nature biomedical engineering

PEER-REVIEW INFORMATION

Shear-stress sensing by PIEZO1 regulates tendon stiffness in rodents and influences jumping performance in humans

Corresponding author: Jess Snedeker

Editorial note

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

Thu 13/08/2020
Decision on Article nBME-20-1797

Dear Prof Snedeker,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Shear sensor mechano-signaling determines tendon stiffness and human jumping performance". The manuscript has been seen by three experts, whose reports you will find at the end of this message. You will see that the reviewers have good words for the work and that they articulate concerns about the degree of support for the claims; in this regard, they provide useful suggestions for improvement. We hope that with significant further work you can address the criticisms. In particular, we would expect that a revised version of the manuscript provides:

* Causative evidence that Piezo1 is a a mechanical shear sensor in tendon cells.

* Thorough characterization of the key phenotypes, and of the effects of collagen crosslinking.

* Evidence of any major effects of heterogeneity in the cellular microenvironment, in tendon types, and in genotype.

* Thorough description of the statistics. Please provide the actual p-values (rather than ranges), and make sure that the overlaid measurements in bar plots are visually clear.

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 25 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

__
Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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

This manuscript evaluates the role of PIEZO1 in the mechanobiologic response of tendons. This study was beautifully done and the data are impressive. The comprehensive dataset includes in vitro experiments with tenocytes, ex vivo fascicle level testing, evaluation of whole tendons from a Piezo1gof mouse, and clinical data from carriers of a human gof Piezo1 mutation. The results suggest that PIEZO1-mediated mechanosensitive signaling plays a role in tendon stiffness. The results are very interesting and have potential for high impact. The link from cell level responses to potential clinical implications are intriguing. A few questions are outlined below that should be addressed.

• Fig 1g: if spontaneous calcium signals are the same magnitude as strain-induced changes, how does a cell respond to mechanical signals? In other words, if a mechanobiologic response to load is in the same range as normal signal flucutations, then how does a cell sense an actual mechanical input?

• Fig 2a: what is the meaning of the mechanical thresholds? How are these values computed and what is their significance? Are all seven signals plotted here from the same fascicle, within the same row? Or are these from different lines of cell/fascicles?

• Fig 2e: what is the meaning of the shear stress threshold? How was this value computed and what is its relevance?

• Fig 2f: this is a nice set of data, but it brings up a related question. The tenocytes for the data in panels d and e come from which type of tendon? One of the same tendons that are shown in panel f, rat tail tendon, or a different tendon altogether? Similarly, what tendon provided the cells shown in panel g?

• Fig 4b: the stiffness values for Yoda1 don't appear to be much higher than control, even though the values are reported to be significantly larger. What are the average values for these two groups and what was the p-value? Is this a meaningful increase for the Yoda1 fascicles compared to control?

• Page 13; lines 1-2: the investigators suggested that the mechanical differences could be due to denser

collagen crosslinking. Why not measure this to check this hypothesis? Various previous studies have quantified mature and immature crosslinks in tendons and other tissues; this type of evaluation could provide significant insight here.

• Fig 5f: it appears that the max jump height values might be larger for the non-carriers compared to the carriers. Is this a significant difference? If so, what does this mean? Why is the CMJ-DCMJ internal comparison within groups more important than the comparison of raw values across groups?

• The heterozygotes and homozygotes were grouped together for the human E765del carriers. Was there any difference in measured properties between these two genotypes?

• The authors mention that the E756del mutation protects against malarial infection; is this mechanism related at all to the mechanism at play in tendon mechanobiology? Or is this believed to be due to a separate/distinct action of this mutation?

• On page 20, the authors suggest that pharmacological activation of PIEZO1 could potentially help in tissue reinforcement and tendon healing. However, it's not clear how this might happen. Perhaps the authors could elaborate on this potential mechanism and expand their thoughts on this topic.

• What is OGB-1? Should be defined.

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

This study by Passini et al., is focused on understanding how tendons sense and adapt to mechanical loads, thereby facilitating tendon function and skeletal movement. This work identifies shear stress, via collagen fascicle sliding, as a critical component of mechanosensing, with Piezo1 identified as a molecule regulator of this process. A combination of in vitro and in vivo studies using Piezo1 gain of function approaches suggests Piezo1 regulates tendon strength and stiffness, while natural Piezo1 gain-of-function mutations in humans are associated with increased jumping height. The data demonstrating strain-regimen calcium responsiveness are very interesting, and the human components adds a novel application of these data.

