful to have some context, as well as to understand if non-bleb rupture is something that may have been previously missed in other contexts.

Significance

General assessment:

This study is a careful analysis of how RNA polymerase inhibition reduces nuclear blebbing. The study demonstrates this very well, using a variety of approaches. However, some limitations are the overstatement of some conclusions (specifically that it is RNA polymerase II when the inhibitor may also affect RNA polymerase III; that the RNA polymerase activity is important at the bleb and involves motor activity).
Advance: This paper is a significant advancement because it shows the role of transcription in the biophysics of the nuclear shape. To my knowledge this is the first report of this phenomena, and thus will be impactful to the nuclear mechanics field.
Audience I think the findings are of broad interest, including beyond the nuclear mechanics field. I think the audience would be the entire cell biology community.
Expertise: My expertise is in cell mechanics, including forces at the the nuclear LINC complex. While I do not work in the field of nuclear blebbing and rupture, I follow this field quite closely.

Reviewer 2

Evidence, reproducibility and clarity

The authors present data supporting the potential involvement of active transcription in the formation of nuclear blebs when the global deacetylase inhibitor valproic acid (VPA) has been applied to cells

Significance

The authors present data supporting the potential involvement of active transcription in the formation of nuclear blebs when the global deacetylase inhibitor valproic acid (VPA) has been applied to cells. While somewhat interesting, this is a rather specific condition that is further restricted by the limited use of experimental approaches. For example, the only deacetylase inhibitor used is VPA. Is this because VPA is the only one to trigger the effect? The authors should expand their approach to include additional inhibitors or, preferably, a directed knockdown tactic that targets the specific HDACs driving their phenomena.
Moreover, the authors imply that VPA works through histone deacetylation yet do not provide direct evidence. It is equally likely that the application of VPA alters the acetylation pattern of a non-histone protein that eventually alters nuclear blebbing. Regardless, the reported findings with VPA were previously reported (Stephens et al. 2018) and the influence of alpha amanitin only represents an incremental advancement in our understanding of nuclear blebs. Adding to the concern is that actinomycin D does not have the same level of influence as alpha amanitin (Figure 2), which suggests the alpha amanitin is having a pleotropic impact on blebbing. To validate that the changes in blebbing in the presence of VPA are dependent upon active transcription, the authors should use the anchor-away technique to remove RNAP from the nucleus thereby avoiding any indirect effects of the drugs (i.e., alpha amanitin) in use. Further adding concern that it is an indirect outcome is the prolonged incubation period (16-24 hours) that is apparently needed to observe the changes (page 5 paragraph 4). If it is active transcription that is causing the change in blebbing, then this should be apparent in a much shorter time frame (<1 hour). In addition to these issues, the authors rely on immunofluorescence signals to measure the levels of various factors including the Ser5 and Ser2 phosphorylation, which is capturing the total levels of these factors and not the DNA bound forms. If the changes in blebbing actually involve transcription initiation, then the authors should include measurements on the DNA-bound factors. As reported the authors conclude that there is no changes in Ser2 and Ser5 phosphorylation yet they report that total RNA levels rise (Figure 1). How is the disconnect between RNA levels and Ser2 and Ser5 phosphorylation occurring? Comparably, they use H3K9ac immunofluorescence as a measure of euchromatin. While the authors might be gaining a view on the total levels of H3K9ac under these experimental conditions, it is not clear whether this is DNA associated or not. Minimally, the authors should perform ATAC-Seq to judge the changes in euchromatin. A final major concern is the lack of a correlation between the blebbing and nuclear ruptures (page 7 paragraph 3; Figure 4). If ruptures are not correlating with the blebbing, what is the relevance of the blebbing?

Reviewer 3

Evidence, reproducibility and clarity

This is an interesting study that shows, for the first time, that inhibition of transcription reduces the occurrence of nuclear blebs in cells that have been pre-treated with valproic acid. The data that supports this is in Figure 2, collected in two different cell types (MEFs and HT1080 cells). The effect appears robust. New data is also provided that a marker of initiation of transcription but not transcriptional elongation is enriched in valproic acid-induced blebs.

Major comments

1. The paper makes general claims about transcription and nuclear shape, when in reality, it is only reporting on the inhibition of transient, small, valproic acid-induced blebs by alpha- amanitin. This scenario under which the experiments were performed, for which there is no obvious physiological counterpart, ought not to be construed to challenge or contrast with the current understanding that the nucleus maintains its shape by resisting cytoskeletal forces. Cytoskeletal forces are well-known to establish nuclear shape; nuclear shape in this context, is generally taken to refer to the gross shape of the nucleus (e.g. elliptical, circular, etc.), and not small local blebs that may form due to F-actin based confinement or other mechanisms. Thus, this interpretation is overstated:

"Surprisingly, we find that while nuclear stiffness largely controls nuclear rupture, it is not the sole

determinant of nuclear shape. This contrasts with previous studies, which suggested that the nucleus maintains its shape by resisting cytoskeletal and/or other external antagonistic forces (Khatau et al., 2009; Le Berre et al., 2012; Hatch and Hetzer, 2016; Stephens et al., 2018; Earle 12 et al., 2020)."

As an aside, the data in the paper does not appear to support the interpretation that "nuclear stiffness largely controls nuclear rupture". It is unclear what the authors mean by this statement.
2. Further to point 2, treatment with alpha-amanitin does nothing to the occurrence of blebbing in normal cells. Thus, the data are specifically applicable to valproic acid-treated cells. As such, the broad interpretations related to nuclear shape and mechanics should be tempered.
3. The motor model for RNA pol II activity assumes that the motor 'repels' nearby chromatin units. It is not clear how this is related to the mechanism of motor action of RNA pol II on chromatin during transcription. The motor model also does not seem to add conclusive insight to the manuscript, as the nuclear shapes predicted are not directly comparable to the experimental shapes which are flat and smooth with only an occasional, single, local bleb. The model offers 'proof of principle', but is not capable of ruling out alternative mechanisms (such as nuclear pressurization by confinement, chromatin decompaction, or changes to osmotic pressure). It may be more appropriate to include the model in the discussion as opposed to presenting it as a new result that can be reliably interpreted through comparisons with experiment.
4. The data in the paper is not strong enough to rule out the more conventional mechanism of nuclear pressurization, which could be caused by F-actin based confinement or chromatin decompaction, or changes to osmotic pressure. Immunostaining of myosin is not a reliable way to compare myosin activity across conditions. It is possible that the long treatment with alpha-amanitin (unto 24 h, Fig. 2) relieves the pressure in the nucleus without measurable changes in the already established cell shape and hence the nuclear shape (height changes in spread cells are small at best -- valproic acid appears to reduce height by ~0.5 microns in Figure 3E which is smaller than the optical resolution along the z-axis of a typical confocal microscope).
5. Further to point 4, the data in Figure 4B and 4D both show a decrease in the mean of the % of ruptured nuclei and rupture frequency (please provide units for this frequency on the Y- axis). With more experiments, perhaps the data would have reached statistical significance?

Minor comments.

1. Confirmatory data, which has already been published in the same cell line in the past, could be moved if possible to supplemental information. Figure 1 seems to be a characterization of the efficacy of alpha-amanitin which is well-known, and therefore does not represent an original finding. It should perhaps be in supplemental information.
2. Did the counting method used to collect data in Figure 4B exclude nuclei that rupture multiple times? This should be specified in the manuscript.
3. This statement should be rephrased: "Since transcription is needed to form and stabilize nuclear blebs, at least some aspect of nuclear shape deformations appears to be non- mechanical" - deformation in the model in Figure 7 is clearly 'mechanical' - driven by motor force.
4. It is important to specify the times for which cells were treated with the various drugs in each figure (and not just in figure 2).

Significance

This paper reports new data that nuclear blebbing induced by treatment with valproic acid can be inhibited by co-treatment with alpha-amanitin. The data provided are reproducible across different cell lines. The data suggest that inhibition of transcription inhibits blebs which are induced by valproic acid treatment, but it does not inhibit blebs in cells untreated with valproic acid. Immunostaining reveals some enrichment of RNA pol II phosphorylated at Ser5 in valproic acid-induced blebs, suggesting an enhancement of transcription-initiation (but not transcriptional elongation) in the bleb. Alpha-amanitin treatment reduces bleb formation and bleb lifetime.

While the data are clearly presented, and interesting in terms of relating transcription to blebbing, the proposed interpretation in terms of a new mechanism of blebbing is not strongly supported by the data or by the computational model. More definitive evidence is required to rule out that

blebbing in valproic acid treated cells is not caused by a pressurization of the nucleus due to valproic acid treatment, which could be released by treatment with alpha-amanitin treatment for upto 24 h. The manuscript generalizes the findings to 'nuclear shape', and interprets them as suggestive of an alternative mechanism of establishment of nuclear shape; this generalization seems unsupported by the data.

Overall, the data provided is novel and interesting to cell biologists, provided more definitive evidence can be provided to rule out other models and to establish the new proposed model for nuclear blebbing. Else, the claims of an alternative mechanism for blebbing could be toned down, and the data on the relation between transcription and blebbing, which is the novel and interesting finding in this paper, could be presented in a more focused way.

