

**Nuclear mechanosensing drives chromatin remodelling in persistently activated fibroblasts**

Corresponding author: Kristi Anseth

**Editorial note**

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This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

**Correspondence**

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Tue 05/05/2020

**Decision on Article nBME-20-0605**

Dear Prof Anseth,

Thank you again for submitting to *Nature Biomedical Engineering* your Article, "Nuclear mechanosensing drives epigenetic remodeling of persistently activated myofibroblasts". The manuscript has been seen by three experts, whose reports you will find at the end of this message. You will see that although the reviewers have some good words for the work, they articulate concerns about the degree of support for the claims, and in this regard provide useful suggestions for improvement. We hope that with significant further work you can address the criticisms and convince the reviewers of the merits of the study. In particular, we would expect that a revised version of the manuscript provides:

- \* Robust and causative evidence for the proposed mechanism of epigenetic remodelling.
- \* Evidence from additional human samples of aortic valve tissue, and use of metrics beyond the chromatin condensation parameter.
- \* Thorough characterization of the persistent-myofibroblast phenotype and epigenetics.
- \* Adequate characterization of the hydrogels.
- \* Complete methodological details.
- \* Fairer background discussion and referencing.

When you are ready to resubmit your manuscript, please [upload](#) the revised files, a point-by-point rebuttal to the comments from all reviewers, the (revised, if needed) [reporting summary](#), and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

- \* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).
- \* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

\* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

\* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

\* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

\* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 20 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*. Because of the COVID-19 pandemic, should you be unable to carry out experimental work in the near future we advise that you reply to this message with a revision plan in the form of a preliminary point-by-point rebuttal to the comments from all reviewers that also includes a response to any points highlighted in this decision. We should then be able to provide you with additional feedback.

We hope that you will find the referee reports helpful when revising the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies  
Chief Editor, [Nature Biomedical Engineering](#)

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Reviewer #1 (Report for the authors (Required)):

Walker and coauthors investigate the effect of mechanical environment on chromatin condensation in cultured valve interstitial fibroblasts. Main findings of the study are that prolonged culture (>7d) of fibroblasts on stiff (~4.5 kPa) polymer substrates activates into stress-fiber smooth muscle alpha actin (SMA) positive myofibroblasts. This phenotype is preserved even after subsequent light-induced softening of the substrates (~1.5 kPa, 2d) which the authors define as 'persistent myofibroblast'. Conversely, shorter pre-exposure to stiff substrate culture (2 d) activates myofibroblasts which are not stable in phenotype after substrate softening, here called 'transient myofibroblast'. This work follows earlier studies of this group using phototunable substrates to reveal mechanical dosing effects on cell phenotypes. While previous works of this group and others have described this phenomenon as 'mechanical memory', they here set out to untangle epigenetic modifications at the basis of this memory.

The authors correlate myofibroblast persistence with chromatin condensation, which in turn associates with actin cytoskeleton-dependent stiffening of the nuclear membrane. This study is overall well done and of high technical quality. The presented data are solid and convincing. However, the study has major flaws in interpreting the data, and provides correlative rather than functional links between mechanics, phenotype persistence, nuclear stiffening and chromatin condensation. The mechanistic insight is limited to processes that have already been shown by others, such as the link between substrate stiffness, adhesions, cytoskeleton integrity and changes in the nuclear envelope. The authors do an excellent job in integrating these previously published aspects into a coherent story but the essential questions how stiff substrate conditions translate into chromatin condensation and transcriptional activity and how these changes are made permanent on soft substrate remain unanswered. It seems critical to establish this missing link to enhance the novelty of the study.

Major:

1. One major concern with the study is the low conceptual novelty and mechanistic insight. While the work appears to establish a correlation between substrate mechanics, nuclear mechanics, and epigenetic changes, all these single aspects have been published before – partly by this group. They and others have published that fibroblast-like mesenchymal stromal cells (MSC) acquire a persistent myofibroblast phenotype (PMID: 24633344, PMID: 27798620). It has also been shown that prolonged culture on stiff substrates results in high chromatin condensation parameter (CCP) values (PMID 31235788; PMID: 26592929) that persist after substrate softening (PMID 30775233). The connection between substrate stiffness, enhanced adhesion through larger matrix contacts, increased actin/myosin stress fiber formation and nuclear stiffening in a myofibroblast/fibrosis context has been amply documented (e.g., see works of the Discher lab). The cytoskeleton-modifying experiments with effect on nuclear mechanics have been done and published. It is irritating that none of these original studies that reduce manuscript novelty are acknowledged, apart from those published by this group.
2. One novel aspect of the study is that trichostatin A (TSA) can mimic stiff substrate-induced memory to some extent with respect of general CCP values. However, the conclusion that HDACs are only partly responsible for chromatin condensation (as opposed to earlier observations of this group with MSCs) is not well substantiated. For instance, gene regions which are affected by tension can be different those affected by HDAC and HAT and two separate mechanism may generate similar results; this possibility must be assessed. Gene expression of HDAC is also not a reliable indicator for activity
3. Along these lines, one essential question remains open: How do fibroblasts manage to keep condensed chromatin and stiff nuclei on soft substrates where adhesions and actin cytoskeleton would typically be weakened, and cell stress reduced?
4. This conundrum points to a possibly fatal artifact generated by the authors' experimental design. The authors are not clear which exact conditions are used in each experiment to produce transient or persistent myofibroblasts. The respective methods section on 'dosing' does not explain dosing but how the gels are softened. This reviewer understood that 7d stiff-2d soft was used to study persistent myofibroblasts and presumably 3d stiff-2d soft to study transient myofibroblasts (as interpreted from figure 1C). The phototunable substrate assay is undoubtedly very elegant and useful. However, it has the inherent problem that transient (5 d) and persistent myofibroblasts (9 d) are cultured for different total periods. This growth difference will be enough to produce substantially different matrix and mechanical environment in each condition that possibly override the hydrogel stiffness switch. The authors will need to create comparable conditions in key control experiments. Prolonged culture on soft substrates (e.g. 2 d stiff, 7 d soft) will correct for growth time artifacts but present other problems related to dosing effects.
5. The authors initially describe that stiff culture results in higher CCP values (Fig.1) which is consistent with prior studies. However, they then continue to follow up on transcripts from open chromatin regions, notably adhesion- and cytoskeleton-related genes. How increased overall chromatin condensation would specifically open these regions remains elusive.
6. Directly linking changes in chromatin structure of adhesion/cytoskeleton-related genes to structural changes in focal adhesions and cytoskeleton in the observed timeframe is highly problematic. Focal adhesion size and actin organization are regulated on a shorter timeline by post-translational modifications (e.g., cofilin phosphorylation). How these would be affected by epigenetic changes within few days remains to be shown. As noted above, different growth times will also skew these results due different times available to express gene products.
7. The ATAC sequencing data is under-analyzed for critical genes in the context, such as different actin isoforms, focal adhesion proteins, actin-turnover proteins, focal adhesion kinases and phosphatases, etc. An obvious omission is to look for SMA as the bona fide myofibroblast protein. From their ATAC-seq data, the authors claim that genes are associated with opening peaks, but they do not validate, e.g., using ATAC-qPCR. Alternatively, ATAC-seq tracks should prove that the region near to the specific gene is open and then the enrichment should be verified using ATAC-qPCR or even traditional qPCR.
8. Understandably, the authors focus on the 80% activated myofibroblast in their study. However, analysis of the CCP values of the remaining 20% non-activated fibroblasts would tell whether the observed phenomena are a direct consequence of substrate mechanics or indirectly associates with myofibroblast activation. As the authors note, chromatin condensation has been described for myofibroblasts independently of (or ignoring) mechanical environment. The experiments with TSA seem to indicate that chemical factors may

compensate for or override mechanics.

9. Data presentation in Fig.1H is somewhat confusing by connecting or fitting the data points for very different conditions. It is understandable that CCP values are higher on stiff than on soft substrates. However, why would CCP values increase in transient myofibroblast upon substrate softening as stated in the text?

10. The correlation of CCP values in cultured valve fibroblasts and tissue samples is a stretch and a functional relation to valve stiffening speculative. If the authors wanted to follow this up, they would need to directly correlate valve stiff regions (e.g., AFM) with nuclear chromatin and lamina staining using statistically significant sample sizes.

11. It is highly problematic that cytochalasin D and Rho kinase inhibition do not affect SMA stress fibers at the reported concentrations. First, this observation is contradictory to numerous published studies. In fact, Rho kinase inhibitors have been reported to mainly affect myofibroblast contraction and have advanced to clinical studies (e.g., publication from the Thannickal group). Second, data presented in Fig.3C, D, G do not make biological sense. Stress fiber SMA is filamentous actin and labelled by phalloidin. In myofibroblasts SMA contributes to the vast majority of F-actin positive signals at light microscopy resolution. If SMA stress fibers change upon drug treatment, F-actin levels will change accordingly - or both do not change. Immunofluorescence assessment does not seem reliable in these experiments and the authors should perform G-/F-actin sedimentation assays. Another curiosity is that CCP values decrease in chemically produced transient myofibroblasts with an intact SMA stress fiber cytoskeleton – assuming that actin stress contributes to nuclear stiffening as one precondition. The conclusion on page 15 is hard to follow.

12. As noted before it remains unanswered why drug-induced relaxation of myofibroblasts is not equivalent to soft-substrate-induced relaxation. Rescue experiments are missing with this respect. For instance, if uncoupling the cytoskeleton from the nucleus prevents persistent myofibroblast formation, is it possible to turn persistent into transient myofibroblast by overexpression of the identified components. Would increasing cytoskeletal tension affect chromatin condensation in soft-cultured cells in the same way stiff environment does?

Minor comments:

1. The schematic drawing Fig.1A should match the experimental values (e.g. 4,5 instead of 3.5 kPa).

2. Fig.2: It is preferred to show the western blot data from one blot rather than single cut bands. This should be re-run with respective loading.

Reviewer #2 (Report for the authors (Required)):

In this paper Anseth and co-workers use a light-activated softening hydrogel demonstrated previously by the group (for instance, *Nat. Mater.*, 13, 645–652, 2014) to study nuclear mechanosensing in myofibroblasts. Fibroblasts were cultured on dynamic substrates that were subsequently softened, and statically soft and stiff hydrogels, with markers for myofibroblasts monitored. Interestingly, they identified “transient” and “persistent” populations of myofibroblasts that depended on the initial culture time on stiff substrates. Analysis of chromatin condensation and treatment with inhibitors of histone modifying enzymes and cytoskeleton disruptors, demonstrates attenuation of the persistent myofibroblast phenotype in favour of the transient. This is an exciting demonstration of controlling epigenetic plasticity with dynamic cell culture materials. Nevertheless, there are several points that need to be addressed prior to publication. While the light-sensitive PEG system has been used before by the group, there is little detail in the paper regarding these hydrogels. The mechanical properties are described but what about peptide density at the cell interface? Some additional information describing the quantity of RGD ligands that a cell adheres to and how this changes on softening is important. Could decreased exposure to RGD peptide on softening influence myofibroblast phenotype?

TSA will lead to open chromatin –how does this relate to myofibroblast (de)activation? The ATAC seq results and actin disruption suggests that mechanotransduction through the cytoskeleton directs myofibroblast persistence. However, broad spectrum HDAC inhibitors will disrupt many aspects of nuclear signalling, with a host of transcription factors with binding partners, etc.. Is the mechanism proposed that physical unwinding of the chromatin by itself regulates this transition from transient to persistent? Additional discussion of the

proposed mechanism would be useful.

The DN KASH experiments are interesting and do suggest that direct connectivity of actin to the nuclear membrane is responsible for guiding the persistent phenotype. However, surely there are many more activities associated with actin-nuclear activity. Are the fibroblasts showing a transient or quiescent phenotype after integration of this construct? The issue here is related to whether the loss of the persistent phenotype is on account of other attenuated activities from the cytoskeleton.

The authors discuss their results in the context of recent work to “unload” fibrotic tissue to attenuate disease and didn’t find a decrease in fibrosis. One clear difference is the planar studies explored in this work, and the 3D nature of fibrotic tissue. Could the dimensionality of the fibrotic matrix—and presumably a very different adhesive-cytoskeletal pattern—decrease the potential for epigenetic reset on account of “unloading”?

Minor points:

The results section begins with a very brief description of the hydrogel system, followed by numbered sections. This first section of the results should be under a subheading with more detail of the biomaterials fabrication and characterisation.

Epigenetic signatures as used in reference to Figure 1 is not strictly correct as they are measuring chromatin compaction. Suggest rewording, e.g. chromatin signatures

HAT is an acronym for Histone Acetyltransferase; please be sure to use consistent wording

Typo in reference 11 – journal name “Developmental Cell” appears

Reviewer #3 (Report for the authors (Required)):

In this manuscript, Walker and colleagues explore the role of epigenetic remodeling in the persistent activation of myofibroblasts in the context of tissue fibrosis. Using an established PEG hydrogel platform, in which the modulus can be dynamically softened by light, they find that longer culture on stiff substrates leads to a greater percentage of cells expressing SMA following softening (i.e. “persistent myofibroblasts”). Persistent myofibroblasts exhibit higher levels of chromatin condensation, and alterations in genome accessibility, relative to transient myofibroblasts. Inhibition of actin and rho reduces persistence and chromatin condensation. Finally, resistance is correlated with higher tension on nesprin, and knockdown of KASH reduces persistence.

This is potentially an important paper that ties mechanical memory to changes in nuclear architecture and the epigenome, and then changes in nuclear architecture and the epigenome to nuclear tension, specifically in the context of fibrosis. However, the major conclusions need stronger support and there are some key inconsistencies/questions with the data and text as shown. These need to be addressed substantially prior to publication.