The primary weaknesses of this manuscript are that the data provided only partially support the authors conclusion that Piezo is the tendon mechanosensory, there is insufficient mechanistic characterization of key phenotypes, and there is some concern as to the rigor/ consistency of characterization of key experimental models. As such, the advance to the field is seen as moderate.

Major issues:
1. While there is a clear mechanical phenotype in the Piezo1 GOF mice, the characterization of the Piezo1 KO is lacking. More specifically, the Piezo1 KO data in Figure 3 only demonstrates changes in Ca2+ signaling- it is unclear if Piezo1 KO/ LOF also alters tendon mechanics and does not support Piezo1 as the shear stress sensor.

2. As all LOF experiments were conducted in vitro rather than in vivo, direct comparisons to the GOF are not possible. In addition, the Yoda1 data only indicate that pharmacological activation of Piezo1 increases tendon stiffness, but there is no mechanical component to these assays (e.g. comparisons between Yoda1 treatment +/- mechanical stimulation). As such, these data simply demonstrate that activation of Piezo1 contributes to enhanced mechanical properties, but do not define Piezo1 as the mechanosensor.

3. Given that this is a global gain of function, the phenotype cannot necessarily be attributed to a tendon-specific effect of Piezo1GOF. For example, Piezo1 activation via short-term Yoda1 treatment increases bone mass, which would likely increase tendon mechanical properties simply by virtue of having to transmit heavier forces- this should be discussed in greater detail.

4. Related to mechanism, on pg 19 lines 1-5 the authors state "Instead, we suspect that a denser collagen cross-linking network causes the phenotypes." Given the importance of this potential contribution to the tendon phenotype, direct assessment of cross-linking (e.g. via LOX etc) should be conducted.

5. Fig 3- The authors are isolating a heterogeneous population of cells- how does their knockdown (shown by mRNA isolated from the total tendon cell population in 3C) relate to the data shown in 3E at the single cell level? Do all tendon cells express all calcium channels? How can they be sure that the differences that they're showing in 3E are not just an efficiency issue (ie. not all cells express all calcium channels, not all cell

types are targeted equally by their CRISPR? Also, what are the kinetics of the various Ca channels- ie. do they all respond to stimulus on the same time scale (they only measured for 5sec)? Rather than total # of calcium events as the outcome, could the % change in intracellular calcium levels at baseline compared to stimulated be shown instead? This would overcome the issue with comparing total population properties to single cell properties.

6. Multiple tendons are used throughout this study. Given the clear heterogeneity between tendons this variable could have potentially important effects. Are there differences in the effects of PIEZO1 GOF between types of tendons (positional vs load bearing, tail (in vitro studies) vs. Achilles/flexor for the in vivo)?

7. Unrelated to the science, this reviewer would urge careful thought as to the description of human subjects on page 20, lines 3-8 and how these statements may be perceived.

Minor
1. In Figure 5F, I don't believe this was directly quantified, but the data suggest that E756del carriers may have reduced jumping height as baseline. How this fits in to the data interpretation should also be clarified.
2. Figure 2, while important from a technical development perspective, does not make a substantive contribution to the authors conclusion, and could be considered as supplemental information.

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

Summary: The following manuscript utilizes in vitro, in situ, and human studies to pursue a detailed multi-hierarchical level hypothesis on how mechano-signaling determines tendon stiffness and human jumping performance. Utilizing calcium imaging in both tendon cell culture and in situ explant culture the authors demonstrated that tenocytes most likely detect mechanical forces through shear stress induced by collagen fiber-sliding. CRISPR/Cas9 screening in human and rat tenocytes identified PIEZO1 as the crucial shear sensor. In rodents, elevated mechano-signaling resulted in increased tendon stiffness and strength both in vitro by Yoda1 activation and in vivo by a Piezo1 gain-of-function mutation (a 16% average increase in normalized jumping height). The results of these multiple studies lead to the conclusion that PIEZO1-mediated mechano-signaling regulates tendon stiffness and impacts human athletic performance.

Degree of Advance: This study has an amazing depth to it utilizing a hierarchal level of advanced cellular characteristic tools, tissue characterization, and human subject analysis. This article highly advances the state of tendon mechanobiology and health. The conclusions are original.