Author response to reviewers' comments

Manuscript number: RC-2022-01761

Corresponding author(s): Andrew Stephens

1. **General Statements [optional]**

Cover letter: We are submitting a Revision Plan for our manuscript, "Transcription inhibition suppresses nuclear blebbing and rupture independent of nuclear rigidity," along with our proposed revised manuscript, and we are seeking editorial input on whether the revision would be suitable for publication in *Journal of Cell Science*. Our manuscript reports the novel finding that inhibition of transcription activity suppresses increases in nuclear blebbing that occur with perturbations to chromatin and lamins, many of which are associated with diseases and conditions like prostate cancer, progeria, and aging. Furthermore, we find that transcription inhibition decreases nuclear bleb formation, stability, and size, all of which directly lead to nuclear ruptures, which are well known to disrupt key nuclear functions.
Nuclear blebbing and ruptures are believed to be due to a less rigid nucleus that succumbs to actin compression and confinement. With our unique micromanipulation measurements, we establish that the effects of transcription inhibition on nuclear shape are independent of changes in nuclear rigidity. Furthermore, transcription inhibition does not alter actin confinement or contraction. These findings suggest that transcription inhibition functions through a new mechanism to affect nuclear shape and rupture. Transcriptional activity has been reported to drive coherent chromatin motion. We apply an established simulation framework to investigate a new proposed biophysical mechanism for bleb formation that originates from transcription-driven chromatin dynamics. Thus, we report that inhibition of transcription activity is a novel method to suppress nuclear blebbing and rupture relevant to human disease and dysfunction.

Reviewers 1 and 3 were largely positive about the manuscript. Reviewer #1 stated "the manuscript was quite interesting, and the data well presented", it is a "significant advancement", and "the first report of this phenomena, and thus will be impactful to the nuclear mechanics field." Reviewer #3 stated that our study is "interesting", "reproducible", and it shows the effect of transcription inhibition on nuclear blebbing "for the first time".

Reviewer 2 was largely concerned with the "rather specific condition" of studying one RNA pol II transcription inhibitor (alpha-amanitin) and one nuclear perturbation that causes nuclear blebbing (histone deacetylase inhibitor VPA). We have directly addressed this concern by providing new data to show that four different transcription inhibitors and four different nuclear perturbations that increase nuclear blebbing show the same effect: transcription inhibition suppresses nuclear blebbing.

The major concerns raised by the reviewers include: 1) the use of a few nuclear blebbing perturbations and transcription inhibitors, 2) time interval of treatments, 3) basis for the simulation modeling, and 4) interpretation and conclusions. To directly address these concerns we

have provided new data and made substantial revisions including: 1) use of four different nuclear blebbing perturbations and four different transcription inhibitors (**Figure 2 and Supplemental Figure 1**), 2) new time-lapse experiments tracking when transcription inhibitors begin to suppress nuclear blebs as well as one inhibitor which takes effect rapidly (**Figure 2 and Supplemental Figure 1**), 3) more complete explanation and justification of the simulation model & limitations and a new figure panel (see **Figure 7C** and "Transcriptional motor activity generates nuclear deformations in active polymer simulations"), 4) changed language throughout & added new text to the Discussion section.

Our results provide a new demonstration of the relationship between structure and function (here, from function to structure), and furthermore, they raise exciting new questions about the molecular, mechanical, and dynamical elements of nuclear shape regulation. We therefore anticipate that our manuscript will be of great interest. We look forward to your response.

2. **Description of the planned revisions**

Insert here a point-by-point reply that explains what revisions, additional experimentations and analyses are planned to address the points raised by the referees.

We do not have any more planned additional experimentations. Below we have provided all of our additional experiments which we feel fully address the reviewers' concerns.

3. **Description of the revisions that have already been incorporated in the transferred manuscript**

Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript. If no revisions have been carried out yet, please leave this section empty.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary: In this manuscript by Berg et al the authors demonstrate that RNA polymerase activity is important for the formation of nuclear blebs. This is an interesting and significant finding because prior work has suggested nuclear bleb formation is a result of changes in nuclear rigidity (lamins) or chromatin (via histone modifications). Overall I thought the manuscript was quite interesting and the data well presented. I think the inclusion of multiple mechanisms of blebbing (VPA treatment, as well as lamin B KO) helps to further support the importance of RNA polymerase/transcription activity in the blebbing process. However, I do have some concerns regarding the conclusions of the data that I think should be addressed as a revision.

We appreciate that Reviewer states that "the manuscript was quite interesting, and the data well presented", it is a "significant advancement", and "the first report of this phenomena, and thus will be impactful to the nuclear mechanics field."

In the points below, the Reviewer specifically suggests that we: 1) clarify possible contributions from RNA pol III, 2) address how global vs. local chromatin motion might contribute to our findings, and 3) discuss the force production capabilities of RNA pol II. We also appreciate the feedback regarding the conclusions and have made the specific changes requested in the revision.

Major Comments:

1. One concern I have is that the alpha-amanitin inhibitor has been shown to also inhibit RNA polymerase III. In an old study (1974 Weinmann PNAS) it appears that the inhibitor starting at 1 to 10 ug/ml. In this study the authors are using 10 uM alpha- amanitin, which is ~ 9 ug/ml and within the range of inhibiting some RNA polymerase
III. Additionally, the other drug (actinomycin D) is even less specific for RNA polymerase II. I would suggest that the authors consider one of the following approaches 1) acknowledge in the manuscript the potential for RNA polymerase III to be important in the blebbing process 2) try a 10-fold lower dose of alpha-amanitin and see if that also inhibits blebbing, 3) try to find a way

to demonstrate that RNA polymerase III activity is not inhibited at the 10 uM alpha-amanitin dosage, or 4) consider an alternate method to perturb RNA polymerase II activity (see Zhang Science Advances 2021 for an auxin-based approach to downregulate RNA polymerase II).

The Reviewer raises the point that alpha-amanitin inhibits both RNA pol II and III. In the revised manuscript, we provide new data to further support that the observed effects arise from RNA pol II. We now include new data from cells treated with the transcription inhibitors flavopiridol (which inhibits RNA pol II elongation) and triptolide (which inhibits RNA pol I and II initiation). These transcription inhibitors also suppress nuclear blebbing in VPA-treated nuclei (**Figure 2C**) as well as three other nuclear blebbing perturbations in chromatin and lamins (**Supplemental Figure 1A**). These new experiments directly show that nuclear bleb suppression by transcription inhibitors can be observed without possible inhibition of RNA pol III by alpha-amanitin.

2. A second concern I have is that the inhibition of RNA polymerase is global. Thus it is difficult to know for sure the biophysical function of the polymerase occurs immediately at the bleb, or instead is somehow affecting the overall chromatin state throughout the entire nucleus. I agree that figure 3 does provide some evidence that major mechanical and biophysical properties of the nuclei are not changed in response to the inhibition of the polymerase. However, micromanipulation experiments are done with isolated nuclei, which may be somehow mechanically altered already by isolation from cells. I feel that there still must be given some consideration in the discussion of the possibility that RNA polymerase activity outside of the bleb may be having some role in the stabilization of the chromatin and blebbing propensity.

We appreciate the Reviewer's insightful comments and we have revised the manuscript to clarify that we do not attribute blebbing purely to local effects. Instead, we argue that global changes in chromatin motion driven by transcription could contribute to nuclear blebs.

We did not intend to communicate that alterations to chromatin or its dynamics were necessarily only local. Indeed, we found that relative levels in RNAP Ser2 and Ser5 phosphorylation were different inside the blebs (**Figure 6**). Nonetheless, transcription was perturbed globally in our experiments, so we realized that blebbing could be driven by global changes (**Figure 1**). We hypothesize that global regulation of transcription can stimulate nuclear blebbing since transcription and its inhibition can, respectively, drive and suppress correlated chromatin motion throughout the entire nucleus (as previously observed by Zidovska et al. (*PNAS* 2013) and Shaban et al. (*NAR* 2018, *Genome Biol.* 2020), among others). We have revised the manuscript to clarify this point (Discussion section, page 15). We have also added new simulation snapshots showing global chromatin motions and how these motions are coupled to nuclear morphology (**Figure 7C**).

In response to the concern that isolated nuclei exhibit different mechanical properties than nuclei inside of cells, we refer to our previously published micromanipulation measurements (Stephens et al. *MBoC* 2017). There, we found that nuclei within the cell and outside of the cell have quantitatively similar spring constants and qualitatively similar force-extension curves. Therefore, we are confident that the lack of change in nuclear stiffness measured by micromanipulation accurately reflects the mechanics of nuclei inside of cells across different perturbations.

3. While I lack expertise to evaluate the basis of the model, I appreciate the model can show that motor activity can influence bulge. But it is not clear in the manuscript that RNA polymerase can generate these kinds of forces. The Liu citation is a model, and does not provide direct evidence that the RNA polymerase can generate force, or forces large enough to be meaningful. To me the model in this paper (Figure 7) felt as if it was only a possible hypothesis of why the RNA polymerase has an effect on blebbing, but I imagine there could be other hypotheses that would cause the same effect. The authors state (in the abstract) that RNA pol II can generate active forces, but I am concerned this is not sufficiently established. Since this motor/force activity of RNA polymerase is not experimentally demonstrated in this paper the authors should either do a better job of including evidence of this from the literature or consider removing this part of the manuscript.

RNA polymerase is capable of exerting forces in excess of 10 pN (*e.g.*, see Wang et al. *Science* 1998; Herbert et al., *Annu Rev Biochem* 2008). The collective activity of many motors (10's of

thousands, e.g., see Zhao et al. *Proc. Natl. Acad. Sci.* 2014) may generate even larger forces. As discussed in our earlier modeling paper, this force scale is consistent with the motor strengths studied in our simulations (Liu et al. *Phys. Rev. Lett.* 2021); in the present work, we present simulation results for motors that generate 0.14 pN forces. Thus, transcription, in principle, could generate forces even larger than the ones we considered in the model.

Additional experiments indicate that at larger length scales, RNA polymerase activity appears to drive coherent motions of chromatin throughout the cell nucleus (Zidovska et al. *PNAS* 2013; Shaban et al. *NAR* 2018; Shaban et al. *Genome Biol* 2020). It is these motions, driven by motors, that appear to drive the formation of nuclear bulges in our model (please see new panel **Figure 7C**).