1. Are the “persistent” myofibroblasts really persistent?

a. Do persistent myofibroblasts represent a distinct state or is it just that the timescale for recovery to a non SMA positive state just longer when cultured on stiff gels first for 7 days? The authors should culture the fibroblasts for a longer period of time after 7 days on stiff gels (e.g. 7d/7d) to evaluate whether the phenotype recovers in this case. This could have implications for claiming the relevance of these findings in human fibrotic disease.

b. Analysis of persistence is based solely on staining for aSMA. Additional molecular analyses should be included to strengthen the identification as myofibroblasts e.g. gene expression of other markers and transcription factor activation.

c. As shown in a recent paper from the Burdick group (Loebel, et al., Nature Materials 2019), cells can deposit matrix locally to form their own microenvironment. Are cells depositing their own matrix, and is this the reason more persistent myofibroblasts occur when cells are cultured on the stiff gels for longer because they are depositing their own matrix? This should be investigated.

d. Another caveat of the longer culture on stiff gels is the potential that cell numbers could be different due to proliferation, which could mediate the different myofibroblast phenotype. This should be quantified and the basic experiment should be redone with proliferation inhibited.

2. The CCP analysis and, in particular the connection of chromatin condensation in healthy/diseased valve tissue is potentially powerful, but there appear to be some flaws in the analysis and the in vivo comparison is quite preliminary.

a. I am very confused by the results of Fig. 1H and how they are described in the manuscript. While CCP goes up for stiff only case (from ~1 to 2), it seems to be constant at ~2 for the stiff-to-soft case, independent

of culture time (Fig. 1H). For example, after 3 days of culture in stiff case (high SMA+), CCP is ~1.0, while 1day stiff/2day soft (lower SMA+) is ~2.0. This seems inconsistent with claim that more persistent fibroblasts have higher CCP than less persistent ones (Fig. 2c), and the text that "After in situ softening in the transient myofibroblast conditions, the CCP increased significantly while the CCP of persistent myofibroblasts remained constant and did not change significantly in response to hydrogel softening." Is this figure mislabeled or are there some additional data that are missing?

b. In vivo analysis was done on only one patient for each condition. This is obviously not sufficient to make any conclusions (i.e. "Notably, myofibroblasts from human aortic valve tissue show similar epigenetic signatures"). The authors need to conduct on a number of patients and compare the averages of the CCP values to make a stronger conclusion about how CCP varies with healthy vs disease condition. The in vivo connection is an important part of this manuscript.

c. Further, with the current data, the normalized CCP values are different in range for the in vivo case (both healthy ~ 1.0 and diseased ~1.2) as compared to the in vitro studies (1.0 – 2.0), yet the authors highlight the increase in CCP in diseased state (though not a very low P-value) as consistent with their results. Its not clear to me whether the relative increase vs. the values themselves is what should be considered. The authors need to explain this.

d. Related to making the in vivo comparison stronger, the authors should look at other metrics beyond CCP to build stronger support for their model and for a more rigorous analysis of the nucleus state. For example, they can easily quantify nuclear characteristics (shape & volume or cross sectional area) and show how these line up.

3. ATAC-seq analysis is very general and under-utilized, adding little to the manuscript in its current form.

a. The authors show that there are differentially accessible peaks between transient and persistent fibroblasts, including more open and more closed peaks. However, the CCP data (I think) seems to indicate that chromatin should be more closed on average in the transient myofibroblasts. How the authors reconcile these seemingly different interpretations?

b. ATACseq data is a good starting point, but it should be confirmed that at least some of the peaks with larger differences between conditions correspond to differences in gene expression.

c. Further, the authors should use standard bioinformatics pipelines to identify transcription factors predicted to act on the opened up sites. Ideally, they could knockdown or knockout these TFs to test for a functional role.

d. ATAC-seq analysis should be done with the actin/myosin inhibitors and the KASH mutants to show that when the persistent state is inhibited, the transient ATAC-seq signature is recovered. This would provide strong evidence for the claim of "distinct chromatin signatures" in persistent fibroblasts.

4. This brings up a major question to me – is myofibroblast persistence mediated through a more closed chromatin architecture? While there is a lot of correlative evidence, the only causative evidence supporting this connection seem to be the TSA inhibition experiments, but these are not very convincing as TSA treatment is such a blunt perturbation. The authors should complement this with treatment with other inhibitors, and genetic knockdowns that more precisely perturb the chromatin state (i.e. histone modifying enzymes and/or TFs identified in 3c).

5. The potential mechanism of epigenetic remodeling through tension on the nucleus would be a very nice result, however, this needs more clarification and support. The nuclear tension values are very different between Fig. 4F and 4I, and the cytoD and rho inhibition tension values seem to be higher than the persistent case of 4F, despite the major impact on persistence of the inhibitor. This suggest to me that the tension sensor might be too noisy to make definitive conclusions. Further, the DNKASH mutant leads to a very small impact on normalized CCP (4K; 10% reduction), but a major impact on the % persistent cells (Fig. 4N). This seems to point towards the nuclear tension as playing only a minor role with regards to epigenetic remodeling. Stronger support is needed to make this connection.

Minor comments.

1. Why are the moduli of 4.5 kPa and ~1.5 kPa chosen? Are these physiologically relevant? Literature reported values for healthy and disease fibrosis conditions should be included with references.

2. Fig. 4O needs to be modified substantially: the ATAC-seq data would seem to suggest there isn't a broad increase in chromatin accessibility in transient myofibroblasts. Also, line 400 of the text 3 indicates that global chromatin structure of healthy myofibroblasts is less accessible than that of diseased myofibroblasts, which contradicts Figures 1J and 4O. Finally Figure 4O implies that nuclear tension first increases in the transient myofibroblast state, but then decreases for persistent myofibroblasts, but based on the data in Figure 4E, tension across Nesprin is higher for persistent myofibroblasts

3. The authors claim that chromatin condensation is associated with stabilization of the actin cytoskeleton. In lines 376-380, the authors mention two potentially conflicting prior findings: one in which actin assembly tends to decondense chromatin vs another in which actin assembly reduces pluripotency in iPSCs by

reducing chromatin accessibility. What explanation may there be for this discrepancy, and where would the results presented in this manuscript fit in?

4. Western blots and quantification of lamin A and lamin C expression in Figure 4C seem to show that lamin C levels decrease in the persistent population as compared to the transient population, which contradicts the text in line 277.

5. In Figure 4M, the sample image of a DN KASH cell seems to have a very different morphology from typical myofibroblasts shown throughout the manuscript. Was this morphology common for this population of mutant cells? Is it possible that cytoskeletal organization is significantly altered or destabilized by nuclear uncoupling, potentially making it easier for cells to de-activate upon softening? The authors should show typical morphologies of DN KASH vs control cells.

6. Figure 1F: Is there any explanation for why 1d-2d stiff-to-soft samples seem to have even fewer persistent myofibroblasts than soft control gels alone?

7. Methods section for the Omni-ATAC procedure mentions adding *Drosophila* nuclei to the reactions. Is this part of the protocol, or just a typo?

Thu 22/10/2020

**Decision on Article NBME-20-0605A**

Dear Prof Anseth,

Thank you for your revised manuscript, "Nuclear mechanosensing drives chromatin remodeling of persistently activated myofibroblasts", which has been seen by the original reviewers. In their reports, which you will find at the end of this message, you will see that the reviewers acknowledge the improvements to the work and that Reviewer #2 and #3 raise further technical criticisms that we hope you will be able to address. In particular, we would expect that the next version of the manuscript provides solid evidence of the claimed direct relationship between chromatin condensation and myofibroblast persistence.

As before, when you are ready to resubmit your manuscript, please [upload](#) the revised files, a point-by-point rebuttal to the comments from all reviewers, the (revised, if needed) [reporting summary](#), and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

As a reminder, please follow the following recommendations:

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We hope that you will be able to resubmit the manuscript within 18 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*.

We look forward to receive a further revised version of the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies  
Chief Editor, [Nature Biomedical Engineering](#)

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Reviewer #1 (Report for the authors (Required)):

Walker and coauthors have submitted a carefully and much improved revision of their study on epigenetic regulation of myofibroblast persistence. Most of my initial comments have been addressed and the authors have added more data now showing critical controls and amended unclear language. There is still one major remaining problem with the study. The authors set out to "investigate mechanisms that lead to persistent myofibroblast activation upon extended exposure to stiff microenvironments (i.e., mechanical dosing)" but



they do not answer this exciting and novel question. Instead they show how matrix and cell mechanics affect nuclear components, chromatin condensation and accessibility. But what is the relevance of chromatin accessibility for maintenance of myofibroblast phenotype (SMA stress fibers) on soft substrates that the authors use as indication of 'persistence'? The point is reiterated below.

1. To emphasize the novelty of their study in the rebuttal letter the authors argue that valvular interstitial fibroblasts regulate myofibroblast persistence differently than MSC for which many of the shown data (Figure 4 onwards) have been produced before. This is a somewhat blunt argument since (a) the authors do not systematically compare MSCs with VICs to substantiate the claim and (b) VICs have been shown to exhibit progenitor 'MSC' properties which, at least in part, seem to support valve calcification and VIC 'osteogenesis' in disease (e.g., PMID: 31506459; PMID: 19218344). The Simmons group has produced a series of studies on (even porcine) VIC mechanobiology which are all not cited but should be considered here. For instance, the group has measured local stiffness variations in heart valves and VIC stiffness on hydrogels (PMID: 22189247; PMID: 23746597). These values would respond to one of the other reviewer's questions on physiological relevance of 2 kPa and 4 kPa PEG gels.

2. Figure 1H: With the soft control now added it is curious and not discussed why persistent (9d) myofibroblasts all have very similar CPP values, regardless of prior experienced substrate stiffness. At least at the global chromatin condensation level, always soft and always stiff grown populations seem indistinguishable.

3. The authors have now performed ATAC-seq analysis of SMA as the myofibroblast hallmark gene in addition to improved analysis of global chromatin opening. The outcome of these new experiments is surprising in that myofibroblast genes would not be particularly affected by chromatin condensation. This relates to my earlier concern that the authors cannot provide the missing link between the phenotype stabilization (i.e., SMA stress fiber persistence in persistent myofibroblasts after soft substrate switch) and epigenetic changes. In other words, they use SMA stress fiber stability as an indication for myofibroblast persistence but cannot show how chromatin condensation is responsible for myofibroblast persistence. Both phenomena, SMA stress fiber stability and chromatin condensation, seem to be very different phenomena that are linked at some level, but it is still unclear how.

Reviewer #2 (Report for the authors (Required)):

The authors have done a good job responding to my concerns raised in the previous review. In my opinion the manuscript should be accepted for publication.

Reviewer #3 (Report for the authors (Required)):

The authors have been mostly responsive to my initial critique in their revised manuscript. However, there are several issues that still need to be addressed prior to publication, particularly regarding the ATAC-seq data.

Major comments:

1. The claim that persistent myofibroblasts have a more condensed chromatin structure with genome wide alterations (w.r.t transient fibroblasts) is not supported by the ATAC-seq data. In Figs. 2A-C, reads for persistent are consistently higher than reads for transient when viewed in different ways, corresponding to an increase in genome accessibility for the persistent case. The authors acknowledge this, but then include Fig. 2D – an analysis of fraction of reads inside peaks (FRIP) and drosophila spike-in reads – to argue the opposite. They find that FRIP and Drosophila reads are greater for persistent case as somehow indicative of the persistent case having fewer chromatin loci accessible to the transposase. First, the argument is very difficult to follow. Second, Figure 2D is not very high-quality data – 3 points of data for each condition that are very heterogeneous – and I wouldn't be confident concluding anything from these data. Third, the data in Figs. 2A-C seem pretty clear that the persistent case has more accessible chromatin (including at specific genomic loci for GAPDH and ACTA2), so at best, the conclusions from this figure are inconclusive. Genome-wide data can be very powerful, but are often quite complex and can defy simple interpretations, so that they need to be complemented by more specific analyses. I had suggested that the authors pursue a more specific analysis

based on these data (PCR of genes/TF analysis) precisely because of this complexity. If the authors cannot do such analyses, I would suggest that authors need to be clear in describing what each of their analyses of the ATAC-seq data show (In abstract, subtitle for the section, fig. 2 title, etc.), instead of deciding to pick conclusions from 1 specific analysis (Fig. 2D) that supports their conclusions, and ignoring other (more convincing) analyses that do not.

2. I had asked the authors to complement the TSA inhibition experiment with more specific inhibitors (e.g. of HDACs) or genetic knockdowns (e.g. of epigenetic modifying enzymes or genes/TFs implicated by ATAC-seq) previously. This was because the TSA inhibition experiments are the only data supporting a mechanistic link between chromatin remodeling and myofibroblast persistence and because TSA is such a blunt perturbation (i.e. its not clear that the chromatin changes impacted by the inhibitor are the same as those that occur during the transition from transient to persistent, particularly given the complexity of the ATAC-seq data). While the authors have added a HDAC activity reporter (which as expected is impacted by TSA inhibition), they haven't actually done the suggested experiment. The authors have not suggested a mechanistic link in the abstract/title, so I don't think the authors have to do any more experiments. However, I do think the title of Figure 3 and the section sub-title("myofibroblast persistence is dependent on changes in chromatin accessibility") extend beyond what the authors show, and the authors should reword these titles to focus on their specific results rather than making sweeping conclusions.

Minor comments:

1. Statistics are needed for 1E, 1F, 1H, 1I.

2. The additional in vivo data is helpful and I agree that the in vivo data on CCP between healthy and diseased matches what is seen in vitro. However, I think the authors have to be careful about overstating their conclusions. In particular, the nuclei in the in vivo case morphologically appear to be different than the in vitro studies (roundness is different, cross sectional area looks smaller, structure of chromatin looks clearly different). I would ask the authors to reword "Notably, myofibroblasts in patients with aortic valve stenosis display a condensed chromatin structure similar to cultured persistent myofibroblasts." to be more precise and focus on the in vivo case showing a similar trend with the CCP metric.

3. The data in 4D looks like it is simply underpowered (n = 2 or 3??), and the effect of inhibitors on average percentage of %SMA+ cells looks to be similar to the effect on normalized CCP (4E). If they had a higher number of experiments, 4D might show statistical significant differences. Thus, I don't think the authors can conclude that treatment of inhibitors does not affect % fibroblasts but does effect CCP organization.