Major Issues: None
Minor Issues / missing statistics / missing citations

1. Page 5, Lines 9 – 11: Regarding the strain rate dependence and multiple versus single calcium signaling responses with higher strain rates. Do the authors believe that the higher strain rates only stimulated the cells to calcium signaling once or did the higher strain rates induce multiple signals, but it just appears as one long calcium signal as the rate was high enough to not allow the cell to re-establish baseline calcium levels. Did the authors measure the length of calcium signaling?
2. Page 5, Line 14: Do you have a reference or more reasoning behind the statement "potential cell-cell communication processes occurring predominately at low strain rate"?
3. Figure 1 d and g: In Figure 1d there are ** and ***, I assume these relate to significant differences, but it is not clear what they mean. In Figure 1g the figure legend states significant differences in calcium although it is not apparent from the figure either through annotation or through the mean and standard deviation levels.
4. Figure 2: Has the value for p-values associated with ** and ***, but not Figure 1 which has the * symbols, whereas Figure 2 has none.
5. Page 15, Line 15-17: "These biomechanical differences (higher stiffness and strength w/o differences in geometry with PIEZO1 compared to wild type) are particularly prominent at high tendon loading" It appears that all of these tests in this section are at the same loading rate (1%/second). How is this statement backed by the current study?
6. Page 15, Line 2: Is it known that being a E756del heterozygous carrier will lead to the PIEZO1GOF mutation or does it require a homozygous carrier?
7. Page 18, Lines 8-10: There is a previous study that found that loaded rat tail tendon under a microscopic device to determine that in-situ tenocytes respond to increased strain with increased calcium signaling. It is quite old, so it may be difficult to locate.
It was originally published as an abstract:
Shirakura, K., Ciarelli, M.J., Arnoczky, S.P., and Whallon, J.H.: Deformation

Induced Calcium Signaling, Trans. Combined Ortho. Res. Soc. 1995, p 94.

This was then re-presented in a book chapter:
Arnoczky S.P., Lavagnino M., Egerbacher M. (2007) The response of tendon cells to changing loads: Implications in the etiopathogenesis of tendinopathy. In: Tendinopathy in athletes, Encyclopedia of Sports Medicine, (eds S.L.Y. Woo, P. Renstrom, S.P. Arnoczky), Oxford, UK: Blackwell Publishing. pp. 46–59. https://doi.org/10.1002/9780470757987.ch4
This article being 25 years old does not have the technology, breadth, or depth of the current article, but would still be worth a citation. The abstract is cited in your reference number 48.
8. Page 25, Line 8: How was the fiber length lfiber= 900 μm determined?

Tue 23/02/2021
Decision on Article NBME-20-1797A

Dear Prof Snedeker,

Thank you for your revised manuscript, "Shear sensor mechano-signaling determines tendon stiffness and human jumping performance". Having consulted with the original reviewers (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 '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

__
Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

P.S. Nature Research journals encourage authors to share their step-by-step experimental protocols on a protocol-sharing platform of their choice. Nature Research's Protocol Exchange is a free-to-use and open resource for protocols; protocols deposited in Protocol Exchange are citable and can be linked from the published article. More details can be found at www.nature.com/protocolexchange/about.

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

The authors have addressed all my concerns. The authors are to be commended for a thorough and elegant study. Well done!

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

The authors have addressed all prior critiques. I commend them on the tremendous amount of additional work that has gone into the revision. Of particular note is the new data using the inducible Scx-Cre to delete Piezo1 in tendon. These experiments much more clearly demonstrate the requirement for Piezo1 in tenocyte mechanosensation, and make an important contribution to the literature.

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

The authors have more than adequately addressed all my minor technical criticisms or questions.

Rebuttal 1

Point-by-Point Response: Passini et al., nBME-20-1797

*The reviewers' comments are cited directly from the decision letter. Below are the individual comments (black) and our responses (**blue**). Of note, the figure referencing in this reply refers to the updated figure numbering in the revised version of the manuscript.*

Points raised by more than one reviewer:

Reviewer #1

• Page 13; lines 1-2: the investigators suggested that the mechanical differences could be due to denser collagen crosslinking. Why not measure this to check this hypothesis? Various previous studies have quantified mature and immature crosslinks in tendons and other tissues; this type of evaluation could provide significant insight here.