Therefore, the aim of the model is to build on established and new results to better understand how transcription could alter nuclear morphology. Our model is adapted from earlier models, which could reproduce observations of chromatin-based nuclear rigidity, (Stephens et al. *MBoC* 2017, Banigan et al. *Biophys J* 2017, Strom et al. *eLife* 2021), some aspects of nuclear morphology (Banigan et al. *Biophys J* 2017, Lionetti et al. *Biophys J* 2020), and possibly explain how nonequilibrium motor activity (such as RNA pol II) can drive coherent chromatin dynamics (Liu et al. *PRL* 2021), which have been observed in live-cell imaging experiments (e.g., Zidovska et al. *PNAS* 2013; Shaban et al. *NAR* 2018; Shaban et al. *Genome Biol*. 2020, among others). The precise form of the motor activity is not the focus of our model (or the previous motor model in Liu et al. PRL 2021). Instead, our simulation result indicates that the relatively small motor forces that generate coherent chromatin dynamics could explain the surprising observation that transcription is a critical component of nuclear blebbing.

To address the Reviewer's comment, we have added additional text to the Introduction and the Results sections to support the inclusion of motors to model the possible effects of transcription on chromatin dynamics and nuclear shape.

In the Introduction (page 4), we now write:

Simulations suggest that chromatin connectivity combined with the forces generated by polymerase motor activity (~10 pN per polymerase (Herbert et al. 2008)) could generate these dynamics (Liu *et al.*, 2021).

In the Results section (page 10), we write:

We consider motors that generate sub-pN forces, well below the 10 pN forces that may be generated by individual RNA polymerases (Herbert et al. 2008).

Additionally, we have updated Table 1 to include the simulated motor strength.

Minor Comments:

1. Did the authors do any analysis to see if the increased RNA transcription with VPA treatment (Figure 1B) has any spatial relationship to where the bleb occurs? Could an analysis of this be done similar to Figure 6 (with a bleb/body ratio)?

The Reviewer raises an interesting point about measuring RNA localization relative to the bleb. We measured RNA intensity in the bleb and the nuclear body for wild type cells only. We find that RNA levels are significantly decreased in the bleb (80% of body signal, $p < 0.05$), but not as much as the DNA signal (68% of body signal). This data agrees with the idea that RNA pol II Ser2 phosphorylation (representing elongation) is also decreased in the bleb (Figure 6). We now include this data in the text and Supplementary data.

2. Is there anything known about lamin B1 KO cells as to whether or not they have increased transcription? Or could the authors do an analysis like they did with VPA treatment to check this?
If they were to have increased transcription this would further support the authors' proposed mechanism of transcription itself (or RNA polymerase activity) driving blebbing).

In the revised manuscript, we show that several nuclear perturbations that are known to decrease nuclear stiffness and cause increased nuclear blebbing also rely on active transcription. Lamin B1 knockout or knockdown cells have been shown to result in changes in transcription. However, it was difficult to find data that shows whether the overall level of transcription changes. Collaborators of ours have *unpublished* data that indicates that twice as many genes are upregulated as downregulated upon lamin B1 knockdown, but this still does not assess the total level of transcription within the nucleus. Alternatively, increasing transcription via other means is fraught with off-target effects, which would require many additional complementary experiments. We thank the Reviewer for this interesting suggestion, but we believe this is beyond the scope of this manuscript, in which we have focused on showing that transcription inhibition suppresses bleb formation.

3. Figure 1D, the VPA ser2 image appears much brighter than the untreated image. Yet the graph shows they are similar. Perhaps a more representative image should be used?

The image used reflects the data that Ser2 signal is brighter (by ~10%) in VPA-treated cells but is not significantly altered compared to wild type (unt), and thus it is an accurate reflection of the data.

4. Can the authors comment if there is less DNA at the bleb site? In Figure 6 A this appears to be the case (based on the VPA image). If true, is the alpha-amanitin treatment rescuing this such that there is more DNA at the bleb (maybe causing the bleb to be smaller?).

We find that there is less DNA signal intensity per unit area in the nuclear bleb as compared to the nuclear body (bleb has ~60% the signal of the body; see teal dots/data in **Figure 6B**). This agrees with previously published work from our lab (Stephens et al. 2018 *MBoC*).

Alpha-amanitin treatment does not rescue this effect. Decreased DNA enrichment in the bleb remains with alpha-amanitin treatment ($p > 0.05$, comparing across all 4 conditions in **Figure 6B**).

5. What is the significance of bleb vs non-bleb nuclear rupture? Is there anything known in the literature as to how these ruptures may be different in terms of biophysics, impact to DNA, repair? It would be helpful to have some context, as well as to understand if non-bleb rupture is something that may have been previously missed in other contexts.

The Reviewer asks a valid and interesting question that this manuscript only begins to address. In general, we believe that ruptures occurring with blebs vs. without blebs may reflect aspects of the underlying mechanism(s) of blebbing and rupture, in the presence or absence of transcription. We offer a few further thoughts below.

1) Non-bleb nuclear ruptures have been reported in a few papers by our group (Stephens et al., 2019 *MBoC*) and others (Chen et al., 2018 *PNAS*), but much is still unknown.

2) Non-bleb nuclear rupture is part of normal nuclear behavior, as it accounts for ~20% of nuclear ruptures in wild type and perturbed cells (VPA and LMNB1-/-).

3) Overall, we think that bleb-based and non-bleb-based ruptures may occur through different mechanisms. The simplest difference is that bleb-based nuclear ruptures follow the nucleus' ability to form blebs, whereas non-bleb-based nuclear rupture occurs in cases where there is less bleb formation, suggesting that factors other than the ability to form blebs may also be important for rupture. In the current study, we observed that bleb-based nuclear ruptures (and bleb formation) require transcription. In another manuscript from our lab under review, bleb-based nuclear ruptures (and nuclear blebbing) can be suppressed by actin contraction inhibition and increased by increased actin contraction (Pho et al., biorxiv 2022).

Additionally, we note it was reported that non-bleb-based nuclear ruptures, at least some of which are driven by microtubule prodding, result in increased levels of DNA damage (Earle et al. *Nat Mater* 2020), as has been observed for bleb-based ruptures (Stephens et al., 2019 *MBoC*; Xia et al. *J Cell Bio* 2018). Thus, nuclear rupture in general is thought to lead to DNA damage. However, total levels of DNA damage due to rupture may be controlled by different cellular processes.

In the revision, we have clarified our motivation for quantifying ruptures with and without blebs. We have also added a few remarks, drawn from the above comments, to the Discussion section (pages 11-14).

Reviewer #1 (Significance (Required)):
General assessment:
This study is a careful analysis of how RNA polymerase inhibition reduces nuclear blebbing. The study demonstrates this very well, using a variety of approaches.
However, some limitations are the overstatement of some conclusions (specifically that it is RNA polymerase II when the inhibitor may also affect RNA polymerase III; that the RNA polymerase activity is important at the bleb and involves motor activity). Advance: This paper is a significant advancement because it shows the role of transcription in the biophysics of the nuclear shape. To my knowledge this is the first report of this phenomena, and thus will be impactful to the nuclear mechanics field.
Audience: I think the findings are of broad interest, including beyond the nuclear mechanics field. I think the audience would be the entire cell biology community. Expertise: My expertise is in cell mechanics, including forces at the the nuclear LINC complex. While I do not work in the field of nuclear blebbing and rupture, I follow this field quite closely.

We greatly appreciate the Reviewer's statement that "To my knowledge this is the first report of this phenomena, and thus will be impactful to the nuclear mechanics field." We thank the Reviewer for their thoughtful comments and suggestions, which have helped to improve the manuscript.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

The authors present data supporting the potential involvement of active transcription in the formation of nuclear blebs when the global deacetylase inhibitor valproic acid (VPA) has been applied to cells

Reviewer #2's greatest concern throughout the review was that we focused on the use of VPA as a model for generating increased nuclear blebbing and 24-hour treatment with alpha-amanitin as a transcription inhibitor. In the revised manuscript, we provide new data to show that nuclear blebbing generated by a variety of different nuclear perturbations (VPA, DZNep, LMNB1-/-, and LA KD **Figure 2D** and **Supplemental Figure 1A**) is reliant on active transcription in two different cell lines (MEF and HT1080, **Figure 2 A and B**). This is supported by use of four different transcription inhibition drugs, which work over varying time periods (24 hrs in alpha-amanitin, triptolide, or flavopiridol; actinomycin D for 1.5 hrs **Figure 2C**). We also timelapse imaged during drug treatment to show that transcription inhibitors for which we used 24-hour incubation times, can suppress nuclear blebs within 8 hours (**Supplemental Figure 1B**). We also show that nuclear bleb formation and stability in wild type is transcription dependent (**Figure 5**). We believe the new data added in our revised manuscript addresses the concerns of the Reviewer that the findings were specific to VPA and alpha-amanitin together only.

Reviewer #2 (Significance (Required)):

The authors present data supporting the potential involvement of active transcription in the formation of nuclear blebs when the global deacetylase inhibitor valproic acid (VPA) has been applied to cells.

While somewhat interesting, this is a rather specific condition that is further restricted by the limited use of experimental approaches. For example, the only deacetylase inhibitor used is VPA. Is this because VPA is the only one to trigger the effect? The authors should expand their approach to include additional inhibitors or, preferably, a directed knockdown tactic that targets the specific HDACs driving their phenomena.

The Reviewer is concerned that we have used limited experimental approaches by focusing on VPA

treatment to induce nuclear blebs and alpha-amanitin overnight treatment to suppress nuclear blebbing. VPA treatment is a well-established perturbation to induce nuclear blebbing via HDAC inhibition, and it is similar to a variety of other nuclear perturbations that also induce blebs (Stephens et al. *MBoC* 2018, 2019; Kalinin et al. *MBoC* 2021; Pho et al. biorxiv 2022).