Tue 02/02/2021

**Decision on Article NBME-20-0605A**

Dear Prof Anseth,

Thank you for your revised manuscript, "Nuclear mechanosensing drives chromatin remodeling of persistently activated myofibroblasts". Having consulted with Reviewers #1 and #3 (whose comments you will find at the end of this message), I am pleased to say that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*, provided that the points specified in the attached instructions file are addressed.

When you are ready to submit the final version of your manuscript, please [upload](#) the files specified in the instructions file.

For primary research originally submitted after December 1, 2019, we encourage authors to take up [transparent peer review](#). If you are eligible and opt in to transparent peer review, we will publish, as a single supplementary file, all the reviewer comments for all the versions of the manuscript, your rebuttal letters, and the editorial decision letters. If you opt in to transparent peer review, in the attached file please tick the box 'I wish to participate in transparent peer review'; if you prefer not to, please tick 'I do NOT wish to participate in transparent peer review'. In the interest of confidentiality, we allow redactions to the rebuttal letters and to the reviewer comments. If you are concerned about the release of confidential data, please indicate what specific information you would like to have removed; we cannot incorporate redactions for any other reasons. If any reviewers have signed their comments to authors, or if any reviewers explicitly agree to release their name, we will include the names in the peer-review supplementary file. [More information on transparent peer review is available.](#)

Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies  
Chief Editor, [Nature Biomedical Engineering](#)

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Reviewer #1 (Report for the authors (Required)):

Walker and coworkers have further improved their manuscript and responded to all my remaining concerns. They are strongly encouraged to include Figure R1 into the manuscript data (not 'preliminary', of course).

While regulation of chromatin condensation by cytoskeletal stress is well supported, data elucidating the inverse regulation (i.e., how condensed, or open chromatin regulate myofibroblast features) are still scarce.

However, the authors now better explain their assumption that chromatin condensation prevents persistent myofibroblasts from proper mechano-sensing. Any good study will generate new questions that need a follow-up and the work should now be acceptable as it stands.

Reviewer #3 (Report for the authors (Required)):

I am satisfied with the revised manuscript.

Rebuttal 1

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**Manuscript ID:** NBME-20-0605A

**Authors:** Cierra J. Walker, Claudia Crocini, Daniel Ramirez, Anouk R. Killaars, Joseph C. Grim, Brian A. Aguado, Kyle Clark, Mary A. Allen, Robin D. Dowell, Leslie A. Leinwand, Kristi S. Anseth

We would like to thank the editorial staff and the reviewers for their thoughtful and comprehensive comments on our manuscript. We have worked diligently to address all of the points raised by the reviewers in our revised resubmission. The reviewers' comments have been italicized and listed below in a blue font, and point-by-point responses and revisions are in black text. Any references to line numbers are based on the revised submission, and edits to the manuscript are highlighted in yellow. We thank the reviewers for their critiques and guidance and believe that this input has significantly improved our contribution.

**Reviewer #1 (Remarks to the Author):**

*Walker and coauthors investigate the effect of mechanical environment on chromatin condensation in cultured valve interstitial fibroblasts. Main findings of the study are that prolonged culture (>7d) of fibroblasts on stiff (~4.5 kPa) polymer substrates activates into stress-fiber smooth muscle alpha actin (SMA) positive myofibroblasts. This phenotype is preserved even after subsequent light-induced softening of the substrates (~1.5 kPa, 2d) which the authors define as 'persistent myofibroblast'. Conversely, shorter pre-exposure to stiff substrate culture (2 d) activates myofibroblasts which are not stable in phenotype after substrate softening, here called 'transient myofibroblast'. This work follows earlier studies of this group using phototunable substrates to reveal mechanical dosing effects on cell phenotypes. While previous works of this group and others have described this phenomenon as 'mechanical memory', they here set out to untangle epigenetic modifications at the basis of this memory.*

*The authors correlate myofibroblast persistence with chromatin condensation, which in turn associates with actin cytoskeleton-dependent stiffening of the nuclear membrane. This study is overall well done and of high technical quality. The presented data are solid and convincing. However, the study has major flaws in interpreting the data, and provides correlative rather than functional links between mechanics, phenotype persistence, nuclear stiffening and chromatin condensation. The mechanistic insight is limited to processes that have already been shown by others, such as the link between substrate stiffness, adhesions, cytoskeleton integrity and changes in the nuclear envelope. The authors do an excellent job in integrating these previously published aspects into a coherent story but the essential questions how stiff substrate conditions translate into chromatin condensation and transcriptional activity and how these changes are made permanent on soft substrate remain unanswered. It seems critical to establish this missing link to enhance the novelty of the study.*

We thank the reviewer for their positive comments. While this work builds upon prior results from our lab and others, this contribution describes a novel mechanism to prevent myofibroblast persistence by manipulating chromatin state. We use materials with dynamically tunable stiffnesses, ATAC-seq, and small molecule interventions. Moreover, we demonstrate through

inhibition studies and genetic overexpression that nuclear mechanosensing determines the chromatin state of persistent myofibroblasts.

The reviewer is correct in that links have been shown between substrate stiffness, adhesions, and cytoskeletal stability that translate to changes in nuclear stiffening and chromatin condensation. These studies were instrumental in our experimental design and informed our hypotheses. However, none of these studies demonstrated a mechanism that drives a disease-relevant (persistent) myofibroblast phenotype. Our findings should be of broad interest to the fibrosis community (e.g., lung, heart, valve, kidney) and the bioengineers studying mechanosensing. Our experiments quantify the role that matrix stiffness plays in driving irreversible, myofibroblast activation through nuclear mechanosensing, which has not been reported in the context of fibrotic disease. Prior literature has focused on the transient myofibroblast phenotype, which we show is distinct from the persistent myofibroblast phenotype. Our findings, therefore, represent a significant advancement, and provide new mechanistic insight into the role of nuclear mechanosensing on fibrotic disease progression.

*Major:*

*1. One major concern with the study is the low conceptual novelty and mechanistic insight. While the work appears to establish a correlation between substrate mechanics, nuclear mechanics, and epigenetic changes, all these single aspects have been published before – partly by this group. They and others have published that fibroblast-like mesenchymal stromal cells (MSC) acquire a persistent myofibroblast phenotype (PMID: 24633344, PMID: 27798620). It has also been shown that prolonged culture on stiff substrates results in high chromatin condensation parameter (CCP) values (PMID 31235788; PMID: 26592929) that persist after substrate softening (PMID 30775233). The connection between substrate stiffness, enhanced adhesion through larger matrix contacts, increased actin/myosin stress fiber formation and nuclear stiffening in a myofibroblast/fibrosis context has been amply documented (e.g., see works of the Discher lab). The cytoskeleton-modifying experiments with effect on nuclear mechanics have been done and published. It is irritating that none of these original studies that reduce manuscript novelty are acknowledged, apart from those published by this group.*

We apologize for the oversight. Our original submission focused the Introduction and motivation on the literature in the fibrosis community. The intent was to be focused, but we did not mean to diminish the contributions from the mesenchymal stem cell (MSC) community (and are aware of many of these key publications). We also appreciate the opportunity to better communicate the novelty of our results in our revised contribution, especially in the context of previous studies on multipotent MSCs vs. terminally differentiated valvular fibroblasts. Although infiltrating cells, including MSCs, can contribute to fibrosis, persistently activated resident myofibroblasts are thought to be the primary contributors to fibrosis. Moreover, our study reveals important differences between persistent myofibroblasts from MSCs and aortic valve-derived myofibroblasts, further highlighting the originality of our work. In the revision, we expanded the Discussion to place our findings in the context of the MSC literature, which should also broaden interest (lines 427 – 439).

We believe that our results are not merely a correlation, but rather define a mechanism that links the chromatin state of persistent myofibroblasts to altered nuclear mechanosensing via cytoskeleton tension. As the reviewer states, parts of this mechanism have been previously published or hypothesized, but never linked in a quantitative way. In addition, the concept of myofibroblast persistence relying on nuclear mechanosensing, especially in the context of cardiac and valve disease, is novel, and literature reports linking *in vitro*, mechanistic experiments to *in vivo*, clinically relevant samples are limited. This highlights the novel mechanistic insight of our work, both its biological context and its completeness from molecular to tissue level.

The reviewer asks about the novelty of the nuclear mechanics in the myofibroblast/fibrosis context relative to prior work from the Discher group. We agree that their work is outstanding; studying the influence of the ECM on nuclear mechanics and laying a foundation for nuclear mechanosensing, especially with respect to the role of nuclear lamina. Also, we agree that there is body of literature about nuclear mechanosensing and its effects on chromatin state (PMID: 28043971), and we apologize if our statements implied that we discovered this mechanism. Instead, we found a profound link between nuclear mechanosensing and fibrotic processes, specifically those that control the persistence of the myofibroblast phenotype in diseased tissue. As mentioned in the introduction, many studies have investigated myofibroblasts, without considered their transient versus persistent nature. Prior studies generally focus on comparing cells cultured on stiff hydrogels (myofibroblasts) versus soft hydrogels (fibroblasts) (PMID: 22461426). Much less is known about persistent myofibroblasts, especially with respect to their persistence in diseased tissue and the role of nuclear mechanics in this process. Uniquely, our study uses a materials system that allows us to generate two populations of myofibroblasts: transient and persistent. We believe that our results and experimental methods will provide a strong basis to understand the chronic nature of fibrotic disease and we use valve fibrosis as one example. Regardless, we included additional citations and further discussion of prior work in this field (lines 488-492).

*2. One novel aspect of the study is that trichostatin A (TSA) can mimic stiff substrate-induced memory to some extent with respect of general CCP values. However, the conclusion that HDACs are only partly responsible for chromatin condensation (as opposed to earlier observations of this group with MSCs) is not well substantiated. For instance, gene regions which are affected by tension can be different those affected by HDAC and HAT and two separate mechanism may generate similar results; this possibility must be assessed. Gene expression of HDAC is also not a reliable indicator for activity*

The above-mentioned literature with MSCs only used gene expression of HDACs as an indicator of activity (PMID: 30775233). Recognizing that gene expression is not necessarily an indicator for activity, we built upon our gene expression studies and performed an HDAC assay to assess its activity within the nucleus (SI Fig. 6). Interestingly, HDAC activity increases in persistently activated myofibroblasts compared to transient myofibroblasts. This is the opposite of what occurs in MSCs (PMID: 30775233) where MSCs decrease expression of HDACs over time. This finding further highlights a major difference between MSCs and fibroblasts when cultured in stiff microenvironments for extended periods of time. Without our investigation here, it's possible this differential response would not have been found, if others assume MSCs and fully differentiated fibroblasts share the same mechanisms. This is not completely surprising, since MSCs have the



potential to be either multipotent, or differentiate into various lineages. We have included this finding in the revised manuscript, Figure 3D and SI Fig. 6.

The reviewer raises another good point - perhaps HDACs and nuclear tension work through separate mechanisms. To investigate this, we A) studied nuclear tension in TSA-treated persistent myofibroblasts, B) measured HDAC activity with cytoskeleton inhibitors, and C) measured HDAC activity in tension-disrupted DNKASH cells.

A) When persistent myofibroblasts are treated with TSA, nuclear tension remains elevated, similar to DMSO-treated persistent myofibroblasts. This result indicates that TSA likely reverses myofibroblast persistence by reducing HDAC activity, but not by reducing nuclear forces. We included this finding in the revised manuscript, SI Fig. 13D.

B) When persistent myofibroblasts are treated with actin cytoskeleton inhibitors, HDAC activity is reduced and other morphometric parameters (nuclear roundness and cell area) approach those of transient myofibroblasts. These findings are part of SI Fig. 11.

C) To investigate if high nuclear tension increases HDAC activity and promotes myofibroblast persistence, we measured HDAC activity in DNKASH cells, which have reduced nuclear forces (PMID: 21652697). DNKASH and control cells were cultured under conditions to induce persistence (7 days on stiff hydrogels) and compared to cells cultured under conditions that yield transient myofibroblasts (1 day stiff hydrogels). After 7 days, HDAC activity increased in control cells, but remained constant in DNKASH cells. This result suggests that tension across the nuclear membrane may control HDAC activity, which further alters chromatin architecture, and ultimately myofibroblast persistence. This result is included in Figure 5L.

Our results indicate that cytoskeletal-nuclear tension controls HDAC activity, which then contributes to chromatin condensation.

*3. Along these lines, one essential question remains open: How do fibroblasts manage to keep condensed chromatin and stiff nuclei on soft substrates where adhesions and actin cytoskeleton would typically be weakened, and cell stress reduced?*

We apologize for the confusion and included soft hydrogel controls for critical experiments throughout the manuscript, and we now include them in the supplemental data (SI Fig. 2, 3, 6, 7, and 13) to address this reviewer's point. The chromatin of cells cultured on soft hydrogels remains closed, and the HDAC activity does not significantly change over time (Figure 1H, SI Fig. 6), whereas the chromatin of cells on stiff hydrogels is initially open, but then closes over time with an increase in HDAC activity. Note that the nuclei of these cells on soft hydrogels are NOT stiff, as measured by the nesprin tension sensor and lamin AC (SI Fig. 13). In fact, the nuclei on soft hydrogels remain rather compliant from d1 to d7.

Thus, one interesting finding from these data is that chromatin structure does NOT correlate with ECM stiffness or activation, but rather myofibroblast transience or persistence. Here, we focused on what occurs during the transformation of a transient myofibroblast to a persistent myofibroblast

on stiff hydrogels, rather than the process by which cells maintain a closed chromatin structure on soft hydrogels. However, we can speculate that maintenance of a closed chromatin structure in cells on soft hydrogels does not rely on the same nuclear mechanosensing mechanisms employed by cells on stiff hydrogels, since nuclear tension does not change over time and HDAC activity is not increased.