Reviewer #2

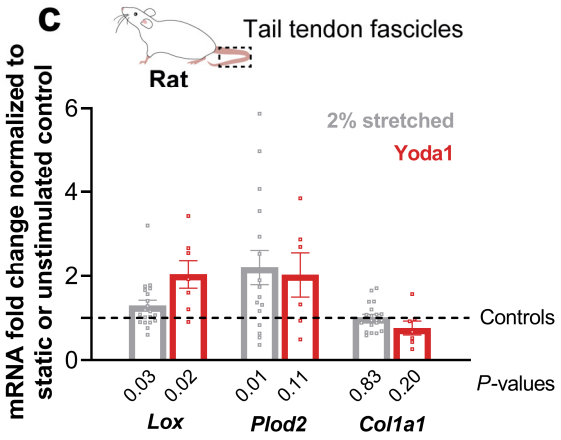
4. Related to mechanism, on pg 19 lines 1-5 the authors state "Instead, we suspect that a denser collagen cross-linking network causes the phenotypes." Given the importance of this potential contribution to the tendon phenotype, direct assessment of cross-linking (e.g. via LOX etc) should be conducted.

We thank the reviewers for this comment. We have added additional data that now firmly support our previous speculation that PIEZO1 regulates collagen crosslinking and that presence of crosslinks are elevated in tissues with the augmented mechanics we observe.

As suggested by reviewer #2, we performed a wide range of additional experiments to investigate whether PIEZO1 activity regulates the collagen crosslinking enzymes, such as LOX and LH2 (encoded by *Plod2*). All data returned from our multi-pronged effort were consistent with PIEZO1 mediation of enzymatic crosslinking. We would like to note that crosslink quantification is extremely challenging, particularly in mature tissues. "Gold-standard" mass spec-based measurement of homogenized / digested tissues are not only technically challenging, but are highly variable and prone to biases related to tissue processing protocols (more cross-linked tissues are more difficult to process). Further, it is unclear from the literature which crosslinks one should/must analyze (there are many species formed downstream of known enzymes) and/or to what quantitative degree crosslinking would/could underlie the mechanical differences we report (20% increased stiffness). Nonetheless, we were able to find significant PIEZO1 mediated

differences in gene expression, thermal denaturation, and crosslink-associated fluorescence as discussed below.

In a first series of additional experiments, we found that stimulation of PIEZO1 through Yoda1 leads to an upregulation of *Lox* but not of *Col1a1* gene expression in rat tail tendon fascicles. Similarly, mechanical stimulation to 2% stretch – corresponding to the mechanical threshold identified in our calcium imaging experiments – leads to an upregulation of crosslinking enzymes but not of *Col1a1* (**NEW Figure 5c,** page 17). These data likely explain why recurrent pharmacological PIEZO1 activation in tenocytes of tendon explants leads to mechanical adaptation (reinforcement) at the tissue level (Figure 5b).



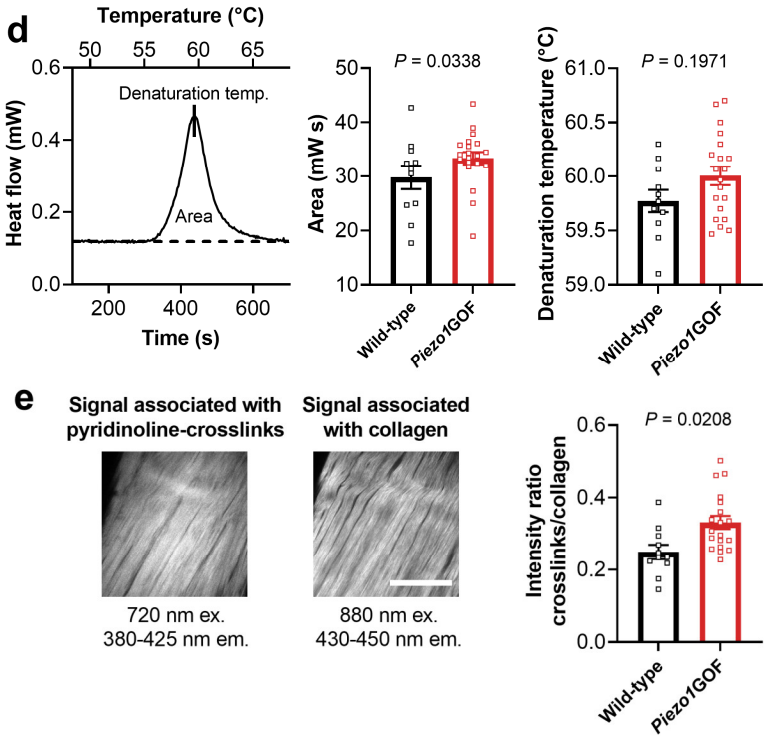
NEW Figure 5c: *mRNA expression of genes encoding collagen crosslinking enzymes (Lox and Plod2) and type I collagen (Col1a1) in tendon explants 48 h after four-times stretching to 2% (normalized to static control using the 2^{-ddCT} method, n=20, 7 rats) or 5 µM Yoda1 stimulation (normalized to sham control using the 2^{-ddCT} method, n=8, 4 rats), one sample t-test.*