Nonetheless, to clearly address the Reviewer's concerns we have provided new data which shows that four different nuclear perturbations are suppressed by transcription inhibition and that four different transcription inhibitors suppress nuclear blebbing. In addition to these perturbations, we also note that transcription inhibition affects bleb formation and stability in wild type cells. Below we outline the diverse experimental approaches that support the major conclusion of our manuscript.

Our data shows that transcription inhibition suppresses nuclear blebbing through data for:

1. Multiple cell lines (MEF and HT1080, **Figure 2, A and B**) – original data
2. Multiple transcription inhibitors (**Figure 2C** and **Supplemental Figure 1**):
    a. Alpha-amanitin (RNA pol II and III degradation) – original data
    b. Triptolide (RNA pol I and II initiation inhibition) – **new data**
    c. Flavopiridol (RNA pol II elongation inhibition) – **new data**
    d. Actinomycin D (DNA intercalation) – original data
3. Multiple perturbations that cause nuclear blebbing (**Figure 2D and Supplemental Figure 1**):
    a. VPA histone deacetylase inhibitor, which increases euchromatin and chromatin decompaction; used because it is the most highly studied treatment by our lab (Stephens et al., 2017, 2018, 2019 *MBoC*; Pho et al., 2022 biorxiv) – original data
    b. DZNep histone methyltransferase inhibitor, which decreases heterochromatin and chromatin decompaction (Stephens et al., 2018, 2019 *MBoC*) – **new data**
    c. Lamin B1 null cells (LMNB1-/- or LB1-/-) (many previous works, including Stephens et al. *MBoC* 2018) – original data
    d. Lamin A constitutive knockdown cells (LA KD) (Vahabikashi et al., 2022 *PNAS*) – **new data**
4. Nuclear bleb formation and stabilization in wild type cells is dependent on transcription in addition to VPA (**Figure 5**). – original data
5. Time dependence of suppression of nuclear blebbing requested by Reviewers 2 & 3:
    a. Actinomycin D treatment of 1.5 hrs is sufficient to suppress nuclear blebs (**Figure 2C**) – original data
    b. Transcription inhibition with alpha-amanitin, triptolide, and flavopiridol all show an increased rate of nuclear bleb reabsorption in the first 8 hrs of treatment for both VPA and LMNB1-/- perturbations (**Supplemental Figure 1B**) – **new data.**
        i. This new data indicates that even formed blebs require active transcription to remain blebbed for long times
        ii. This new data also shows that the effect of transcription inhibition on nuclear blebbing does not require 24 hours of treatment.

Moreover, the authors imply that VPA works through histone deacetylation yet do not provide direct evidence. It is equally likely that the application of VPA alters the acetylation pattern of a non-histone protein that eventually alters nuclear blebbing.

The Reviewer questions whether histone deacetylation due to VPA treatment is responsible for nuclear blebbing. As the Reviewer notes in their next point below, histone deacetylation (e.g., by VPA or TSA treatment) as a mechanism for nuclear blebbing was previously established by work from our lab (Stephens et al., 2018 and 2019 *MBoC*) and others (Kalinin et al. MBoC *2021*). This was described and referenced in the original manuscript's introduction.

To summarize previous work, inhibition of histone deacetylation by VPA induces chromatin decompaction (Stypula-Cyrus et al. *PLoS One* 2013, Lleres et al. *J Cell Bio* 2009), increasing histone acetylation/euchromatin (Göttlicher et al. *EMBO J* 2001; Krämer et al. *EMBO J* 2003). In turn, this softens the nucleus (Stephens et al. *MBoC* 2017; Shimamoto et al. *MBoC* 2017), which succumbs to nuclear blebbing (Stephens et al., *MBoC* 2018). Softening and blebbing effect can also be induced by histone hyperacetylation via TSA or histone demethylation via DZNep (Stephens et al., *MBoC* 2018). This effect can be reversed by chromatin compaction via increased histone

methylation/heterochromatin formation (Stephens et al. *MBoC* 2019).

In the present work, we measured histone acetylation (H3K9ac) in both VPA and VPA+alpha-amanitin perturbations to ensure that alpha-amanitin does not simply reverse the increase in VPA-based histone acetylation and thereby decrease nuclear blebbing, which it does not (**Figure 3, A and B**).

Altogether, inhibition of histone deacetylation by VPA as a mechanism for nuclear blebbing is established by the previous literature. The present work builds on those results to uncover a surprising new driver of nuclear blebbing which is transcirption. Therefore, we consider it to be unnecessary to provide further confirmatory measurements of VPA-treated cells beyond what is already provided in the manuscript. Finally, we point to the inclusion of new data from three other nuclear perturbations that cause nuclear blebbing that can be suppressed by transcription inhibition (**Figure 2**).

Regardless, the reported findings with VPA were previously reported (Stephens et al. 2018) and the influence of alpha amanitin only represents an incremental advancement in our understanding of nuclear blebs.

The finding that alpha-amanitin inhibits nuclear blebbing implies that a previously unknown mechanism/pathway, involving an essential genomic process, is critical to nuclear shape regulation. We therefore strongly disagree with the Reviewer that bleb inhibition upon alpha-amanitin treatment represents an incremental advance.

Moreover, the existing literature generally argues that nuclear blebbing is caused by actin- based compression and confinement. It is widely believed that the cytoskeleton deforms the nucleus, which can herniate a nuclear bleb in softer nuclei. Here, we show that with transcription inhibition there are no overt changes to actin contraction (**Supplemental Figure 2**), actin confinement (**Figure 3E**), and nuclear mechanics (**Figure 3G**). However, levels of blebbing change anyway! This will be a new and surprising result to those who believe the current prevailing narrative from the literature. We have now shown for the first time that transcription is also needed to form and stabilize nuclear blebs; to our knowledge, this was almost entirely unknown until now.

Further supporting our belief in the significance of our findings, Reviewer #1 and Reviewer #3 clearly state that our work is novel and important:

> Reviewer #1 “To my knowledge this is the first report of this phenomena, and thus will be impactful to the nuclear mechanics field.”
>
> Reviewer #3 “This is an interesting study that shows, for the first time, that inhibition of transcription reduces the occurrence of nuclear blebs in cells that have been pre- treated with valproic acid.”

To address the Reviewer’s concern, we have revised the manuscript to clarify that active transcription is required to form nuclear blebs across all of the perturbations now presented in this manuscript. Furthermore, we have clarified that transcription inhibition appears to suppress blebbing without altering other cellular components and properties (actin, nuclear stiffness) that are widely believed to control blebbing (see Results page 7, Results page 10, Discussion page 14).

In addition to these issues, the authors rely on immunofluorescence signals to measure the levels of various factors including the Ser5 and Ser2 phosphorylation, which is capturing the total levels of these factors and not the DNA bound forms. If the changes in blebbing actually involve transcription initiation, then the authors should include measurements on the DNA-bound factors.

We are measuring Ser5 and Ser2 phosphorylation of RNA polymerase to track the actively DNA transcribing population. These markers appear on DNA-bound RNAP. Ser5 and Ser7 of RNAP are phosphorylated during initiation, and subsequently dephosphorylated during transcription elongation, while Ser2 is added at that time (Hsin and Manley 2012 *Genes Dev*). Ser2 is removed at transcription termination. Therefore, we expect immunofluorescence to measure DNA-bound RNAP.

As reported the authors conclude that there is no changes in Ser2 and Ser5 phosphorylation yet they report that total RNA levels rise (Figure 1). How is the disconnect between RNA levels and Ser2 and Ser5 phosphorylation occurring?

The Reviewer raises a question about how VPA treatment increases RNA levels but not levels of active RNA pol Ser2 and Ser5. While this is an interesting question, without a dedicated investigation, we can only speculate, at best; this question is beyond the scope of the paper focused on how transcription inhibition suppresses nuclear blebbing. The point of this data is to show that treatment with alpha-amanitin alone and along with VPA causes decreases in both RNA and RNA pol II Ser2 and 5 confirming transcription inhibition.

A final major concern is the lack of a correlation between the blebbing and nuclear ruptures (page 7 paragraph 3; Figure 4). If ruptures are not correlating with the blebbing, what is the relevance of the blebbing?

The Reviewer is asking for a clarification of the importance of nuclear blebbing in relation to nuclear ruptures. We have revised the manuscript to add new text to the Figure 4 legend clarifying the measurements and to the Discussion section describing the importance of this data (Discussion pages 12-13 and page 14). We discuss this in more detail below.

We would like to clarify that blebbing and nuclear rupture are not uncorrelated, as suggested by the Reviewer. We and others have shown that nuclear blebs are sites of high curvature that result in nuclear ruptures. In the present manuscript, timelapse imaging of nuclear bleb formation has been observed to result in nuclear rupture within minutes in all imaged cases (**Figure 5**). This data in the manuscript agrees with previous published data from our lab of bleb formation to rupture in >95% of the time (Stephens et al., 2019 *MBoC*). Furthermore, stabilized nuclear blebs persist for hours (**Supplemental Figure 1B**) and undergo more rupture, as shown in **Figure 4D**. Therefore, ruptures remain correlated with nuclear blebs in our study.

What we have shown, however, is that the percentage of cells that undergo at least one nuclear rupture during the time lapse is not statistically significantly decreased from VPA- treated levels by the addition of alpha-amanitin (**Figure 4B**). This appears to be due to two factors: 1) a basal level of nuclear rupture (see wild type data in **Figure 4**) and 2) an increase in the level of non-bleb-based nuclear rupture. However, importantly, non-bleb-based ruptures appear to occur less frequently for cells that undergo nuclear ruptures. Of the cells that exhibit nuclear rupture, those with non-bleb-based ruptures on average undergo only a single rupture over a 3-hour timelapse whereas those undergoing bleb-based rupture undergo an average of > 2 ruptures over the same time (**Figure 4D**).