*4. This conundrum points to a possibly fatal artifact generated by the authors' experimental design. The authors are not clear which exact conditions are used in each experiment to produce transient or persistent myofibroblasts. The respective methods section on 'dosing' does not explain dosing but how the gels are softened. This reviewer understood that 7d stiff-2d soft was used to study persistent myofibroblasts and presumably 3d stiff-2d soft to study transient myofibroblasts (as interpreted from figure 1C). The phototunable substrate assay is undoubtedly very elegant and useful. However, it has the inherent problem that transient (5 d) and persistent myofibroblasts (9 d) are cultured for different total periods. This growth difference will be enough to produce substantially different matrix and mechanical environment in each condition that possibly override the hydrogel stiffness switch. The authors will need to create comparable conditions in key control experiments. Prolonged culture on soft substrates (e.g. 2 d stiff, 7 d soft) will correct for growth time artifacts but present other problems related to dosing effects.*

We apologize for any confusion as to the conditions used for achieving transient or persistent myofibroblasts. Lines 130-134 and the schematics within each figure were intended to make this clear, but we now provide more clarification as to the timeline for each condition, as well as added more details in each the figure legends to aid in interpretation of the data. For example, in Figure 1, myofibroblasts cultured on stiff hydrogels for either 1 or 3 days are considered transient. Myofibroblasts cultured on stiff hydrogels for either 7 or 9 days are considered persistent. The material softening then allowed us to determine if the myofibroblast populations were transient or persistent.

However, we agree with the reviewer's point that there is an inherent time difference to generate the transient versus persistent myofibroblasts. Indeed, evolution of the persistent myofibroblast phenotype is a time-dependent process. To address this point, we included control results for fibroblasts cultured on the soft hydrogel for up to 9 days (Fig. 1H-I, SI Fig. 2, 3, 6, 7, and 13) and this direct comparison further confirms that the phenomenon is in fact caused by stiffness, rather than culture time.

We also acknowledge that soft controls may not completely account for growth differences between soft and stiff cultures. Thus, we also analyzed our persistence timeline as a function of initial cell seeding density (SI Fig. 1D). We did not observe any significant differences, even over a 4-fold difference in cell density, suggesting that our results are independent of cell density or growth. Additionally, while MSCs are very active and deposit matrix molecules in culture, valve-derived fibroblasts require an ascorbic acid supplement to crosslink and deposit a collagen matrix (PMID: 6308103). We did not include ascorbic acid in our media. Even if fibroblasts were as active as MSCs, we would not expect that ECM molecules deposited would significantly alter the mechanical properties of our hydrogel over the course of 9 days. Our gels are 2-3 orders of magnitude stiffer than any cell secreted matrix, so the cells are largely sensing the mechanics of the synthetic hydrogel. Further, we do not observe any significant collagen deposition on our

hydrogels, even after 9 days (SI Fig. 1C). As such, we believe any ECM deposited from valve fibroblasts would not override the hydrogel stiffness switch.

*5. The authors initially describe that stiff culture results in higher CCP values (Fig.1) which is consistent with prior studies. However, they then continue to follow up on transcripts from open chromatin regions, notably adhesion- and cytoskeleton-related genes. How increased overall chromatin condensation would specifically open these regions remains elusive.*

This is a good point. Since the initial submission, we obtained additional ATAC-seq replicate datasets and further analyzed our existing datasets using new analytical methods. We now have an altered interpretation of our collective ATAC-seq results. Based on our original and more limited ATAC-seq data set, we were unable to observe any genome-wide differences in chromatin accessibility between the transient versus persistent myofibroblasts. However, after additional replicates and improved analysis of the data, we can now confirm that there are **global** chromatin accessibility differences. Our improved analysis uses spiked-in *Drosophila melanogaster* nuclei that serves as an important internal control. This allowed us to better normalize the sequencing coverage across the experimental conditions. In addition, we manually curated and annotated a set of genome segments from the current pig genome assembly (susScr11) to flag them as ‘blacklisted’. We then filtered those regions out from our analysis. These blacklisted regions are known to produce a substantial degree of mapping artifacts, which can, in turn, alter subsequent total mapped reads-dependent normalization approaches. After adding this to our analysis, it became clear that the persistent myofibroblast phenotype has compacted chromatin, but we did not find evidence that there are specific genes whose chromatin is preferentially open or closed, not even those genes related to adhesion nor cytoskeleton formation or maintenance. Instead, the effect is global. We have updated Fig. 2 with the new ATAC-seq datasets and analysis.

*6. Directly linking changes in chromatin structure of adhesion/cytoskeleton-related genes to structural changes in focal adhesions and cytoskeleton in the observed timeframe is highly problematic. Focal adhesion size and actin organization are regulated on a shorter timeline by post-translational modifications (e.g., cofilin phosphorylation). How these would be affected by epigenetic changes within few days remains to be shown. As noted above, different growth times will also skew these results due different times available to express gene products.*

We agree with the reviewer that focal adhesion signaling occurs over a shorter timescale than epigenetic changes. Indeed, we hypothesize that focal adhesions and the cytoskeleton are the initiators for responding to stiff hydrogels, inducing myofibroblast activation (transient myofibroblasts). Cytoskeletal stabilization over time is what causes chromatin condensation and determines the persistent myofibroblast phenotype (Fig. 4). Chromatin condensation reinforces the myofibroblast phenotype, rather than causing it. Further demonstration of this point is included by treating fibroblasts on soft substrates with Garcinol, a histone acetylase inhibitor (SI Fig. 8). Garcinol causes chromatin condensation and accelerates myofibroblast persistence but does not promote myofibroblast activation on soft hydrogels. High CCP levels, such as those observed in soft controls, does not induce activation ( $\alpha$ -SMA expression), thus confirming that ‘closed’ chromatin state is responsible for persistence and not activation. Like many pathological processes, we believe this is a positive feedback loop that further exacerbates the contractile, myofibroblast

phenotype making the cells permanently activated. To clarify this point, we include experiments using TSA and garcinol (now Fig. 3 and SI Fig. 8) and added to the discussion (lines 458-462).

*7. The ATAC sequencing data is under-analyzed for critical genes in the context, such as different actin isoforms, focal adhesion proteins, actin-turnover proteins, focal adhesion kinases and phosphatases, etc. An obvious omission is to look for SMA as the bona fide myofibroblast protein. From their ATAC-seq data, the authors claim that genes are associated with opening peaks, but they do not validate, e.g., using ATAC-qPCR. Alternatively, ATAC-seq tracks should prove that the region near to the specific gene is open and then the enrichment should be verified using ATAC-qPCR or even traditional qPCR.*

We agree with the reviewer and completed additional ATAC-seq experiments and analyses. As mentioned in Point #5 above, we changed some of our analysis to improve the interpretation of our ATAC-seq datasets. This allowed re-normalizing our data in a way that it now accounts for potential genome-wide changes in the magnitude of the read coverage signal. Regarding the reviewer's suggestion to check on any chromatin accessibility changes at those genes that are known to be implicated in cytoskeleton dynamics, we observe that though most of those genes show a slight increase in signal in the persistent state (Fig. 2A, shows specifically ACTA2/  $\alpha$ SMA), this change in signal is not specific nor enriched in any gene ontology classification. Rather, our results suggest a global decrease in chromatin accessibility that affects all genes in a similar fashion. We cannot not rule out the existence of groups of genes that are specifically affected (i.e., through a concrete chromatin remodeler or transcription factor that only affects them), given that our attempts to use a statistical analysis to find such genes failed because 1) there is a great degree of noise across the replicates, and 2) most genes do seem to change (Fig. 2B,C), which may drown out the signal from any putative set of genes that may be differentially regulated.

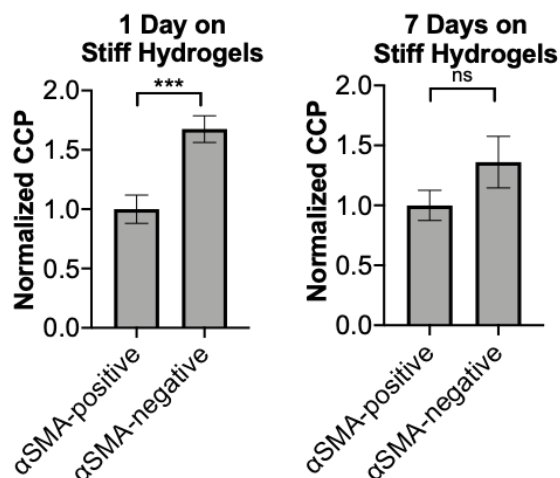
Regarding the reviewer's point on validating ATAC-seq changes with ATAC-qPCR, we agree that any genome-wide sequencing assay requires orthogonal validation when it claims to have found specific loci of interest. In our case, however, we did not find any specific ATAC-seq peak changes between the transient and persistent myofibroblast conditions. Mostly, there are many peaks that are changing slightly in their signal of chromatin accessibility. Also, even for the few peaks that showed a promising change in their ATAC-seq signal magnitude, they were not found at TSS, but rather in intergenic regions. To accurately predict the target of those putative regulatory regions would require us to conduct chromatin conformation capture assays, which are beyond the scope of this current study.

*8. Understandably, the authors focus on the 80% activated myofibroblast in their study. However, analysis of the CCP values of the remaining 20% non-activated fibroblasts would tell whether the observed phenomena are a direct consequence of substrate mechanics or indirectly associates with myofibroblast activation. As the authors note, chromatin condensation has been described for myofibroblasts independently of (or ignoring) mechanical environment. The experiments with TSA seem to indicate that chemical factors may compensate for or override mechanics.*

If we are interpreting the reviewer's comment correctly, but we believe the question is if chromatin condensation depends on substrate stiffness or myofibroblast activation. We appreciate the suggestion of measuring CCP in the 20% un-activated cells on stiff hydrogels. We measured the

CCP of  $\alpha$ SMA<sup>+</sup> and  $\alpha$ SMA<sup>-</sup> cells cultured on stiff hydrogels for 1 or 7 days (see below). The CCP of  $\alpha$ SMA<sup>+</sup> cells was significantly higher than that of  $\alpha$ SMA<sup>-</sup> cells. This difference between  $\alpha$ SMA<sup>+</sup> and  $\alpha$ SMA<sup>-</sup> cells disappeared by 7 days of culture. These results mirror our findings in Figure 1H, which show un-activated fibroblasts (soft hydrogels) have increased CCP compared to myofibroblasts at early times (1-3 days), but not at late times (7-9 days). To us, this indicates that chromatin dynamics (measured by CCP) depend upon myofibroblast activation and its persistence, and not substrate stiffness.

Our experiments with cytoskeleton inhibitors and DNKASH expression clearly show that chromatin condensation results from tension exerted on the nuclear lamina by the stabilized actin cytoskeleton. Further, HDAC activity depends on nuclear tension (Fig. 5L). TSA opens chromatin because it reduces HDAC activity (Fig. 3D) and by-passes cytoskeleton-driven nuclear tension. However, TSA treatment does not impact nuclear tension (SI Fig. 13D).



**Figure i:** CCP measured from  $\alpha$ SMA positive or  $\alpha$ SMA negative cells cultured on stiff hydrogels for 1 day or 7 days.

*9. Data presentation in Fig. 1H is somewhat confusing by connecting or fitting the data points for very different conditions. It is understandable that CCP values are higher on stiff than on soft substrates. However, why would CCP values increase in transient myofibroblast upon substrate softening as stated in the text?*

The new Figure 1H may provide some clarification. In the revised manuscript, this figure was modified to include soft hydrogel controls. Note that CCP values are lower on stiff hydrogels compared to soft hydrogels at early time points (d1-d3). However, over time, the CCP values of cells on stiff hydrogels increase to a level that is similar to those on soft hydrogels. So, it appears that upon substrate softening, transient myofibroblast increase their CCP values “back” to levels that are observed on soft hydrogels.

This further highlights how fibroblasts behave differently from MSCs in terms of chromatin condensation (PMID: 30775233). While it may be intuitive to assume fibroblasts and MSCs would have similar mechanisms of nuclear sensing and CCP, it is not the case.

*10. The correlation of CCP values in cultured valve fibroblasts and tissue samples is a stretch and a functional relation to valve stiffening speculative. If the authors wanted to follow this up, they would need to directly correlate valve stiff regions (e.g., AFM) with nuclear chromatin and lamina staining using statistically significant sample sizes.*

We agree that our human data are limited in terms of sample size, and this is due to 1) the expense of obtaining human samples and 2) the lack of availability of healthy patient valve tissue. Nonetheless, we increased our sample size for diseased patients to 7 (using available tissues from Origene company) and added morphometric analyses of the nuclei (see below, Fig. 1K). We also added a supplemental figure analyzing results from each patient sample, and a description of the medical history of patients (SI Figure 4). We acknowledge that this limits some of the quantitative aspects of our conclusions, and we note this in our text in lines 515-518.

Since aortic valve disease is associated with increased tissue stiffness (PMID: 22222074), we did not measure tissue stiffness using AFM. We acknowledge that our human data are speculative, and at most correlative, we do believe that it highlights the need to investigate these mechanisms in the context of human disease.

*11. It is highly problematic that cytochalasin D and Rho kinase inhibition do not affect SMA stress fibers at the reported concentrations. First, this observation is contradictory to numerous published studies. In fact, Rho kinase inhibitors have been reported to mainly affect myofibroblast contraction and have advanced to clinical studies (e.g., publication from the Thannickal group). Second, data presented in Fig.3C, D, G do not make biological sense. Stress fiber SMA is filamentous actin and labelled by phalloidin. In myofibroblasts SMA contributes to the vast majority of F-actin positive signals at light microscopy resolution. If SMA stress fibers change upon drug treatment, F-actin levels will change accordingly - or both do not change. Immunofluorescence assessment does not seem reliable in these experiments and the authors should perform G-/F-actin sedimentation assays. Another curiosity is that CCP values decrease in chemically produced transient myofibroblasts with an intact SMA stress fiber cytoskeleton – assuming that actin stress contributes to nuclear stiffening as one precondition. The conclusion on page 15 is hard to follow.*

We appreciate the comments and are aware of this literature. We agree that prior results show that cytochalasin D and Rho kinase inhibition affect  $\alpha$ SMA stress fibers. To be clear, our study is quantifying  $\alpha$ SMA as “stress-fiber positive cells”, and that includes cells that have either 10 or 500 stress fibers. These concentrations were chosen very carefully to not utterly obliterate  $\alpha$ SMA stress fibers (SI Fig. 10E), but rather reduce intracellular, cytoskeletal tension. At higher concentrations of cytochalasin D and Y27632, there is a reduction in  $\alpha$ SMA<sup>+</sup> positive cells. The data even indicate that there is a “trend” towards fewer  $\alpha$ SMA<sup>+</sup> cells with Cytochalasin D and Y27632 inhibition (Fig. 4E), although not significant. In the revised manuscript, we comment in the results section (Lines 299) and note our dose curve for  $\alpha$ SMA in the supplemental information (SI Fig. 10E).