These data are now reported in the results section of the revised manuscript (page 15, lines 17-22), as follows:

> *Moreover, at the gene expression level, Yoda1 stimulation of the fascicles induces an upregulation of the collagen crosslinking enzyme LOX but not of type I collagen (Fig. 5c). A similar gene expression profile was also observed after four-times stretching to 2% (Fig. 5c), which corresponds to the mechanical threshold identified in our calcium imaging experiments (Fig. 1d). This shows a notable degree of overlap between pharmacological PIEZO1 stimulation and mechanical stimulation of tendon fascicles.*

Furthermore, collagen crosslinking has been shown to increase the thermal stability of collagen tissues (Zeeman, Dijkstra et al. 1999). In turn, increased thermal stability is widely employed as a proxy indication of crosslinking extent. Indeed, using differential scanning calorimetry we found

that tendons of *Piezo1*GOF demonstrate an increased transition enthalpy – area between the denaturation curve and the baseline – and thus are thermally more stable (**NEW Figure 6d**, page 19). We then applied two-photon imaging as an orthogonal and well-accepted approach for semi-quantitative assessment of collagen-crosslinking. Elevated fluorescence in tendons of *Piezo1*GOF was observed which suggests the involvement of pyridinoline-crosslinks (**NEW Figure 6e**, page 19). We consider that these data, together with the transmission electron microscopy evidence of highly similar collagen ultrastructure, provides very strong support that elevated collagen crosslinking underpins the elevated mechanical properties of tendons in *Piezo1*GOF mice.



NEW Figure 6d and e: *(**d**) Differential scanning calorimetry measurements with Achilles tendons demonstrate an increased transition enthalpy, corresponding to the area between the denaturation curve and baseline (shown as a dashed line) in Piezo1GOF mice (n=10 mice, 8 heterozygous and 2 homozygous) compared to wild-type littermate controls (n=6 mice). (**e**) Two-photon imaging of Achilles tendons to assess autofluorescence associated with the collagen crosslinks pyridinoline, and second harmonic generation signal associated with the collagen matrix (Marturano, Xylas et al. 2014), n=11 Piezo1GOF mice (8 heterozygous and 3 homozygous) and n=7 wild-type littermate mice, (scale bar, 100 µm). Unless indicated otherwise, statistics was*

performed with linear mixed effects models (mouse ID as random effect and litter as fixed effect). Data are means±SEM.

These data are now reported in the results section of the revised manuscript (page 19, lines 2-11), as follows:

> *To address this, we performed differential scanning calorimetry measurements and assessed the tissue thermal stability, which depends on the crosslink density (Zeeman, Dijkstra et al. 1999). Tendons from Piezo1GOF mice showed an elevated transition enthalpy, demonstrating an increased thermal stability compared to their wild-type littermates (Fig. 6d). Hence, this finding indicates an elevated crosslink density in tendons from Piezo1GOF mice. Moreover, using two-photon microscopy we observed an increased autofluorescence signal associated with the pyridinoline-crosslinks compared to the second harmonic generation signal associated with the collagen matrix (Figure 6e) (Marturano, Xylas et al. 2014). While little is known about the specific contribution of individual crosslinks to the mechanical properties of tendons, our data suggest a denser pyridinoline-crosslink network in tendons of Piezo1GOF mice.*

Reviewer #1

• Fig 5f: it appears that the max jump height values might be larger for the non-carriers compared to the carriers. Is this a significant difference? If so, what does this mean? Why is the CMJ-DCMJ internal comparison within groups more important than the comparison of raw values across groups?

Reviewer #2

1. In Figure 5F, I don't believe this was directly quantified, but the data suggest that E756del carriers may have reduced jumping height as baseline. How this fits in to the data interpretation should also be clarified.

Of note, previous Figure 5 is now Figure 7 in the revised manuscript.