Altogether, these data point to a correlation between blebbing and rupture, where blebbing can promote nuclear rupture, but is not essential for rupture. Therefore, observations of blebs are important in that they correspond to increases in nuclear rupture and corresponding nuclear dysfunction, such as DNA damage. The observation of non-bleb- based rupture, while not entirely a new (Chen et al. *PNAS* 2018, Stephens et al. *MBoC* 2019, Pho et al. bioRxiv 2022), is interesting because it may be driven by a different mechanism; transcription is not essential for nuclear ruptures in the absence of nuclear blebs but promotes rupture in the presence of blebs. These results add to our knowledge of the factors regulating nuclear integrity and shape, and we anticipate that they will be further investigated in future studies.

Finally, beyond these findings, we speculate that blebbing itself may be harmful to cell nuclear function. Previous studies have observed that nuclear deformations can cause DNA damage (Shah et al. *Curr Biol* 2021), chromatin reorganization (Jacobson et al. *BMC Biol* 2018, Golloshi et al. *EMBO J* 2022), and alterations to mechanotransduction (reviewed in Kalukula et al. *Nat Rev Mol Cell Biol* 2022). The extent to which the changes associated with these “nuclear deformations” require blebbing, rupture, or both is under investigation by various labs. Furthermore, previous studies (Shimi et al. *Genes Dev* 2008; Pfleghaar et al. *Nucleus* 2015) along with the present study (RNA Pol Ser2 and Ser5; **Figure 6**) have shown that chromatin content and, possibly, functionality is different within the nuclear bleb. Data in another manuscript in preparation from our lab, further suggests that there is limited exchange of biomolecular content between the nuclear body and bleb.

Therefore, while we cannot conclusively claim that blebs are themselves deleterious to function, there is a growing body of suggestive evidence that this is the case.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

This is an interesting study that shows, for the first time, that inhibition of transcription reduces the occurrence of nuclear blebs in cells that have been pre- treated with valproic acid. The data that supports this is in Figure 2, collected in two different cell types (MEFs and HT1080 cells). The effect appears robust. New data is also provided that a marker of initiation of transcription but not transcriptional elongation is enriched in valproic acid-induced blebs.

We thank Reviewer #3 for positive comments that our study is “interesting”, “reproducible”, and data that shows the effect of transcription on nuclear blebbing “for the first time”.

This Reviewer asks for clarifications on 1) how transcription is a new mechanism for nuclear bleb formation and not part of the traditional view, 2) the generality of our conclusions (similar to Reviewer #2) since we report “on the inhibition of transient, small, valproic acid- induced blebs by alpha-amanitin”, and 3) the insight the modeling provides. We have provided new data and made changes to the manuscript to address all the Reviewer’s comments.

Major comments

1. The paper makes general claims about transcription and nuclear shape, when in reality, it is only reporting on the inhibition of transient, small, valproic acid-induced blebs by alpha-amanitin. This scenario under which the experiments were performed, for which there is no obvious physiological counterpart, ought not to be construed to challenge or contrast with the current understanding that the nucleus maintains its shape by resisting cytoskeletal forces. Cytoskeletal forces are well-known to establish nuclear shape; nuclear shape in this context, is generally taken to refer to the gross shape of the nucleus (e.g. elliptical, circular, etc.), and not small local blebs that may form due to F-actin based confinement or other mechanisms. Thus, this interpretation is overstated:

"Surprisingly, we find that while nuclear stiffness largely controls nuclear rupture, it is not the sole determinant of nuclear shape. This contrasts with previous studies, which suggested that the nucleus maintains its shape by resisting cytoskeletal and/or other external antagonistic forces (Khatau et al., 2009; Le Berre et al., 2012; Hatch and Hetzer, 2016; Stephens et al., 2018; Earle 12 et al., 2020)."

The Reviewer appears to be concerned with two issues in this comment. First, the Reviewer is concerned about our use of the word shape, which could be interpreted too generally, rather than as categorizing the blebbing and rupture phenomena that we observe in this study. We appreciate the Reviewer’s feedback and have made changes to this sentence as well as the paper in general to clarify that we are focused on nuclear blebs. Second, there is the issue of to what degree our results modify our understanding of the role of nuclear stiffness in nuclear blebbing and rupture. We discuss this below.

To address the Reviewer’s comment that the results are limited to “the inhibition of transient, small, valproic acid-induced blebs by alpha-amanitin” we provide new data and context for our results. The revised manuscript includes 1) new data using four transcription inhibitors and four nuclear blebbing perturbations and 2) original data showing that nuclear blebs are persistent rather than small and transient, and they alter gross nuclear shape. Our results are relevant to a wider range of blebbing/rupture and bleb/rupture suppression scenarios, as exemplified by the different nuclear perturbations, transcription inhibitors, cell types tested in our experiments, and long lifetimes for nuclear blebs. More specifically:

1) The Reviewer notes that our original studies were done with VPA and alpha-amanitin, similar to Reviewer #2 concerns. We provide new data to now show that 4 different transcription inhibitors can suppress nuclear blebbing across 2 chromatin and 2 lamin perturbations (**Figure 2** and **Supplemental Figure 1**). Thus, our new data supports the idea that transcription is broadly

required for nuclear blebbing.
2) The Reviewer states that blebs are small and transient, and that "shape" is meant to reflect the gross shape (e.g., circular). In fact blebs are long-lived as we show with new data that most (>95%) of VPA and LMNB1-/- blebs, remain at the end of an 8-hour timelapse (**Supplemental Figure 1B**). Furthermore, on average, nuclear blebs account for 15% of the nuclear size in VPA-treated cells (**Figure 6E**). While not measured in this paper, many studies have shown that nuclear blebs cause gross circularity to decrease significantly and that changes in circularity are associated with nuclear rupture (e.g., Stephens et al. *MBoC* 2018, Xia et al. *JCB* 2018). Most recently, we show nuclear blebs decreased nuclear circularity significantly in another manuscript under review (Pho et al., 2022 biorxiv).

The Reviewer also argues that our data showing the importance of transcription in nuclear blebbing "ought not to be construed to challenge or contrast with the current understanding that the nucleus maintains its shape by resisting cytoskeletal forces." We acknowledge that our results are not sufficient to rule out the broad assertion made by the Reviewer. However, our data shows for the first time that nuclear blebbing relies on transcriptional activity, while we measure no change in actin contraction or confinement or nuclear stiffness (respectively, **Supplemental Figure 2** and **Figure 3, C-E**). Consequently, these results *are* a challenge to the current understanding, which must be updated by our results and future experiments. At the same time, we note that this manuscript's Discussion section acknowledges that we have data in another preprint in which inhibition of actin contraction decreases nuclear blebbing to near 0% in wild type and perturbations (Pho et al., 2022 biorxiv). Together, these observations suggest a complicated picture in which multiple factors are jointly responsible for regulating nuclear blebbing and rupture.

As an aside, the data in the paper does not appear to support the interpretation that "nuclear stiffness largely controls nuclear rupture". It is unclear what the authors mean by this statement.

We originally intended that comment to state the previous understanding in the literature, but we realize it was unclear. We appreciate the Reviewer's feedback and have revised the text.

2. Further to point 2, treatment with alpha-amanitin does nothing to the occurrence of blebbing in normal cells. Thus, the data are specifically applicable to valproic acid- treated cells. As such, the broad interpretations related to nuclear shape and mechanics should be tempered.

The Reviewer is concerned that we cannot support the claim that this effect is broad and general; these concerns are also raised by Reviewer #2. We have provided new data and highlight original data to support that this effect is in fact broad and general, and moreover, that the data supports a role for transcription in nuclear blebbing.

We specifically address the Reviewer's statement: "treatment with alpha-amanitin does nothing to the occurrence of blebbing in normal cells". In the original manuscript, we provided data that showed that wild type nuclear bleb formation and stability are suppressed upon transcription inhibition (**Figure 5**) even though the percentage of wild type nuclei exhibiting a bleb is not changed by alpha-amanitin treatment (**Figure 2**). We also provided data showing that the predominant type of nuclear rupture changes with alpha-amanitin treatment, including in wild type cells (blebbed vs. not, **Figure 4C**). Thus, while the effects of transcription inhibition are most easily visible in VPA-treated cells, they are also present in wild type cells in how blebs are formed and stabilized (**Figure 5**). We have revised the manuscript to better highlight this important point.

In addition, we again emphasize that our results extend beyond VPA-induced blebs. Our revised manuscript now includes new data of 4 different perturbations (to chromatin histone modifications and lamins A and B) that induce nuclear blebs, which can be suppressed by 4 different transcription inhibitors (**Figure 2** and **Supplemental Figure 1**). As previously noted by both Reviewers 1 and 3, this effect is reproducible in different cell lines. This new data directly addresses the concern that the effect is only applicable to VPA and alpha amanitin.

Nonetheless, we agree with the Reviewer that we cannot support broader claims that nuclear mechanical properties are unaltered by transcription inhibitors across all scenarios, as we only

measured this change in VPA-treated cells. Micromanipulation force experiments are detailed and time consuming, making it difficult to include data for multiple perturbations. We chose VPA because we have the most measurements of this perturbation which have remained consistent over the life of micromanipulation force measurements.
Therefore, we have revised our statements on nuclear mechanics in the revised manuscript (page 14).

The motor model for RNA pol II activity assumes that the motor 'repels' nearby chromatin units. It is not clear how this is related to the mechanism of motor action of RNA pol II on chromatin during transcription.

The point of the model is not to precisely reproduce the manner in which transcribing RNA pol II exerts forces on the chromatin fiber. Instead, we have developed a coarse-grained model to study how the collective activity of molecular motors might drive chromatin dynamics and consequently, changes in nuclear shape, either global or local.