To address the second point, we highlight that CCP values do not decrease in chemically produced transient myofibroblasts, but instead remain constant with time, in the transition from transient to persistent myofibroblasts (Fig. 4E). This result shows that cytoskeleton inhibition prevents CCP condensation and thus transition to persistence. In other words, myofibroblasts treated with

cytoskeleton inhibitors are transient, even when exposed to a stiff environment for up to 7 days. In Fig. 4E, non-treated vehicle controls are the ‘conventional’ persistent myofibroblasts and their CCP increases over time (consistent with results in Fig 1). Together, these results indicate that intracellular tension drives a time-dependent chromatin condensation during myofibroblast persistence. To clarify we altered the text (Lines 301-302, Lines 304-306).

*12. As noted before it remains unanswered why drug-induced relaxation of myofibroblasts is not equivalent to soft-substrate-induced relaxation. Rescue experiments are missing with this respect. For instance, if uncoupling the cytoskeleton from the nucleus prevents persistent myofibroblast formation, is it possible to turn persistent into transient myofibroblast by overexpression of the identified components. Would increasing cytoskeletal tension affect chromatin condensation in soft-cultured cells in the same way stiff environment does?*

If we understand correctly, this reviewer is asking if cytochalasin D or Y27632 treatment is similar or not to the condition of hydrogel softening. Our results suggest that persistent myofibroblasts require two components to reverse persistence and then activation (because a myofibroblast can be transient, but still activated): substrate softening/intracellular stress relaxation AND chromatin opening. For instance, once persistence is achieved, we do not observe a change in myofibroblast activation or persistence with either softening or drug treatment (Fig. 1E). Or, once persistence is established, we do not observe a reversal of the myofibroblast phenotype if we treat persistent myofibroblasts with only TSA (no softening) (Fig 3E). However, we can either 1) prevent persistence by preventing intracellular tension via drugs (Fig 4), or we can 2) reverse persistence by reducing intracellular tension via softening AND resetting the chromatin structure (Fig 3F-H).

Transforming transient myofibroblasts into persistent myofibroblasts by increasing cytoskeletal tension could be helpful; however, we don't think believe one specific component of the actin cytoskeleton is responsible. Additionally, we demonstrate that the transition from transient to persistent myofibroblasts is dependent on time as components of the actin cytoskeleton are already expressed in transient myofibroblasts, but it is the cumulative tension on the nucleus that determines persistence.

#### Minor comments:

*1. The schematic drawing Fig.1A should match the experimental values (e.g. 4,5 instead of 3.5 kPa).*

Thank you for pointing this out. We used a representative rheological trace rather than the average. However, we have now changed the rheological trace of Fig. 1A to the mean of several rheological measurements, which now match the experimental values reported in Fig. 1B.

*2. Fig.2: It is preferred to show the western blot data from one blot rather than single cut bands. This should be re-run with respective loading.*

Single bands were shown since samples were not run on neighboring bands of the western blot. However, since we modified Figure 2 to better reflect the new ATAC sequencing data and analysis, the western blot representative images are no longer relevant and have been removed.

## Reviewer #2 (Remarks to the Author):

*In this paper Anseth and co-workers use a light-activated softening hydrogel demonstrated previously by the group (for instance, Nat. Mater., 13, 645–652, 2014) to study nuclear mechanosensing in myofibroblasts. Fibroblasts were cultured on dynamic substrates that were subsequently softened, and statically soft and stiff hydrogels, with markers for myofibroblasts monitored. Interestingly, they identified “transient” and “persistent” populations of myofibroblasts that depended on the initial culture time on stiff substrates. Analysis of chromatin condensation and treatment with inhibitors of histone modifying enzymes and cytoskeleton disruptors, demonstrates attenuation of the persistent myofibroblast phenotype in favour of the transient. This is an exciting demonstration of controlling epigenetic plasticity with dynamic cell culture materials. Nevertheless, there are several points that need to be addressed prior to publication.*

We thank the reviewer for their comments to strengthen our contribution. We performed additional experiments that are summarized below:

*1. While the light-sensitive PEG system has been used before by the group, there is little detail in the paper regarding these hydrogels. The mechanical properties are described but what about peptide density at the cell interface? Some additional information describing the quantity of RGD ligands that a cell adheres to and how this changes on softening is important. Could decreased exposure to RGD peptide on softening influence myofibroblast phenotype?*

To address these points, we now include information about the monomer chemical structure in the supplementary information (SI Fig. 1). The ligand density has been shown to be very important for directing cellular phenotype (PMC1303831). However, based on our prior experiments we know that these RGD concentration used herein is well beyond any threshold that would influence cellular phenotype with hydrogel softening (PMID: 19747725). Specifically, 5 mM of RGD is a large excess of ligand that allows robust fibroblasts attachment, even with the ~10% change in concentration that occurs with softening (PMID: 25778824). Typically, a 10X or great change in the [RGD] is needed to observe any significant change in gene expression in myofibroblasts.

However, to assess the reviewers' point more quantitatively, we characterized the RGD peptide surface concentration before and after light irradiation (SI Fig. 1B). We substituted 0.1 mM of acrylated-RGD with acrylated fluorescein during gel formation with 365 nM light at 10 mW/cm<sup>2</sup> for 360, 600, 900 seconds. We then measured the released fluorescein as a proxy for the amount of acrylated RGD release from the hydrogels. We found that even after 360 seconds of light exposure, only 12% of the fluorescein was released, implying a similar 12% change would occur in the RGD concentration (i.e., 5 mM to 4.4 mM). Tong et al. found that even a ~30% reduction in RGD density had no significant effect on fibroblasts morphology (10.1021/acsbiomaterials.6b00074). We have added this result in SI Fig. 1.

*2. TSA will lead to open chromatin –how does this relate to myofibroblast (de)activation? The ATAC seq results and actin disruption suggests that mechanotransduction through the cytoskeleton directs myofibroblast persistence. However, broad spectrum HDAC inhibitors will disrupt many aspects of nuclear signalling, with a host of transcription factors with binding*



*partners, etc.. Is the mechanism proposed that physical unwinding of the chromatin by itself regulates this transition from transient to persistent? Additional discussion of the proposed mechanism would be useful.*

This is a good point and we propose that HDACs are the primary mechanism leading to myofibroblast persistence. As part of our manuscript review, we were asked to perform HDAC activity assays, instead of gene expression measurements. We found that HDAC activity was higher in persistent myofibroblasts compared to transient myofibroblasts (SI Fig. 6). Moreover, we found that disrupting the nuclear to cytoskeletal connection with DNKASH prevented any increase in HDAC activity (Fig. 5L). Based on this, we updated proposed mechanisms of action to include the role of the cytoskeleton and nuclear tension in altering HDAC activity and subsequently chromatin accessibility and myofibroblast persistence. The discussion has been modified in lines 501 – 511.

*3. The DN KASH experiments are interesting and do suggest that direct connectivity of actin to the nuclear membrane is responsible for guiding the persistent phenotype. However, surely there are many more activities associated with actin-nuclear activity. Are the fibroblasts showing a transient or quiescent phenotype after integration of this construct? The issue here is related to whether the loss of the persistent phenotype is on account of other attenuated activities from the cytoskeleton.*

To mitigate these concerns, we used an infection control with mCherry (control-infected) to account for viral transduction. Both the DNKASH and mCherry infected cells activated to myofibroblasts, suggesting that mechanoresponsiveness is not different between the two. However, it is worthwhile to note that the orientation of the actin stress fibers is different between the two constructs, where the control-infected fibers are across the nuclear membrane and DNKASH cell fibers are limited to the cellular periphery. The original construct (published in PMID: 21652697) performed qPCR on mechanoresponsive genes and showed they were not affected by the disruption of actin-nuclear connection. Additionally, PMID: 29934494 showed that DNKASH expression did not affect global cytoskeletal defects.

We also performed phenotypic analysis of the DNKASH and control-infected cells and found that DNKASH expression affected chromatin condensation and nuclear roundness, but not cell area (SI Fig. 14). This agrees with the above study. We agree that we cannot discount that the DNKASH prevents cells from becoming persistent through some other means (e.g., attenuated transcription factor activity, etc.) However, prior publications show that DNKASH expressing cells generate lower force in their intranuclear space, which correlates to chromatin mobility and condensation (PMID: 24619297, PMID: 29748381). Both Y27632 and DNKASH disrupt this intranuclear force generation, and our results agree are consistent with these findings. Further, our experiments with cytochalasin D and Y27632 suggest also that the actin to nuclear connection is essential, and we propose the nuclear structure as the mechanism of action in DNKASH cells.

*4. The authors discuss their results in the context of recent work to “unload” fibrotic tissue to attenuate disease and didn’t find a decrease in fibrosis. One clear difference is the planar studies explored in this work, and the 3D nature of fibrotic tissue. Could the dimensionality of the fibrotic*

*matrix—and presumably a very different adhesive-cytoskeletal pattern—decrease the potential for epigenetic reset on account of “unloading”?*

We acknowledge that the 3D microenvironment can be different than our experimental setting; however, the 3D matrix introduces complexity (e.g., diffusion limitations, more difficult assays, spatial heterogeneities in matrix mechanics). Here, we simplified the cellular microenvironment to study matrix stiffness effects in isolation of other confounding factors. However, we modified our discussion to acknowledge some of the limitations of our model (lines 443 – 444).

**Minor points:**

*The results section begins with a very brief description of the hydrogel system, followed by numbered sections. This first section of the results should be under a subheading with more detail of the biomaterials fabrication and characterisation.*

We modified the results section accordingly.

*Epigenetic signatures as used in reference to Figure 1 is not strictly correct as they are measuring chromatin compaction. Suggest rewording, e.g. chromatin signatures*

We agree and have modified the title and wording of our manuscript to address this subtle, but important difference. The new title is: “Nuclear mechanosensing drives chromatin remodeling of persistently activated myofibroblasts”. Line 94 also reflects this change.

*HAT is an acronym for Histone Acetyltransferase; please be sure to use consistent wording*

We thank the reviewer for catching our error.

*Typo in reference 11 – journal name “Developmental Cell” appears.*

Thank you for the catch, we edited the reference accordingly.

### **Reviewer #3 (Report for the authors (Required)):**

*In this manuscript, Walker and colleagues explore the role of epigenetic remodeling in the persistent activation of myofibroblasts in the context of tissue fibrosis. Using an established PEG hydrogel platform, in which the modulus can be dynamically softened by light, they find that longer culture on stiff substrates leads to a greater percentage of cells expressing SMA following softening (i.e. “persistent myofibroblasts”). Persistent myofibroblasts exhibit higher levels of chromatin condensation, and alterations in genome accessibility, relative to transient myofibroblasts. Inhibition of actin and rho reduces persistence and chromatin condensation. Finally, resistance is correlated with higher tension on nesprin, and knockdown of KASH reduces persistence.*

*This is potentially an important paper that ties mechanical memory to changes in nuclear architecture and the epigenome, and then changes in nuclear architecture and the epigenome to nuclear tension, specifically in the context of fibrosis. However, the major conclusions need stronger support and there are some key inconsistencies/questions with the data and text as shown. These need to be addressed substantially prior to publication.*

We thank the reviewer for their positive reaction to our findings, and we have performed new experiments to support our main conclusions.

*1. Are the “persistent” myofibroblasts really persistent?*

*a. Do persistent myofibroblasts represent a distinct state or is it just that the timescale for recovery to a non SMA positive state just longer when cultured on stiff gels first for 7 days? The authors should culture the fibroblasts for a longer period of time after 7 days on stiff gels (e.g. 7d/7d) to evaluate whether the phenotype recovers in this case. This could have implications for claiming the relevance of these findings in human fibrotic disease.*

*b. Analysis of persistence is based solely on staining for  $\alpha$ SMA. Additional molecular analyses should be included to strengthen the identification as myofibroblasts e.g. gene expression of other markers and transcription factor activation.*

*c. As shown in a recent paper from the Burdick group (Loebel, et al., Nature Materials 2019), cells can deposit matrix locally to form their own microenvironment. Are cells depositing their own matrix, and is this the reason more persistent myofibroblasts occur when cells are cultured on the stiff gels for longer because they are depositing their own matrix? This should be investigated.*

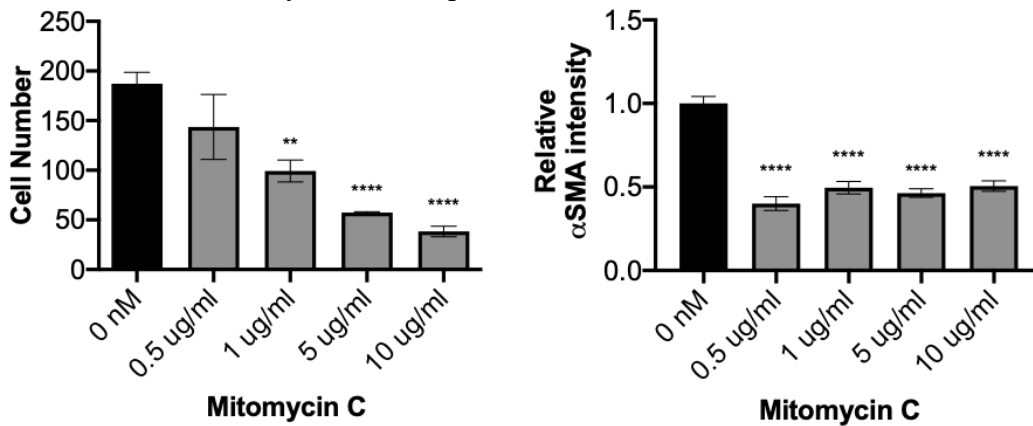
*d. Another caveat of the longer culture on stiff gels is the potential that cell numbers could be different due to proliferation, which could mediate the different myofibroblast phenotype. This should be quantified and the basic experiment should be redone with proliferation inhibited.*

a) Results in SI Fig. 2 show that the myofibroblasts maintain their persistence up to 6 days post softening. Longer experiments are confounded by contact inhibition.