We begin by noting that “best performers” in jumping height also showed plausible explanatory confounding phenotypic features (e.g. high training level). Because of such uncontrolled factors (that were nonetheless well balanced between carrier and non-carrier groups), we anticipated and indeed observed large inter-subject variability in jumping performance. Hence, we chose a study design that allows intra-subject assessment of functional differences in jumping ability. As the tendon phenotype observed in *Piezo1*GOF mice was prominent at high degrees of tendon loading, we chose two jumping modalities with different degrees of tendon-loading (CMJ < DCMJ) and focused on the CMJ-DCMJ internal analysis. This allowed us to isolate the effect of greater tendon loading through intra-subject comparisons, and thereby reduce potential biases.

We have now added this clarification in the revised manuscript (page 21, line 22 and page 22, lines 1-9) as follows:

> *Importantly, as we anticipated high variability in performance between human subjects, we chose a study design which would allow an intra-subject comparison using the two jumping modalities to evoke different levels of tendon loading (CMJ < DCMJ) to properly address differences in tendon-loading exercises between groups. Indeed, the overall performance varied highly between subjects (Fig 7f). However, the intra-subject comparison revealed that E756del carriers performed significantly better in DCMJ, which evokes higher tendon loading, compared to CMJ, while non-carrier controls performed similarly in both jumps (Fig. 7f). Strikingly, by accounting for the inter-subject jumping variability with the normalization of individual DCMJ to CMJ, we found an average performance of 110.9% in E756del carriers that significantly exceeded the average 97.7% reached by non-carriers (Fig. 7g).*

Nonetheless, we performed a statistical analysis with linear mixed effects models (lme4 package in R, subject ID as random effect and leg as fixed effect) to compare the maximal CMJ-height between non-carrier controls and E756del carriers. We found no significant difference between the two groups (**p-value of 0.2513**). Similarly, we compared the maximal DCMJ-height of non-carrier controls and E756del carriers with the same statistical test and found no significant difference (**p-value 0.7722**).

To clarify this better, we have now added the results of this additional analysis in the legend of Figure 7 (page 24, lines 3-9) of the revised manuscript, as follows:

(f) The average jumping heights between non-carriers and E756del carries was similar (CMJ: 13.1±5.4 vs 11.6±4.4 cm (P = 0.25) and DCMJ: 12.8±5.7 vs 12.4±4.6 cm (P = 0.77), mean±SD are represented on the sides). However, intra-subject analysis within non-carriers revealed a similar performance in both jumps (P = 0.28), whereas, within E756del carries performance was significantly better in DCMJ compared to CMJ (P = 0.02, paired analysis). Performances corresponding to the same leg are connected with a line.

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

This manuscript evaluates the role of PIEZO1 in the mechanobiologic response of tendons. **This study was beautifully done and the data are impressive**. The comprehensive dataset includes in vitro experiments with tenocytes, ex vivo fascicle level testing, evaluation of whole tendons from a Piezo1gof mouse, and clinical data from carriers of a human gof Piezo1 mutation. The results suggest that PIEZO1-mediated mechanosensitive signaling plays a role in tendon stiffness. **The results are very interesting and have potential for high impact**. **The link from cell level responses to potential clinical implications are intriguing**. A few questions are outlined below that should be addressed.

We thank the reviewer for his/her positive and constructive feedback.

• Fig 1g: if spontaneous calcium signals are the same magnitude as strain-induced changes, how does a cell respond to mechanical signals? In other words, if a mechanobiologic response to load is in the same range as normal signal flucutations, then how does a cell sense an actual mechanical input?

We thank the reviewer for this comment. Calcium signals demonstrate a large versatility and control an extensive range of cellular processes including, for instance, the cell cycle, cell proliferation, cell death, cell contraction etc. (Berridge, Lipp et al. 2000, Grienberger and Konnerth 2012). This versatility originates from a wide repertoire of different spatio-temporal calcium profiles (Berridge, Lipp et al. 2000, Janssen, Mukherjee et al. 2015).

Upon stretching of the fascicles, we observed a tissue-wide calcium response with a large proportion of the cell population showing calcium signals. For instance, by stretching the fascicle at low strain rate, we found that at 2% tissue strain 50% of the cells showed a calcium signal, with an average of 59 calcium signals per minute detected in the field of view (Figure 1c and d, Movie S1b). In contrast, prior to the stretching, we kept the tendon fascicles in an unstretched position at 0% strain (corresponding to the baseline) and observed only sparce calcium signals (i.e. spontaneous events) in a few cells, with an average of 2.9 calcium signals per minute detected in the field of view (Figure 1c, Movie S1a).