The model itself is based on our earlier models, which were used to recapitulate and understand how changes to chromatin mechanical properties governed nuclear rigidity (Stephens et al. *MBoC* 2017, Banigan et al. *Biophys J* 2017, Strom et al. *eLife* 2021; also see a similar model by Lionetti et al. *Biophys J* 2020) and how nonequilibrium activity due to molecular motors, such as RNA pol II, can drive coherent chromatin dynamics (Liu et al. *PRL* 2021), which have been observed in live-cell imaging experiments (e.g., Zidovska et al. *PNAS* 2013; Shaban et al. *NAR* 2018; Shaban et al. *Genome Biol*. 2020, among others). The current model therefore explores how the newly observed connection between transcription and nuclear blebbing could be explained by known phenomena.

We note that the "repelling" motors used to model RNA pol II activity in the present work are in many ways qualitatively similar to the dipolar "extensile" motors used by other researchers to model motor-driven chromatin dynamics (e.g., see Saintillan et al. *PNAS* 2018). More generally, study of "active matter" over the last 20-30 years (and statistical physics over the last century) has shown that precise details of active molecular agents are often unimportant to the larger-scale behavior of the system (e.g., see Marchetti et al. *Rev Mod Phys* 2013). Thus, we view the repulsive motors as modeling the effective behavior of many RNA pol II within a sub-micron region of chromatin. Better establishing the differences between different choices of motor activities is the subject of a modeling paper in preparation.

To address the Reviewer's concern, we have more clearly stated the scientific foundations of the model, and we have revised our description of the model to clarify that we do not intend to model the behavior of individual RNA pol II by individual repulsive motors (see Results section, page 10).

The motor model also does not seem to add conclusive insight to the manuscript, as the nuclear shapes predicted are not directly comparable to the experimental shapes which are flat and smooth with only an occasional, single, local bleb.

The Reviewer raises two related points with this comment: that bulges and blebs are not directly comparable, and therefore, that the model "does not seem to add conclusive insight to the manuscript."

We agree with the Reviewer that bulges in the simulations are not blebs as they are observed in the experiments. However, it seems likely to us that bulges are necessary precursors to bleb formation; it is difficult to envision how a large local nuclear protrusion could form without first bulging outward from the nuclear body. Furthermore, we disagree with the assertion that nuclei are generally flat and smooth, as qualitative and quantitative analysis of imaging data reveals that nuclei exhibit shape fluctuations and irregularities across multiple scales (see, for example, Chu et al. *PNAS* 2017, Patteson et al. *JCB* 2019, Stephens et al. *MBoC* 2019, Liu et al. *PRL* 2021).

Nonetheless, the observation of bulges but not blebs is a shortcoming of the simulation model. We believe this shortcoming reflects a tradeoff made in developing this model; we chose to develop and study a model with relative simplicity compared to a real cell nucleus. A more complicated model might better capture some aspects of nuclear blebbing at the expense of additional complexity. For example, the current model does not allow lamin- lamin or chromatin-lamin bonds

to rupture, either stochastically or due to high forces. This effect, which is likely present *in vivo*, might be necessary for generating more bleb-like structures in simulations. Developing and refining such a model is an active pursuit within our collaboration, but for the moment, it is beyond the present purpose of the model.

Instead, the purpose of the model is to determine whether the observed effect of transcription inhibition on nuclear blebbing / localized shape deformations can be understood through known biophysical phenomena. Established models – to the extent that they exist – were insufficient because they typically relied on nuclear mechanics, which our experiments provide data that transcription is not changing nuclear mechanical rigidity. The current model demonstrates how *motor activity* within chromatin can alter the structure and dynamics of the lamina. The simulations are certainly not proof that transcription affects nuclear blebbing through the proposed mechanism. However, they are a first-of-their kind demonstration of how nonequilibrium biophysical activity (such as that generated by transcription) within a biopolymer system (chromatin) can emergently alter the geometry of the confining boundary (the lamina). This new result provides a plausible interpretation for the experiments in the manuscript.

In the revised manuscript, we have clarified our modeling approach and objectives in the Results and Discussion sections, and we have more clearly identified and discussed the limitations of the model (Results pages 10-11, Discussion page 15).

The model offers 'proof of principle', but is not capable of ruling out alternative mechanisms (such as nuclear pressurization by confinement, chromatin decompaction, or changes to osmotic pressure). It may be more appropriate to include the model in the discussion as opposed to presenting it as a new result that can be reliably interpreted through comparisons with experiment.

We respectfully disagree with the suggestion to include the model in the Discussion section instead of the Results. As discussed above, the model is new biophysics research and the simulations produced new scientific results, even if the overall interpretation remains open.

However, we have some thoughts about the alternatives suggested by the Reviewer. This is discussed in detail below, but briefly: experimental data, rather than the model itself, suggests that the alternative mechanisms mentioned by the Reviewer do not explain the effects of transcription. After treatment with alpha-amanitin, we do not observe changes to actin-based confinement or contraction (**Figure 3E, Supplemental Figure 2**), and there are no changes to chromatin histone modifications or nuclear rigidity (**Figure 3**). We also are skeptical of osmotic pressure arguments since 1) fluid, ions, and small biomolecules should freely flow through nuclear pores to maintain osmotic pressure balance between the nucleus and the cytoplasm, especially on hours-long time scales, and 2) increasing the osmotic pressure by fragmenting chromatin has previously been observed to have either no effect or a suppressive effect on nuclear stiffness (Stephens et al. *MBoC* 2017, Belaghzal et al. *Nat Genet* 2021), which would potentially increase blebbing (the opposite of the effect suggested by the Reviewer). We have addressed this further in the revised Results section (page 10) and below.

4. The data in the paper is not strong enough to rule out the more conventional mechanism of nuclear pressurization, which could be caused by F-actin based confinement or chromatin decompaction, or changes to osmotic pressure. Immunostaining of myosin is not a reliable way to compare myosin activity across conditions. It is possible that the long treatment with alpha-amanitin (unto 24 h, Fig. 2) relieves the pressure in the nucleus without measurable changes in the already established cell shape and hence the nuclear shape (height changes in spread cells are small at best -- valproic acid appears to reduce height by ~0.5 microns in Figure 3E which is smaller than the optical resolution along the z-axis of a typical confocal microscope).
The Reviewer has proposed several alternative mechanisms and questioned the use of immunostaining and nuclear height measurements in the manuscript. We address each of these below.

Specifically, the Reviewer is concerned that we cannot rule out the more conventionally believed mechanisms of 1) actin confinement, 2) actin contraction 3) chromatin decompaction and/or 4) osmotic pressure. We have revised the text to clarify that our data and data from others strongly

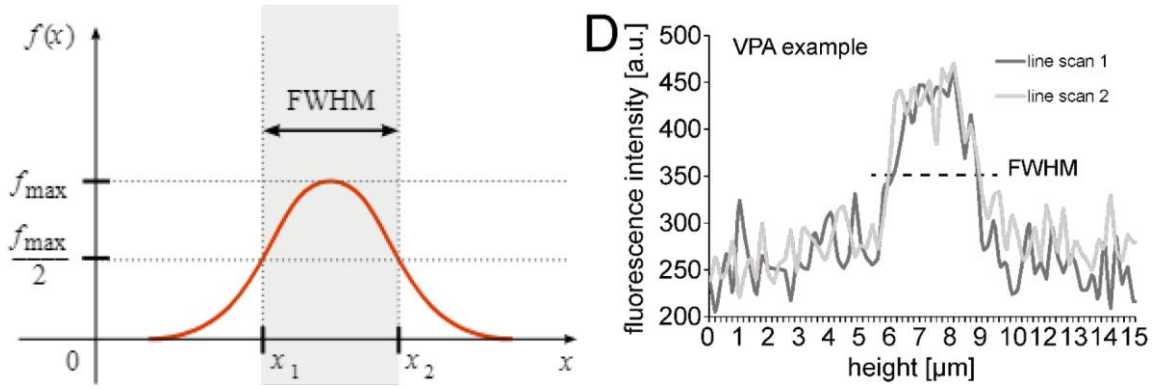
supports that these four “conventional” mechanisms are not responsible for transcription inhibition-based nuclear blebbing suppression (revisions on pages 7, 10, 14).

1) Actin confinement, as measured by nuclear height does not change upon transcription inhibition (**Figure 3, C-E**). Thus, our data supports the idea that transcription inhibition suppresses nuclear blebbing through a different mechanism. The Reviewer objects to this measurement on the basis that even the 0.5 µm change observed for VPA-treated cells is below optical resolution. However, optical resolution is not relevant to this measurement because we are not resolving two objects; rather, we are measuring the size of one object, the nucleus. Furthermore the measured 0.6 µm change is from 3.7 ± 0.2 WT to 3.1± 0.2 VPA the height of the nucleus, and not measuring a distance of 0.6 µm.

When two dots/objects are separated in the same frame or in different z slices, one needs to clearly distinguish two gaussians point spreads from the two objects a distance X apart. That is resolution and that is not the relevant limitation here. We measure the size of one object (the nucleus) using full-width half-maximum, which can quantify changes in nuclear height at scales finer than the optical resolution. For example, the FWHM of a fluorescence bead can be observed to change by just 10’s of nm depending on the light emitted; with small wavelengths, one has smaller FWHM (from the Rayleigh criterion, $\theta = 1.22\lambda/D$, where λ is the wavelength of the light). Our measurements are through a z-stack at 200 nm steps, thus the change in distance from wild type to VPA-treated of 0.5 µm is 2.5 z steps (not smaller than one z step). Finally, we have additional data showing our ability to measure these differences many times over (Pho et al. 2022 biorxiv).

Image left is from: https://en.wikipedia.org/wiki/Full_width_at_half_maximum

Image right is a crop of **Figure 3D** from the manuscript.