b) We also performed western blots for common myofibroblast markers (SI Fig. 2), and we observed persistent protein expression of  $\alpha$ SMA and EDA-fibronectin. All of these markers are consistent with the immunostaining results.

c) In Loebel et al. Nat Mat. 2019 the authors use MSCs grown with full serum (10% FBS), which are known to be highly active and secrete large amounts to matrix. The authors saw significant matrix deposition after 4 days in 3D culture. Unlike 3D, cell-secreted matrix components would be removed with media changes in our 2D system. Also, while valve myofibroblasts are responsible for matrix remodeling and deposition, they are not nearly as secretory as MSCs (PMID: 16740254). Since 70-80% of the ECM secreted by valve fibroblasts is collagen (PMID: 22609448), we tested if the fibroblasts deposited any noticeable collagen on our 2D hydrogels over the timelines used in this study. Picrosirius red staining of transient (1d) and persistent (7d) myofibroblasts on stiff hydrogels did not reveal any noticeable collagen deposition (SI Fig. 1C). At best, some collagen was detected in the intracellular space. Of further note, we used a low growth serum media in our studies (1% FBS), which further limits ECM production (PMID: 6461858). Finally, Loebel *et al.* did not show that nascent protein deposition in 2D influenced MSC behavior. If nascent protein deposition levels influenced myofibroblast persistence, we should see differences in persistence with different initial seeding densities of fibroblasts on hydrogels, since fewer cells would deposit overall less protein. There was not an observed difference in persistence at 10K, 20K, or 40K cells/cm<sup>2</sup> (SI Fig. 1D). These results are included in SI Fig. 1.

d) To minimize proliferation, we routinely culture valve fibroblasts in 1% serum media as this does not compromise viability. While proliferation is reduced, it still occurs (PMID: 28390245). One can inhibit proliferation with mitomycin C, but this completely inhibites myofibroblast activation (result below). Moreover, mitomycin C is a DNA crosslinker, so its use would disrupt our chromatin-based assays (PMID: 16258176). As an alternative strategy, we tested if cell density influences mechanical memory by seeding fibroblasts on hydrogels at densities 10K, 20K, or 40K cells/cm<sup>2</sup> (SI Fig. 1D). No significant differences in persistence were observed. Thus, even with low levels of proliferation that can alter the initial cell density, we believe that ranges of cell density studied do not influence myofibroblast persistence.



**Figure ii:** Fibroblasts treated with a dose curve of mitomycin C to prevent proliferation. Proliferation measured by average cell number per image. Myofibroblast activation measured by αSMA intensity. At doses with a noticeable effect on proliferation also prevent myofibroblast activation. One-way ANOVA with Bonferroni posthoc applied in comparison to 0 nm concentration.

2. The CCP analysis and, in particular the connection of chromatin condensation in

*healthy/diseased valve tissue is potentially powerful, but there appear to be some flaws in the analysis and the in vivo comparison is quite preliminary.*

*a. I am very confused by the results of Fig. 1H and how they are described in the manuscript. While CCP goes up for stiff only case (from ~1 to 2), it seems to be constant at ~2 for the stiff-to-soft case, independent of culture time (Fig. 1H). For example, after 3 days of culture in stiff case (high SMA+), CCP is ~1.0, while 1day stiff/2day soft (lower SMA+) is ~2.0. This seems inconsistent with claim that more persistent fibroblasts have higher CCP than less persistent ones (Fig. 2c), and the text that "After in situ softening in the transient myofibroblast conditions, the CCP increased significantly while the CCP of persistent myofibroblasts remained constant and did not change significantly in response to hydrogel softening." Is this figure mislabeled or are there some additional data that are missing?*

*b. In vivo analysis was done on only one patient for each condition. This is obviously not sufficient to make any conclusions (i.e. "Notably, myofibroblasts from human aortic valve tissue show similar epigenetic signatures"). The authors need to conduct on a number of patients and compare the averages of the CCP values to make a stronger conclusion about how CCP varies with healthy vs disease condition. The in vivo connection is an important part of this manuscript.*

*c. Further, with the current data, the normalized CCP values are different in range for the in vivo case (both healthy ~ 1.0 and diseased ~1.2) as compared to the in vitro studies (1.0 – 2.0), yet the authors highlight the increase in CCP in diseased state (though not a very low P-value) as consistent with their results. Its not clear to me whether the relative increase vs. the values themselves is what should be considered. The authors need to explain this.*

*d. Related to making the in vivo comparison stronger, the authors should look at other metrics beyond CCP to build stronger support for their model and for a more rigorous analysis of the nucleus state. For example, they can easily quantify nuclear characteristics (shape & volume or cross sectional area) and show how these line up.*

a) We have now modified Figure 1H, and we believe the new presentations make the data easier to interpret. First, a soft hydrogel control is included, so one can see directly that cells on the soft hydrogels have a constant CCP, while cells on stiff hydrogels converge toward a similar CPP to those on soft hydrogels levels over the course of 7 days. It should be noted that the final CCP is not significantly different between the stiff and soft hydrogels at day 7+. This result indicates to us that the CCP does not correlate to myofibroblast activation, but rather myofibroblast persistence. Moreover, one can compare the stiff-to-soft conditions in relation to either the constantly stiff or soft controls. For instance, the CCP of stiff-to-soft at transient timepoints (1d2d, 3d2d) reverses back to levels similar to that of soft controls. Conversely, the CCP of stiff-to-soft myofibroblasts at persistent timepoints (7d2d), are similar to both stiff and soft hydrogels. We conclude the following: 1. chromatin condensation changes over time on stiff hydrogels, but not on soft hydrogels. 2. the chromatin phenotype is reversible during transient time points. In short, these results suggest that chromatin condensation correlates with myofibroblast *persistence*, but not with myofibroblast *activation*.

b) To address this point, we increased the patient samples to 8 (Fig 1J-K, Fig 5B) and include a detailed breakdown by patient of our findings in the supplement (SI Fig. 4). We also acknowledge the limitations of our human patient sample analysis in the discussion (lines 515-518).

c) After adding more patients, the CCP values for the persistent myofibroblasts increase to about 1.25, which is not as high as observed in the *in vitro* studies (~1.5-2) but perhaps not unexpected because of the heterogeneities in patient tissue. In contrast, the hydrogel model is uniform, with a defined and discrete increase of stiffness across all cells. Moreover, patient samples likely contain both transient (healthy patients) and persistent (diseased patients), and more transient myofibroblasts would decrease the CPP. We have made added these caveats to our discussion in the revised manuscript. However, as reported in SI Fig. 4, there are interesting patient-specific differences in CCP, nuclear roundness, and lamin AC values. This could be a reflection of disease severity; however, we do not have enough data to state this conclusively.

d) This is an excellent suggestion, and we have now added nuclear roundness analysis for our *in vitro* conditions (Fig. 1I, SI Fig. 7, 11, 14). Interestingly, we found that nuclear roundness correlates with myofibroblast persistence *in vitro* (SI. Fig. 3). We also bolstered our lamin AC nuclear localization analysis by increasing the number of patients analyzed (Fig. 5B). together, the data agrees with our *in vitro* results and shows reduced nuclear roundness and decreased nuclear peripheral lamin AC localization in the nucleus of diseased compared to healthy human valve myofibroblasts.

*3. ATAC-seq analysis is very general and under-utilized, adding little to the manuscript in its current form.*

*a. The authors show that there are differentially accessible peaks between transient and persistent fibroblasts, including more open and more closed peaks. However, the CCP data (I think) seems to indicate that chromatin should be more closed on average in the transient myofibroblasts. How the authors reconcile these seemingly different interpretations?*

*b. ATAC-seq data is a good starting point, but it should be confirmed that at least some of the peaks with larger differences between conditions correspond to differences in gene expression.*

*c. Further, the authors should use standard bioinformatics pipelines to identify transcription factors predicted to act on the opened-up sites. Ideally, they could knockdown or knockout these TFs to test for a functional role.*

*d. ATAC-seq analysis should be done with the actin/myosin inhibitors and the KASH mutants to show that when the persistent state is inhibited, the transient ATAC-seq signature is recovered. This would provide strong evidence for the claim of “distinct chromatin signatures” in persistent fibroblasts.*

a) We include additional ATAC-seq data and analysis, which revealed some new insights. In particular, we clearly see a global chromatin accessibility change, with more compaction in persistent myofibroblasts relative to transient myofibroblasts (Fig. 2). We spiked-in *Drosophila melanogaster* nuclei to serve as an internal experimental control to better normalize the sequencing coverage across the experimental conditions. We also manually curated and annotated a set of genome segments from the current pig genome assembly (susScr11) to flag them as ‘blacklisted’ and filter those regions from our analysis. Blacklisted regions are known to produce a substantial degree of mapping artifacts, which in turn can alter subsequent total mapped reads-dependent normalization approaches. The revised Fig. 2 and analysis is in agreement with our CCP data: a genome-wide decrease in chromatin accessibility in the persistent versus the transient myofibroblasts.

b) We agree that changes in chromatin accessibility involved in gene transcriptional regulation should have an accompanying effect on the transcription of a target gene. However, after with additional experiments and improved analysis of our ATAC-seq datasets, we did not find obvious ATAC-seq peak changes of interest between the transient and persistent myofibroblasts. This is due, in part, to the many peaks that are changing slightly in their signal with chromatin accessibility, but also because the few peaks that changed in their ATAC-seq signal magnitude were not found at TSS, but rather in intergenic regions. To accurately predict the target of those putative regulatory regions would require chromatin conformation capture assays, which was beyond the scope of our current study.

c) The reviewer makes a sound suggestion. Had we found any putative enriched motif (and therefore a transcription factor or chromatin remodeler known to use such motif) in unique peak regions between our two experimental conditions, we would certainly test the putative role of those proteins in persistent myofibroblasts. We did rely on standard bioinformatic pipelines (i.e. find statistically different peaks between our two conditions, scan for enriched motifs in these peaks, match motif in curated motif databases, etc.) and in-house specialized bioinformatic software to detect differential transcription factor activity across experimental conditions, such as TFEA ([doi.org/10.1101/2020.01.25.919738](https://doi.org/10.1101/2020.01.25.919738)), and DASTk (PMID: 29748466). Unfortunately, we did not find enrichment of a putative motif that allowed us to further pursue this candidate. We recognize that absence of evidence is not the same of evidence of absence. We think that the fact that so many peaks change slightly between both conditions, compounded by the fact that our three biological replicates differ significantly in their background noise, made it hard to find such a putative signal, if it exists, and lead us to identify a culprit transcription factor driving the phenotype.

d) While additional ATAC-seq experiments would be insightful, we feel that the genome-wide chromatin condensation is well-captured by our CCP measurements. We also found that nuclear roundness and cell area correlates with myofibroblast persistence, so we included these measurements to bolster our findings.

*4. This brings up a major question to me – is myofibroblast persistence mediated through a more closed chromatin architecture? While there is a lot of correlative evidence, the only causative evidence supporting this connection seem to be the TSA inhibition experiments, but these are not very convincing as TSA treatment is such a blunt perturbation. The authors should complement this with treatment with other inhibitors, and genetic knockdowns that more precisely perturb the chromatin state (i.e. histone modifying enzymes and/or TFs identified in 3c).*

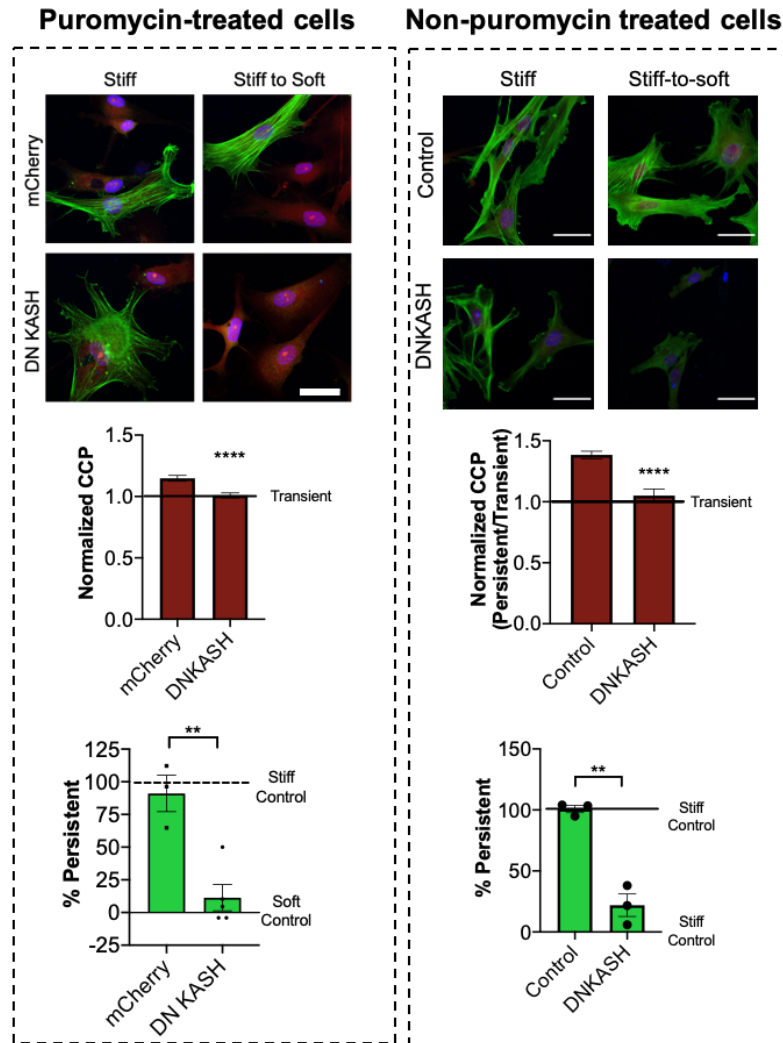
We thank the reviewer for this suggestion, and we further investigated the mechanistic link between mechanical matrix cues and chromatin condensation by studying HDAC activity. We found that HDAC activity increases in persistent myofibroblasts. As TSA is a HDAC inhibitor, we believe that it reverses persistence (Fig. 3) by overriding cytoskeleton-induced HDAC activation. Similarly, Garcinol (HAT inhibitor) encodes persistence by increasing HDAC activity (SI Fig. 8). Finally, our ATAC-seq experiments confirm that the chromatin condensation occurs genome-wide. Together, these results strongly support the hypothesis that myofibroblast persistence is mediated by overall changes of chromatin architecture.