Hence, the tissue-wide calcium profile at baseline and during stretching are completely different. It is likely that stretch-induced biological responses of the tissue are driven by the simultaneous calcium elevations occurring tissue-wide, with the frequency of the calcium signals plausibly

playing a role (Janssen, Mukherjee et al. 2015). Nonetheless, the link between calcium profiles and specific downstream pathway activations remains poorly understood in tenocytes. This is a future research area of great interest.

To clarify this better, we have now added the following sentences to the revised manuscript (page 25, lines 10-14):

> *Upon tissue stretching, we observed that tenocytes exhibit simultaneous calcium signals occurring tissue-wide, while in an unstretched condition they only show a few sparce spontaneous calcium signals. Thus, the tissue-wide spatio-temporal calcium profile differs clearly during a mechanical stimulus, likely triggering a mechanobiological response of the tissue.*

• Fig 2a: what is the meaning of the mechanical thresholds? How are these values computed and what is their significance? Are all seven signals plotted here from the same fascicle, within the same row? Or are these from different lines of cell/fascicles?

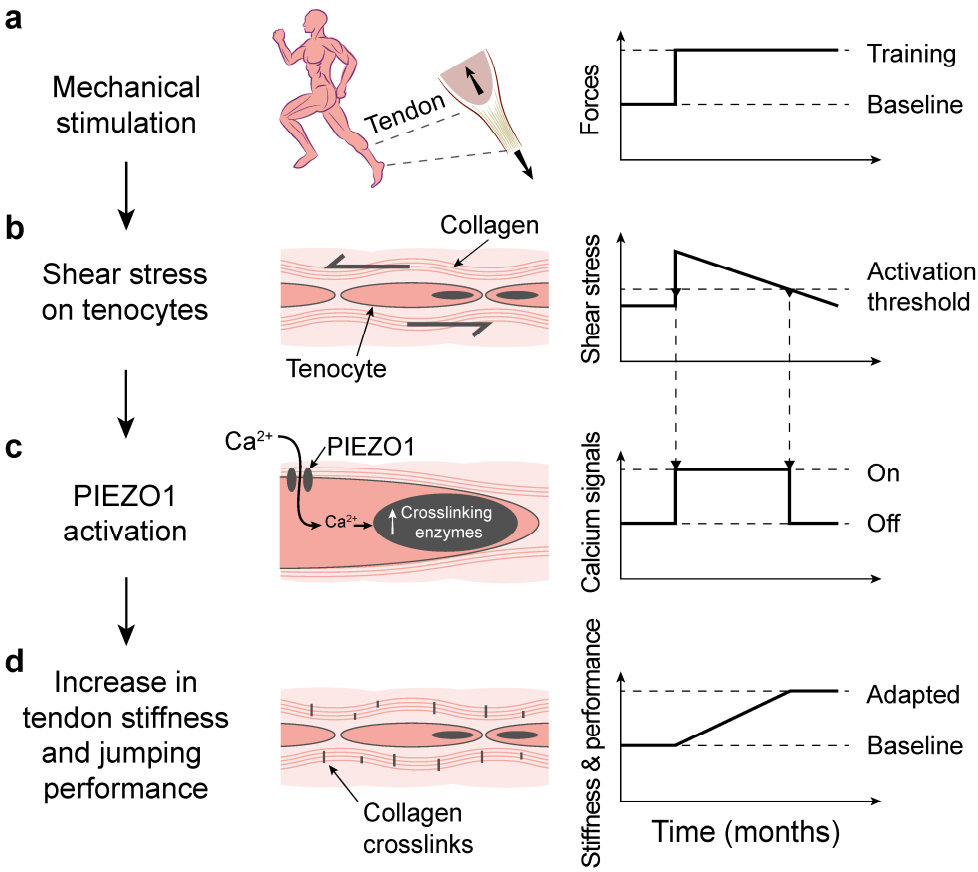
We thank the reviewer for these questions. The calcium images acquired during stretching at low strain rate (Figure 1b-d, n=7 fascicles) were further analyzed to quantify the fiber sliding. Therefore, from those seven fascicles we obtained both the calcium response, from which we calculated the mechanical threshold, as well as the fiber kinematics.