2) Actin contraction, as measured by γMLC2, does not change either (**Supplemental Figure 2**). However, we know that actin contraction is a major determinant of nuclear blebbing (Mistriotis et al., 2019 *JCB* and Pho et al., 2022 biorxiv). Therefore, our data support that transcription affects blebbing in some other way than actin contraction.

The Reviewer disputes this finding by stating that “immunostaining of myosin is not a reliable way to compare myosin activity across conditions.” Published reports show that γMLC2 immunostaining is a reliable way to measure actin contractility changes (Wan et al. *MBoC* 2012; Ramachandran et al. *Mol Vision* 2011; Duan et al. *Cell Cycle* 2016; Nishimura et al. *PLOS One* 2020). We have another preprint showing that alterations to actin contraction as measured by immunostaining of phosphorylated myosin light chain 2 (γMLC2) determine nuclear blebbing, independent of changes in actin confinement (Pho et al., 2022 biorxiv).

There, we clearly show that changes in γMLC2 immunostaining can measure changes in actin contraction due to well-established modulators. Similarly, the ROCK inhibitor Y27632 in **Supplemental Figure 2** can be viewed as a positive control in that γMLC2 immunostaining is clearly decreased after treatment with the inhibitor.

3) Chromatin decompaction via H3K9ac and chromatin-based nuclear rigidity are not rescued by transcription inhibition. New data also shows that levels of heterochromatin H3K9me2,3 do not change upon transcription inhibition (**Figure 3B**). The new data presented in this manuscript shows that transcription inhibition also suppresses blebbing in DZNep-treated cells (**Figure 2D**), where chromatin compaction by heterochromatin formation is inhibited (Stephens et al. *MBoC* 2019). Together, these experiments suggest that transcription inhibition is not suppressing nuclear blebs through increases in heterochromatin-based chromatin compaction.

Furthermore, the lack of change in the measurement of nuclear stiffness via micromanipulation (**Figure 3G**) provides a complementary metric suggesting that chromatin compaction is unchanged, at least in the case of VPA + alpha-amanitin.

Altogether, these results are inconsistent with transcription inhibition suppressing blebs through alterations to chromatin compaction.

4) Osmotic pressure is the least or not at all established of the four "traditional" mechanisms. The Reviewer proposes that transcription inhibitors, such as alpha-amanitin, could relieve osmotic pressure within the nucleus. We disagree with this explanation in that it is implausible for the nucleus to maintain an osmotic pressure imbalance in VPA-treated cells over long periods of time. Fluid, ions, and small biomolecules likely can flow through nuclear pores to maintain osmotic balance between the nucleoplasm and cytoplasm, especially over the hours long duration of VPA treatment. Furthermore, we are skeptical that VPA treatment, even with its chromatin-decompacting effects, significantly increases osmotic pressure because nuclear stiffness actually *decreases* after VPA treatment (Stephens et al. MBoC 2017, 2018, 2019; Krause et al. Phys Bio 2013; Shimamoto et al. MBoC 2017; Hobson et al. MBoC 2020) . Increased osmotic pressure *should* cause the nucleus to be stiffer. Moreover, nuclei in VPA-treated cells consistently undergo blebbing and rupture, which would naturally relieve any pressure imbalance. Thus, the notion that the measurements after hours VPA or VPA+aam treatment (**Figures 2-5**) are the result of a steady-state change in osmotic pressure is simply inconsistent with the experimental data.

We note that in cases of acute osmotic shock, where the osmotic pressure balance of the nucleus may be altered, the nucleus changes in size (e.g., see Finan et al., 2009 *Ann Biomed Eng*), which we do not observe in our experiments. Our measurements of nuclear area (**Figure 6C**) and height (**Figure 3E**) show no change nuclear size upon transcription inhibition (for more on the issue of height measurement, see the previous point).

To further address concerns about overnight treatment causing off-target effects, we have provided new data from a shorter treatment duration in the manuscript. The new data shows that within 8 hours, blebs exhibit more reabsorption after alpha-amanitin, triptolide, and flavopiridol treatment in both VPA-treated and LMNB1-/- cells (**Supplemental Figure 1B**). Additionally, we note that actinomycin D decreased nuclear blebbing in 1.5 hours, and thus did not require overnight treatment.

In summary, our original and new data clearly show that transcription contributes to nuclear blebbing. Transcription inhibition does not change other factors (such as actin-based confinement or contraction, changes in chromatin compaction, or osmotic pressure), that have been shown or may be thought to contribute to nuclear blebbing. The revised manuscript addresses this issue through the inclusion of new data, as discussed above.

5. Further to point 4, the data in Figure 4B and 4D both show a decrease in the mean of the % of ruptured nuclei and rupture frequency (please provide units for this frequency on the Y-axis). With more experiments, perhaps the data would have reached statistical significance?

The Reviewer is asking for clarification on the data included in **Figure 4 B and D** reporting the percentage of cells that display a nuclear rupture.

We have revised the manuscript to clarify that **Figure 4B** is the percentage of all nuclei that show at least one nuclear rupture. The measurement unit, percent (listed as "[%]"), is shown on the y-axis. The revised manuscript also clarifies that **Figure 4D** reports, for the nuclei that rupture, the average number of times a nucleus ruptures during the 3-hour time-lapse.

The Reviewer stats that “with more experiments, perhaps the data would reach statistical significance?” To address this comment, we have altered the text to explain that % of all nuclei that rupture at least once does not significantly decrease by t-test but does show a non-statistically significant decrease. The data in **Figure 4B** shows that VPA causes 18.5 +/-2.7 % rupture and VPA+alpha-amanitin causes 12.4 +/- 1.5 % rupture. Student’s t-test is p =0.08 which is not statistically significant (p > 0.05) for six biological replicates each consists of n = 100-300 cells. We feel the data speaks for itself without us doing more experiments with the sole purpose of getting a lower p value. The stronger data is in **Figure 4D**, which clearly shows less nuclear ruptures per nucleus. We appreciate the Reviewer’s perspective and have modified the text in the Results and Discussion sections to reflect these important points (pages 8 and 14).

Minor comments.

1. Confirmatory data, which has already been published in the same cell line in the past, could be moved if possible to supplemental information. Figure 1 seems to be a characterization of the efficacy of alpha-amanitin which is well-known, and therefore does not represent an original finding. It should perhaps be in supplemental information.

We understand the Reviewer’s point but would like to leave Figure 1 as a main text figure to provide a clearer story for all readers of our manuscript.

2. Did the counting method used to collect data in Figure 4B exclude nuclei that rupture multiple times? This should be specified in the manuscript.

No, Figure 4B is the percentage of nuclei that rupture, which includes nuclei that rupture any number of times as a single nucleus that ruptures. We have revised the Figure 4 legend to clarify this point.

3. This statement should be rephrased: "Since transcription is needed to form and stabilize nuclear blebs, at least some aspect of nuclear shape deformations appears to be non-mechanical" - deformation in the model in Figure 7 is clearly 'mechanical' - driven by motor force.

We appreciate the Reviewer’s feedback and have rewritten the text changes this to “independent of the bulk mechanical strength of the nucleus”.

4. It is important to specify the times for which cells were treated with the various drugs in each figure (and not just in figure 2).

We appreciate the Reviewer’s feedback and have added this information to each figure legend.

Reviewer #3 (Significance (Required)):

This paper reports new data that nuclear blebbing induced by treatment with valproic acid can be inhibited by co-treatment with alpha-amanitin. The data provided are reproducible across different cell lines. The data suggest that inhibition of transcription inhibits blebs which are induced by valproic acid treatment, but it does not inhibit blebs in cells untreated with valproic acid. Immunostaining reveals some enrichment of RNA pol II phosphorylated at Ser5 in valproic acid-induced blebs, suggesting an enhancement of transcription-initiation (but not transcriptional elongation) in the bleb. Alpha-amanitin treatment reduces bleb formation and bleb lifetime.

While the data are clearly presented, and interesting in terms of relating transcription to blebbing, the proposed interpretation in terms of a new mechanism of blebbing is not strongly supported by the data or by the computational model. More definitive evidence is required to rule out that blebbing in valproic acid treated cells is not caused by a pressurization of the nucleus due to valproic acid treatment, which could be released by treatment with alpha-amanitin treatment for upto 24 h. The manuscript generalizes the findings to 'nuclear shape', and interprets them as suggestive of an alternative mechanism of establishment of nuclear shape; this generalization seems unsupported by the data.

Overall, the data provided is novel and interesting to cell biologists, provided more definitive evidence can be provided to rule out other models and to establish the new proposed model for nuclear blebbing. Else, the claims of an alternative mechanism for blebbing could be toned down, and the data on the relation between transcription and blebbing, which is the novel and interesting finding in this paper, could be presented in a more focused way.

We appreciate that the Reviewer points out that "the data are clearly presented and interesting" and "reproducible across different cell lines." The Reviewer's main concerns appear to be with: 1) the effect of transcription inhibition on blebbing that is not induced by VPA, 2) alternatives or limitations to our proposed interpretation of the results, and 3) describing our results as applicable to "nuclear shape" in general.

We have addressed each of these concerns in detail in the above response and the revised manuscript. To summarize:

1) We have included new data to show that four different transcription inhibitors combined with four different nuclear perturbations exhibit the same effects (**Figure 2** and **Supplemental Figure 1**). Furthermore, we have clarified in the revised manuscript that even wild type ("untreated") nuclei exhibit changes to blebbing dynamics (decreased stability, increased reabsorption) after transcription inhibition (**Figure 5**). Furthermore, concerns about time intervals was addressed by time lapse imaging showing that bleb reabsorption (return to normal shape) increases six-fold in the first 8 hours of transcription inhibitor treatment (**Supplemental Figure 1B**).
2) The original manuscript, new data, and previous data from the literature provides evidence that alternative mechanisms involving "pressurization" (discussed above), the actin cytoskeleton (**Figure 3E** and **Supplemental Figure 2**), and chromatin and nuclear rigidity (**Figure 3**) do not explain the observed effects of transcription inhibition. We discuss this in detail in the revised manuscript and the above response. Furthermore, we have revised our presentation and discussion of the simulation model to describe its relevance more clearly to the results, support its inclusion in the manuscript, and provide appropriate caveats on our computational findings.
3) We have revised the manuscript to clarify that our results primarily concern nuclear blebbing and rupture. The Reviewer is correct that the current investigation does not particularly focus on larger-scale shape such as circularity/ellipticity.