*5. The potential mechanism of epigenetic remodeling through tension on the nucleus would be a very nice result, however, this needs more clarification and support. The nuclear tension values are very different between Fig. 4F and 4I, and the cytoD and rho inhibition tension values seem to be higher than the persistent case of 4F, despite the major impact on persistence of the inhibitor. This suggest to me that the tension sensor might be too noisy to make definitive conclusions. Further, the DNKASH mutant leads to a very small impact on normalized CCP (4K; 10% reduction), but a major impact on the % persistent cells (Fig. 4N). This seems to point towards the nuclear tension as playing only a minor role with regards to epigenetic remodeling. Stronger support is needed to make this connection.*

To clarify, the difference the nuclear tension values between the figures is because the method for measuring nuclear tension (PMID: 30141038) cannot be compared across experiments due to variability in microscope setting (i.e., laser intensity fluctuations, etc). To avoid confusion, we normalized the nuclear tension values, as they are relative rather than absolute (Fig. 5F&I). While the data is noisy, this is expected from the literature (PMID: 30141038); which states that “Many times FRET differences are minimal between conditions.” We have also observed some large cell-to-cell variations in FRET within the same condition. However, we performed careful control experiments to ensure that we were measuring changes in nuclear tension (SI Fig. 13). This nuclear tension data is not our only measure of nuclear forces - the lamin AC data supports the conclusion that nuclear tension is altered in persistent myofibroblasts compared to transient myofibroblasts (Fig. 5A, SI Fig. 13).

Finally, we acknowledge that the differences between the control (mCherry) and DNKASH cells were not very large. This was due in part to the lentiviral transduction protocol on the control cells (mCherry), since we did not observe the typical >1.2 fold increase in CCP. The transduction protocol leads to transient damage to the cells and they typically need to recover from the isolation procedure for a few days before handling. Because of this, we prolonged the fibroblast culture on TCPS to adjust for the transduction protocol. We also used puromycin to rid the cell population of non-infected cells, which can also influence cell phenotype (see below). To test for this, we transduced cells with mCherry (Control) or DNKASH, but did not treat with puromycin. We verified expression of control and DNKASH constructs using positive fluorescence with the population showing ~90% expression (SI Fig. 14). We saw that this greatly improved the cell phenotype (see below), and the CCP difference between mCherry and DNKASH cells was much larger (control = ~1.4). The influence on persistence was maintained, and a higher percentage of cells not treated with puromycin were activated on stiff hydrogels compared to the puromycin treated cells (see below). Together, this new data bolsters our conclusion that nuclear tension is playing a role in epigenetic remodeling in persistent myofibroblasts.





**Figure iii:** Comparison of control (mCherry) and DNKASH cells that were treated with puromycin or not. Puromycin affected cellular phenotype (see in representative images), which likely affected the CCP values.

*Minor comments.*

1. Why are the moduli of 4.5 kPa and ~1.5 kPa chosen? Are these physiologically relevant? Literature reported values for healthy and disease fibrosis conditions should be included with references.

The soft hydrogel modulus was chosen based on similarity to healthy, porcine aortic valve tissue (~1.1 kPa, SI. Fig. 1) and on previous studies from our group (PMID: 28390245). Since fibrotic aortic valves are about 2-3 fold stiffer than healthy valves (PMID: 22222074), we chose the high end of this difference at 4.5 kPa. As shown in Figure 1, this leads to consistent and reliable myofibroblast activation on stiff hydrogels as compared to soft hydrogels. We have clarified this in the revised manuscript (line 107).

2. Fig. 4O needs to be modified substantially: the ATAC-seq data would seem to suggest there

*isn't abroad increase in chromatin accessibility in transient myofibroblasts. Also, line 400 of the text 3 indicates that global chromatin structure of healthy myofibroblasts is less accessible than that of diseased myofibroblasts, which contradicts Figures 1J and 4O. Finally Figure 4O implies that nuclear tension first increases in the transient myofibroblast state, but then decreases for persistent myofibroblasts, but based on the data in Figure 4E, tension across Nesprin is higher for persistent myofibroblasts*

We apologize for our original typographical errors. We have read through the text thoroughly to address this contradiction and correct other small errors. Text in line 400 (now Line 512) agrees with the presented data.

Second, we agree that Figure 4O may not have been clear, likely because soft hydrogel controls were not included in the main figures (Figure 1). The schematic has been modified to illustrate only chromatin condensation, rather than both chromatin condensation and nuclear tension (now Fig. 1L). We also added a new graphical abstract (Figure 6) which hopefully presents our main conclusions in a clearer, more detailed manner.

*3. The authors claim that chromatin condensation is associated with stabilization of the actin cytoskeleton. In lines 376-380, the authors mention two potentially conflicting prior findings: one in which actin assembly tends to decondense chromatin vs another in which actin assembly reduces pluripotency in iPSCs by reducing chromatin accessibility. What explanation may there be for this discrepancy, and where would the results presented in this manuscript fit in?*

As seen in Figure 1H, we observe that with myofibroblast activation (1-3 days on stiff hydrogels), chromatin is highly accessible compared to soft hydrogels. During myofibroblast activation high levels of actin assembly (but not necessarily stabilization) are associated with increased chromatin accessibility (PMC6816600). When the actin network stabilizes, it instead promotes chromatin condensation (PMID: 30979898). The results presented in this manuscript highlight the dual role of actin on chromatin, where actin assembly first increases accessibility and then decreases accessibility upon its stabilization. We hope the revised text clarifies this distinction (Lines 471-477). It is worthwhile to note that actin's role on chromatin is likely cell-type dependent. MSCs, which also show a persistent-like phenotype with extended exposure to stiffness, maintain high levels of chromatin accessibility compared to soft hydrogel controls (PMID: 30775233). We include this discussion on lines 479-482.

*4. Western blots and quantification of lamin A and lamin C expression in Figure 4C seem to show that lamin C levels decrease in the persistent population as compared to the transient population, which contradicts the text in line 277.*

We thank the reviewer for catching the typo. Line 342 should now reflect the data.

*5. In Figure 4M, the sample image of a DN KASH cell seems to have a very different morphology from typical myofibroblasts shown throughout the manuscript. Was this morphology common for this population of mutant cells? Is it possible that cytoskeletal organization is significantly altered or destabilized by nuclear uncoupling, potentially making it easier for cells to de-activate upon softening? The authors should typical morphologies of DN KASH vs control cells.*

The DNKASH cells have a different morphology from other control-infected myofibroblasts. Upon close inspection of the images, most  $\alpha$ SMA fibers do not cross or attach to the nucleus, while it is common for the control-infected. This observation was somewhat expected, since the DNKASH prevents actin attachment to the nucleus. While DNKASH affects cytoskeletal to nuclear attachment, it does not affect the entirety of the cytoskeleton. As the reviewer suggests, we analyzed other phenotypic markers of persistent myofibroblasts, nuclear roundness and cell area. While DNKASH did prevent changes in nuclear roundness, it did not affect cell area (SI Fig. 14). This implied to us that DNKASH cells are still capable of producing a stabilized cytoskeleton network; however, the stabilized network does not affect the nucleus.

*6. Figure 1F: Is there any explanation for why 1d-2d stiff-to-soft samples seem to have even fewer persistent myofibroblasts than soft control gels alone?*

This is a good point. We hypothesize that mechanisms governing reversal of myofibroblast persistence are “over-compensating” in their reversal of myofibroblast programming. While not shown here, we have gene expression data that shows that common markers of myofibroblasts ( $\alpha$ SMA, CTCF, integrins, etc) are even lower with softening (1d2d) than soft hydrogel controls. We believe this is an interesting observation about mechanisms and time scales of de-activation, and we hope to follow up on in future studies. Here, we were focused on the myofibroblast persistence questions.

*7. Methods section for the Omni-ATAC procedure mentions adding Drosophila nuclei to the reactions. Is this part of the protocol, or just a typo?*

Spiking in *Drosophila* nuclei is not part of a standard omni-ATAC procedure nor was it a typo on our part. We included *Drosophila* nuclei as an experimental control to test our hypothesis on the degree of compaction of chromatin between our two experimental conditions. We elaborate more on this method in the second to last paragraph of the Figure 2 (results section). In brief, if the accessibility of the chromatin from the pig genome becomes significantly different between our two conditions, by adding another distinct genome to the ATAC-seq transposition reaction, we can infer (and successfully test) if the fraction of *Drosophila* mapped reads would change between these two datasets. If one pig genome is less accessible, then the transposase enzyme will insert less in the pig genome and more in the fly genome; and vice-versa for a more accessible pig genome.

**Rebuttal 2**

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The reviewers' comments have been italicized and listed below in a blue font, and point-by-point responses and revisions are in black text. Any references to line numbers are based on the revised submission, and edits to the manuscript are highlighted in yellow.

*Reviewer #1 (Report for the authors (Required)):*

*Walker and coauthors have submitted a carefully and much improved revision of their study on epigenetic regulation of myofibroblast persistence. Most of my initial comments have been addressed and the authors have added more data now showing critical controls and amended unclear language. There is still one major remaining problem with the study. The authors set out to “investigate mechanisms that lead to persistent myofibroblast activation upon extended exposure to stiff microenvironments (i.e., mechanical dosing)” but they do not answer this exciting and novel question. Instead they show how matrix and cell mechanics affect nuclear components, chromatin condensation and accessibility. But what is the relevance of chromatin accessibility for maintenance of myofibroblast phenotype (SMA stress fibers) on soft substrates that the authors use as indication of ‘persistence’? The point is reiterated below.*

We thank the reviewer for his/her positive comments, and we believe that we have addressed the author's remaining questions in our revised manuscript, as detailed below.

*1. To emphasize the novelty of their study in the rebuttal letter the authors argue that valvular interstitial fibroblasts regulate myofibroblast persistence differently than MSC for which many of the shown data (Figure 4 onwards) have been produced before. This is a somewhat blunt argument since (a) the authors do not systematically compare MSCs with VICs to substantiate the claim and (b) VICs have been shown to exhibit progenitor ‘MSC’ properties which, at least in part, seem to support valve calcification and VIC ‘osteogenesis’ in disease (e.g., PMID: 31506459; PMID: 19218344). The Simmons group has produced a series of studies on (even porcine) VIC mechanobiology which are all not cited but should be considered here. For instance, the group has measured local stiffness variations in heart valves and VIC stiffness on hydrogels (PMID: 22189247; PMID: 23746597). These values would respond to one of the other reviewer's questions on physiological relevance of 2 kPa and 4 kPa PEG gels.*

We appreciate the reviewer's comments, and for the opportunity to clarify our argument. Our experiments were designed to study the differences in chromatin-related mechanical memory in VICs, but we were struck by the major differences between mechanical memory mechanisms in MSCs (publications from many groups, including our own). While we did not repeat the MSC experiments to provide our own direct comparison between VICs and MSCs, we did compare trends between the two based on published results, including our own prior work. For example, our group showed irreversible MSCs have *decreased* HDAC activity compared to reversible MSCs. In VICs, we show persistent (irreversible) myofibroblasts have *increased* HDAC activity compared to transient (reversible) cells. We agree that VICs, like other fibroblasts, share mesenchymal cell properties with MSCs, including expression of vimentin and a spindle-like morphology. We also acknowledge VICs can trans-differentiate into myofibroblasts or osteoblast-like cells, which is similar to the multi-potency of MSCs. To acknowledge the reviewer's point, we added text noting the similarities between VICs and MSCs to our Discussion (lines 458-460). However, despite phenotypic similarities between VICs and MSCs,

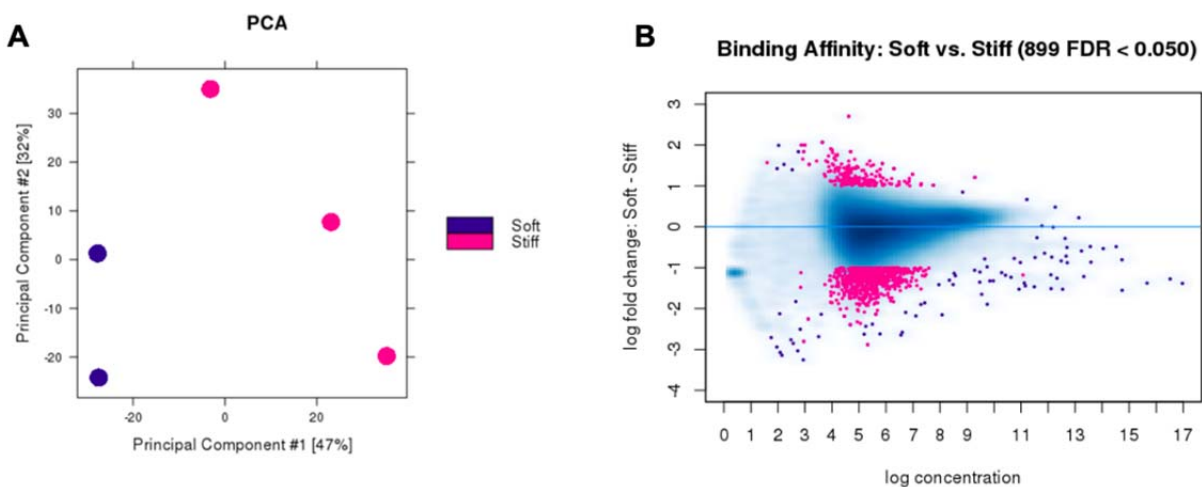
our findings show that VICs and MSCs have different HDAC activity profiles and that the evolution of the irreversible, persistent phenotype occurs through different mechanisms. We believe these results are compelling and will add to the growing body of literature demonstrating links between epigenetics and persistent cellular phenotypes.