In summary, our data clearly indicate that transcription contributes to nuclear blebbing and rupture. Previously suggested mechanisms of blebbing are generally inconsistent with the observed effect in combination with our other measurements. The model investigates a plausible new, complementary mechanism, which in itself represents an advance in biophysical modeling and ties the manuscript together.

We thank the Reviewer for their thorough critique, which we have now addressed. We believe that the new experimental data and analysis and computational modeling in our manuscript significantly advances our overall understanding of nuclear blebbing, even as it raises new questions to be addressed by future work.

4. **Description of analyses that authors prefer not to carry out**

Please include a point-by-point response explaining why some of the requested data or additional analyses might not be necessary or cannot be provided within the scope of a revision. This can be due to time or resource limitations or in case of disagreement about the necessity of such additional data given the scope of the study. Please leave empty if not applicable.

Reviewer #2
Major point
Adding to the concern is that actinomycin D does not have the same level of influence as alpha amanitin (Figure 2), which suggests the alpha amanitin is having a pleotropic impact on blebbing. To validate that the changes in blebbing in the presence of VPA are dependent upon active transcription, the authors should use the anchor-away technique to remove RNAP from

the nucleus thereby avoiding any indirect effects of the drugs (i.e., alpha amanitin) in use. Further adding concern that it is an indirect outcome is the prolonged incubation period (16-24 hours) that is apparently needed to observe the changes (page 5 paragraph 4). If it is active transcription that is causing the change in blebbing, then this should be apparent in a much shorter time frame (<1 hour).

The Reviewer is worried about possible differences between transcription inhibitors actinomycin D and alpha amanitin. To further address these concerns in the revised manuscript, we now present new data for VPA without transcription inhibitor and VPA with transcription inhibition via four different transcription inhibitors (**Figure 2C**). Inhibitors include alpha-amanitin (RNA pol II degradation), triptolide (transcription initiation inhibition), flavopiridol (transcription elongation inhibition), and actinomycin D (DNA intercalation). All VPA plus transcription inhibitor treatments result in a significant decrease in nuclear blebbing relative to VPA treatment alone ($p < 0.01$), while all VPA plus inhibitor treatments have similar levels of nuclear blebbing ($p > 0.05$, **Figure 2C**). Thus, there is no significant difference in the degree of nuclear blebbing suppression between the four different transcription inhibitors used.

Furthermore, the Reviewer raises concerns about the time interval from the start of transcription inhibitor treatment to suppression of nuclear blebbing. We agree that considering this time interval is valuable. However, we need to consider that the time interval for each of the different transcription inhibitors to take effect is different (Bensaude 2011 *Transcription*). Alpha-amanitin inhibits transcription in 4-8 hours (10 µM, Nguyen et al., 1996 *NAR*), triptolide (1 µM, Chen et al. 2014 *Genes Dev*) and flavopiridol (0.5 µM, Chen et al., 2005 *Blood*) work in 2-4 hours, and actinomycin D works in about 1 hour (10 mg/mL, Lai et al. 2019 *Methods*). These times are now mentioned in the manuscript (**Figure 2** legend and Methods section).

It was not, however, known in advance how long it would take for transcription inhibition to have an effect on nuclear morphology. Therefore, the time to observe bleb suppression could have been longer than these treatment durations. As mentioned above, treatment with actinomycin D for 1.5 hours results in a similar decrease in nuclear blebbing as compared to the other inhibitors with 24-hour treatment (**Figure 2C**). To further address these concerns, we provide new data in the revised manuscript showing tracking of nuclear bleb reabsorption during the first 8 hours of treatment with alpha amanitin, triptolide, and flavopiridol via live cell imaging. Nuclear bleb reabsorption for both VPA and LMNB1-/- perturbations goes from ~5 % to 30% or greater during the first 8 hours of treatment with each of the transcription inhibitors (**Supplemental Figure 1B**), consistent with the time required to fully inhibit transcription. This supports our conclusion that transcription is essential to stabilizing nuclear blebs.

Comparably, they use H3K9ac immunofluorescence as a measure of euchromatin. While the authors might be gaining a view on the total levels of H3K9ac under these experimental conditions, it is not clear whether this is DNA associated or not.
Minimally, the authors should perform ATAC-Seq to judge the changes in euchromatin.

The Reviewer questions the use of H3K9ac immunofluorescence as measurement of euchromatin levels, particularly in VPA-treated cells. The relationship between VPA and chromatin decompaction / euchromatin levels has been previously established (e.g., Stypula-Cyrus et al. *PLoS One* 2013, Felisbino et al. *J Cell Biochem* 2014, Lleres et al. *J Cell Bio* 2009). New data in **Figure 3B** shows that heterochromatin marker H3K9me2,3 also is not altered by alpha-amanitin treatment. In the case VPA + alpha-amanitin treatment, micromanipulation and nuclear height measurements provide further evidence that chromatin decompaction remains, since chromatin-based force response is unchanged from VPA treatment alone (**Figure 3, E and G**).

Again, we note that our manuscript focuses on the effects of transcription on nuclear blebbing and rupture, which were not previously reported and differ from the current understanding in the literature. Furthermore, ATAC-seq is a major undertaking that is simply not appropriate for further proving an auxiliary point about a previously established effect.

In summary, the original manuscript addresses this point. The specific experiment requested by the Reviewer is not necessary and is far beyond the scope of this study.

Original submission

First decision letter

MS ID#: JOCES/2023/261547

MS TITLE: Transcription inhibition suppresses nuclear blebbing and rupture independent of nuclear rigidity
AUTHORS: Isabel K Berg, Marilena L Currey, Sarthak Gupta, Yasmin Berrada, Bao V Nguyen Viet, Mai Pho, Alison Patteson, Jen Schwarz, Edward J Banigan, and Andrew D Stephens
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

Overall we are enthusiastic about the fit of your manuscript for publication at the Journal of Cell Science based on the largely positive assessment of the three reviewers who assessed your work for Review Commons and the additional revisions made in response to this initial round of review. The manuscript has been strengthened through the addition of new data and edits to the text, addressing the majority of the key points raised by the expert reviewers. Based on the reviews, there are a few minor revisions that we would like to ask you to make. I hope that you will be able to carry these out easily because I would like to be able to accept your paper.

1. In response to issues raised about the underpinnings of the model and the experimental support for the values chosen, please add a column to the Table 1 of simulation parameters for citation(s) or justification such that the reader will be able to assess the prior work or other basis for the values employed.

2. While I agree that the request for ATAC-seq, for example, is beyond the scope of what is required, in response to comments about potential changes to histone modifications/chromatin compaction, please edit the manuscript to ensure that it reflects on the explicit the experiments carried out and not beyond. For example, this statement should be edited "there are no changes to chromatin histone modifications or nuclear rigidity" to clarify that you are referring to H3K9 modifications, as other changes to histone methylation and acetylation that alter chromatin compaction are not experimentally addressed. Similarly, when referring to observations of H3K9Ac explicitly please indicate as such or state that you are using this modification as a proxy for euchromatin.

3. Last, please also ensure that the intensities in the pre-rupture NLS-GFP images presented (Figs. 4A, 5A) are not saturated, even if this requires applying two different contrast levels to the post-rupture images for comparison.

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

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points above. If you do not agree with any of the criticisms or suggestions please explain clearly why this is so.

First revision

Author response to reviewers' comments

Response to Reviewers

1. In response to issues raised about the underpinnings of the model and the experimental support for the values chosen, please add a column to the Table 1 of simulation parameters for citation(s) or justification such that the reader will be able to assess the prior work or other basis for the values employed.
We have added a column to Table 1 to both provide a justification for this parameter value as well as citations.
2. While I agree that the request for ATAC-seq, for example, is beyond the scope of what is required, in response to comments about potential changes to histone modifications/chromatin compaction, please edit the manuscript to ensure that it reflects on the explicit the experiments carried out and not beyond. For example, this statement should be edited "there are no changes to chromatin histone modifications or nuclear rigidity" to clarify that you are referring to H3K9 modifications, as other changes to histone methylation and acetylation that alter chromatin compaction are not experimentally addressed. Similarly, when referring to observations of H3K9Ac explicitly please indicate as such or state that you are using this modification as a proxy for euchromatin.
We have revised the manuscript to state the specific histone modifications assayed as requested. Find changes highlighted in red in the abstract and results section “Nuclear rigidity is not altered by transcription inhibition.”

3. Last, please also ensure that the intensities in the pre-rupture NLS-GFP images presented (Figs. 4A, 5A) are not saturated, even if this requires applying two different contrast levels to the post-rupture images for comparison.
We have revised both figures to include non-saturated images as requested.

Second decision letter

MS ID#: JOCES/2023/261547
MS TITLE: Transcription inhibition suppresses nuclear blebbing and rupture independent of nuclear rigidity
AUTHORS: Isabel K Berg, Marilena L Currey, Sarthak Gupta, Yasmin Berrada, Bao V Nguyen Viet, Mai Pho, Alison Patteson, Jen Schwarz, Edward J Banigan, and Andrew D Stephens
ARTICLE TYPE: Research Article

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I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. Where referee reports on this version are available, they are appended below.