Finally, we apologize for the oversight in not recognizing the key and relevant work from the Simmons group. We added appropriation citations, as well as a discussion of their VIC mechanobiology findings (lines 556-557).

*2. Figure 1H: With the soft control now added it is curious and not discussed why persistent (9d) myofibroblasts all have very similar CPP values, regardless of prior experienced substrate stiffness. At least at the global chromatin condensation level, always soft and always stiff grown populations seem indistinguishable.*

This is a good point. We posit that persistent myofibroblasts (9d-stiff) and fibroblast controls (1d-9d soft) have similar CCPs because both cell types reach a “steady-state” once the cellular phenotype is stabilized. We speculate that only chromatin regions required for that particular “steady-state” are accessible, so both persistent myofibroblasts and fibroblasts display a more closed chromatin structure, albeit with different regions accessible for transcription. In contrast, a transient myofibroblast is in a dynamic state and can either transition to a persistent myofibroblast or a quiescent fibroblast; it is this flexibility that necessitates a more open chromatin structure. We contextualized our results in our discussion (lines 481-488).

In a parallel study that supports some of the inferences above, we performed an ATAC-seq analysis that compared the open chromatin sites between persistent myofibroblasts (9 days *stiff*) and fibroblasts (9 days *soft*). There are significant differences in chromatin accessibility (Figure R1), which suggests that persistent myofibroblasts and fibroblasts have differential chromatin landscapes governing their respective phenotypes, which is not readily observed in a CCP analysis of global chromatin accessibility. We did not include this result in the manuscript, since we aimed to focus on the differences between transient and persistent myofibroblasts, but this could be added at the recommendation of the editor.



**Figure R1:** Preliminary ATAC-sequencing analysis of persistent myofibroblasts (9 days stiff) and fibroblasts (9 days soft). **A)** Principal component analysis (PCA) of persistent myofibroblasts and fibroblasts sequencing samples using DiffBind. **B)** MA plot of significantly differentially accessible regions (pink) identified using DiffBind between persistent myofibroblasts and fibroblasts (FDR < 0.05, log fold change > abs(1)).

*3. The authors have now performed ATAC-seq analysis of SMA as the myofibroblast hallmark gene in addition to improved analysis of global chromatin opening. The outcome of these new experiments is surprising in that myofibroblast genes would not be particularly affected by chromatin condensation. This relates to my earlier concern that the authors cannot provide the missing link between the phenotype stabilization (i.e., SMA stress fiber persistence in persistent myofibroblasts after soft substrate switch) and epigenetic changes. In other words, they use SMA stress fiber stability as an indication for myofibroblast persistence but cannot show how chromatin condensation is responsible for myofibroblast persistence. Both phenomena, SMA stress fiber stability and chromatin condensation, seem to be very different phenomena that are linked at some level, but it is still unclear how.*

We apologize if our original manuscript was unclear about this critical point. Indeed, we believe our data show a mechanism between  $\alpha$ SMA stress fiber stability (in persistently activated myofibroblasts) and chromatin condensation. Specifically, we show that: i) chromatin accessibility decreases in *persistent* compared to *transient* myofibroblasts, demonstrated with both CCP and ATAC-seq analyses (Fig 1, 2); ii) myofibroblast persistence can be reversed by *decreasing chromatin condensation* via HDAC inhibition, while persistence can be programmed by *increasing chromatin condensation* via HAT inhibition (Fig 3); and iii) myofibroblast persistence is completely avoided by uncoupling the cytoskeleton-to-nuclear attachment that controls chromatin condensation (Fig 4, 5). Collectively, these interventions demonstrate that the mechanisms of myofibroblast persistence are chromatin condensation and accessibility.

We speculate that chromatin condensation in *persistent* myofibroblasts does not necessarily feed the stabilized myofibroblast phenotype by encoding increased expression of  $\alpha$ SMA, but instead prevents the *persistent* myofibroblasts from sensing and responding to the substrate mechanics, especially softening that is sensed by *transient* myofibroblasts. In fact, when we open the chromatin of *persistent* myofibroblasts by TSA treatment, they remain  $\alpha$ SMA positive, which makes them very similar to *transient* myofibroblasts ( $\alpha$ SMA-positive and open chromatin). However, *transient* myofibroblasts sense the substrate softening and respond by reverting to fibroblasts. Thus, to clearly demonstrate the link between chromatin and myofibroblast *persistence*, we used garcinol (HAT inhibitor) to accelerate chromatin condensation in *transient* myofibroblasts. With garcinol treatment, the myofibroblasts no longer respond to matrix softening, and are no longer *transient*, but *persistent*. To emphasize these findings, we moved the garcinol data from supplemental information to Figure 3 (H-N).

We acknowledge that our study does not elucidate all of the mechanistic steps of the outside-in signaling that lead to changes in chromatin and ultimately controls myofibroblast persistence, although our results reveal what is necessary. Our ATAC-sequencing dataset indicates that myofibroblast persistence is a complex phenotype, but this rich dataset should prove useful for future work. In our Discussion, we elucidate specific areas worthy of future investigations, including which proteins/signaling pathways link chromatin accessibility to irreversibility (lines 559 – 561).

*Reviewer #2 (Report for the authors (Required)):*

*The authors have done a good job responding to my concerns raised in the previous review. In my opinion the manuscript should be accepted for publication.*

We thank the reviewer for his/her time, positive comment, and support for publication of our work.

*Reviewer #3 (Report for the authors (Required)):*

*The authors have been mostly responsive to my initial critique in their revised manuscript. However, there are several issues that still need to be addressed prior to publication, particularly regarding the ATAC-seq data.*

We thank the reviewer for the opportunity to further improve and clarify our study, particularly regarding the ATAC-seq data analysis and presentation. Additionally, we took steps to clearly articulate the main conclusions without overstating them, and similarly revised the results sections, as suggested.

*Major comments:*

*1. The claim that persistent myofibroblasts have a more condensed chromatin structure with genome wide alterations (w.r.t transient fibroblasts) is not supported by the ATAC-seq data. In Figs. 2A-C, reads for persistent are consistently higher than reads for transient when viewed in different ways, corresponding to an increase in genome accessibility for the persistent case. The authors acknowledge this, but then include Fig. 2D – an analysis of fraction of reads inside peaks (FRIP) and drosophila spike-in reads– to argue the opposite. They find that FRIP and Drosophila reads are greater for persistent case as somehow indicative of the persistent case having fewer chromatin loci accessible to the transposase. First, the argument is very difficult to follow. Second, Figure 2D is not very high-quality data – 3 points of data for each condition that are very heterogeneous – and I wouldn't be confident concluding anything from these data. Third, the data in Figs. 2A-C seem pretty clear that the persistent case has more accessible chromatin (including at specific genomic loci for GAPDH and ACTA2), so at best, the conclusions from this figure are inconclusive. Genome-wide data can be very powerful, but are often quite complex and can defy simple interpretations, so that they need to be complemented by more specific analyses. I had suggested that the authors pursue a more specific analysis based on these data (PCR of genes/TF analysis) precisely because of this complexity. If the authors cannot do such analyses, I would suggest that authors need to be clear in describing what each of their analyses of the ATAC-seq data show (In abstract, subtitle for the section, fig. 2 title, etc.), instead of deciding to pick conclusions from 1 specific analysis (Fig. 2D) that supports their conclusions, and ignoring other (more convincing) analyses that do not.*

We thank the reviewer for bringing this issue to our attention. We agree that analysis of ATAC-seq data can be complex and attention must be paid to every detail for proper interpretation.



In light of the reviewer's comments, we added additional data analysis and figures to our original ATAC-seq analysis.

The major assumption of all next generation sequencing analysis pipelines is that under different experimental conditions the overall yields of the samples (DNA or RNA) are identical per cell. When this assumption is not true (as in our case), spike-in controls are needed to properly analyze the data (1). In the last version of this paper, we included the fraction of mapped *Drosophila* reads per sample, but we did not normalize the pig data to these spike-in controls. This made the ATAC results harder to interpret.

We now present the data normalized to the spike-in controls (as recommended in Reference 1). Replicate 1 was removed as it was not possible to apply normalization to the dataset since spike-ins were not initially included. The normalized data are in agreement with our initial hypothesis that persistent myofibroblasts have a more closed chromatin state. Additionally, we have added a schematic and explain why we believe there is an increase in the fraction in reads in peaks (FRIP). Finally, we reorganized the figures and text in Figure 2 to reflect this additional analysis.

To provide more specific details to the reviewer, our ATAC-seq reactions were done in the presence of a fixed amount of *Drosophila* nuclei spike-in. Because we sequenced to a fixed depth, the reads will be distributed across all available chromatin. If the pig cells' chromatin accessibility were to be globally reduced (as in the case of the persistent myofibroblasts), the transposase would have less access to the pig genome, and this would result in a greater bias towards the unaffected fly chromatin. We initiated the analysis with an equal amount of identical *Drosophila* nuclei input in each condition, but upon sequencing, we observed large differences in the fraction of *Drosophila* reads recovered. We subsequently normalized all samples based on the premise that the *Drosophila* reads should be equivalent between samples. In other words, we rescaled the persistent and transient ATAC-seq datasets to equalize the *Drosophila* spike-in (1).

The resulting spike-in normalized datasets are now displayed in Fig. 2 and SI Fig. 5. The results in these figures are consistent with our other observations, namely that the persistent myofibroblasts display less accessible chromatin globally. As recommended by the reviewer, we have included a diagram in Fig. 2D that helps explain how the loss of spurious chromatin accessibility provokes an increase of the FRIP score. We have also normalized the FRIP scores of transient myofibroblasts relative to the persistent myofibroblasts, so the trend of a lower FRIP score in the transient condition is apparent.

1. Chen K, Hu Z, Xia Z, Zhao D, Li W, Tyler JK. The Overlooked Fact: Fundamental Need for Spike-In Control for Virtually All Genome-Wide Analyses. *Mol Cell Biol.* 2016;36:662–667.

*2. I had asked the authors to complement the TSA inhibition experiment with more specific inhibitors (e.g. of HDACs) or genetic knockdowns (e.g. of epigenetic modifying enzymes or genes/TFs implicated by ATAC-seq) previously. This was because the TSA inhibition experiments are the only data supporting a mechanistic link between chromatin remodeling and myofibroblast persistence and because TSA is such a blunt perturbation (i.e. its not clear that the*

*chromatin changes impacted by the inhibitor are the same as those that occur during the transition from transient to persistent, particularly given the complexity of the ATAC-seq data). While the authors have added a HDAC activity reporter (which as expected is impacted by TSA inhibition), they haven't actually done the suggested experiment. The authors have not suggested a mechanistic link in the abstract/title, so I don't think the authors have to do any more experiments. However, I do think the title of Figure 3 and the section sub-title ("myofibroblast persistence is dependent on changes in chromatin accessibility") extend beyond what the authors show, and the authors should reword these titles to focus on their specific results rather than making sweeping conclusions.*

We thank the reviewer for these suggestions. We did not perform the above-mentioned experiments with specific HDAC inhibitors or genetic knockdowns. Our rationale was that as presented in SI Fig. 7 of mRNA analysis of HDACs, there was no clear candidate to target, and we could not exclude multiple modifiers working in concert to decrease chromatin accessibility. Identifying a specific HDAC or other modifier would require an in-depth investigation, which we felt was beyond the scope of this work.

We agree that other inhibitor studies would further support the mechanistic link between chromatin remodeling and myofibroblast persistence. We had performed a garcinol (HAT inhibitor) experiment to complement the TSA (HDAC inhibitor) experiments, and when taken together, the results demonstrate that myofibroblast persistence is dependent on changes in chromatin accessibility. To highlight this additional study, the garcinol data are now part of Fig. 3. We would like to emphasize that we carried out these experiments following a well-accepted mechanistic paradigm of both inhibiting and activating chromatin accessibility. We hope this new presentation of our data clarifies the conclusion that myofibroblast persistence depends on chromatin accessibility. We also changed the sub-title as suggested, which now reads "Myofibroblast persistence is linked to chromatin accessibility".

*Minor comments:*

*1. Statistics are needed for 1E, 1F, 1H, 1I.*

Thank you for the suggestion, and we included statistics for these results in Figure 1 and SI Figure 3.

*2. The additional in vivo data is helpful and I agree that the in vivo data on CCP between healthy and diseased matches what is seen in vitro. However, I think the authors have to be careful about overstating their conclusions. In particular, the nuclei in the in vivo case morphologically appear to be different than the in vitro studies (roundness is different, cross sectional area looks smaller, structure of chromatin looks clearly different). I would ask the authors to reword "Notably, myofibroblasts in patients with aortic valve stenosis display a condensed chromatin structure similar to cultured persistent myofibroblasts." to be more precise and focus on the in vivo case showing a similar trend with the CCP metric.*

We agree and have reworded our conclusion with regard to in vitro and in vivo results to be more specific to the measured outputs (lines: 37-38, 95, 151, 358).

*3. The data in 4D looks like it is simply underpowered ( $n = 2$  or  $3??$ ), and the effect of inhibitors on average percentage of  $\alpha$ SMA+ cells looks to be similar to the effect on normalized CCP (4E). If they had a higher number of experiments, 4D might show statistical significant differences. Thus, I don't think the authors can conclude that treatment of inhibitors does not affect %fibroblasts but does effect CCP organization.*

The  $\alpha$ SMA analysis relies on hydrogels as an 'n', while CCP analysis uses cells as an 'n'. We performed two additional replicates of 4D, so now the  $n \geq 4$  hydrogels. The results remain non-significant; however, we revised our conclusion to be more specific to our conditions tested (line 318-319, 330-331).