The mechanical thresholds (highlighted with red squares in Figure 2a) correspond to the level of tissue stretch at which 50% of the cells responded with a calcium signal. We added this clarification to the legend of Figure 1 (page 7, lines 5-9) and the legend of Figure 2 (page 9, lines 5-6). In addition, to better clarify the meaning of the mechanical thresholds, we have now added the following sentences to the revised manuscript (page 5, lines 16-20):

> *Compared to identified limits of tissue damage (Stauber, Blache et al. 2020) and in vivo measurements of tendon tissue strains (Snedeker, Ben Arav et al. 2009), the identified mechanical thresholds were found to reside near the upper limits of the physiological range. This suggests that the mechanical thresholds likely represent a "limit switch" for mechanical load induced tissue deformation that triggers tissue adaptation.*

Fiber sliding was calculated from the relative displacement of adjacent fibers. The images of each fascicle contained between 9 and 12 fibers. Therefore, for a fascicle that contained, for example, 9 fibers we obtained 8 relative inter-fiber displacements, which were then averaged and represented as mean ± s.e.m. in Figure 2a. This was performed and plotted for each of the seven fascicles. We added this clarification to the legend of Figure 2 (page 9, lines 2-5).

To better clarify the link between the different discoveries made at different levels – molecular, cellular, tissue and functional – we added a summary figure (**NEW Figure 8,** page 28) that illustrates the proposed mechanism of tendon mechanotransduction.



NEW Fig. 8. Proposed mechanism of tendon mechanotransduction that adapts the tissue and influences physical performance. *(**a**) Mechanical loading of tendons during, for instance, training (**b**) causes shear stress on tissue-resident tenocytes. (**c**) Such stimulus is sensed by PIEZO1 – a mechanosensitive ion channel – that triggers intracellular calcium signals and leads to an upregulation of collagen crosslinking enzymes. (**d**) As a consequence, the stiffness of tendons increases, affecting the physical performance.*

• Fig 2e: what is the meaning of the shear stress threshold? How was this value computed and what is its relevance?

The shear stress data of Figure 2e were fitted with a nonlinear fit (Hill slope). The shear stress threshold was defined as the level of shear stress at which 50% of the cells respond with a calcium signal. We have now added this clarification to the figure legend of Figure 2e (page 10, line 3-4).

Interestingly, this 50% threshold was at 3.3 Pa which falls well within the range of the calculated shear stress that occurs during tissue stretching (Fig. 2b). This suggests that a shear stress of 3.3 Pa could be an optimal mechanobiological stimulus for tenocytes. And shear stresses exceeding this threshold might trigger tissue adaptation processes.

• Fig 2f: this is a nice set of data, but it brings up a related question. The tenocytes for the data in panels d and e come from which type of tendon? One of the same tendons that are shown in panel f, rat tail tendon, or a different tendon altogether? Similarly, what tendon provided the cells shown in panel g?

We are thankful for this comment. We have now clarified this better and specified the source of the cells in the figure legend of Figure 2 (page 9, lines 13 and page 10, lines 2, 8 and 9). Tenocytes used in Figure 2d and 2e were isolated from human flexor digitorum tendons and are also depicted in Figure 2f. The isolated cell shown in Figure 2g originated from a human semitendinosus tendon, whereas the tissue-resident cell was imaged in a rat tail tendon fascicle. These data reflect our cumulative experience that the mechanical thresholds for shear flow induced calcium signals is well conserved and consistent across tendon cells from different anatomical origins and species.

• Fig 4b: the stiffness values for Yoda1 don't appear to be much higher than control, even though the values are reported to be significantly larger. What are the average values for these two groups and what was the p-value? Is this a meaningful increase for the Yoda1 fascicles compared to control?

Of note, previous Figure 4 is now Figure 5 in the revised manuscript.

After 16 days of culture, we observed an average Δ stiffness in the control group of –2.22 N/%. In the Yoda1 group, however, we found an average Δ stiffness of +1.94 N/%. To statistically

compare the control versus the Yoda1 group, we performed a Mann-Whitney test and found a p-value of 0.0221. We have now added the exact p-values in the figure and the average Δ values in the figure legend (Figure 5, page 17, lines 6-7).

The average Δ stiffness of +1.94 N/% observed in the Yoda1 group after 16 days of culture corresponds to a stiffness increase of 4.1%. Interestingly, in humans, a 14-weeks exercise intervention (4 times a week) with high-magnitude tendon strain induced a 36% increase in the Achilles tendon stiffness (Arampatzis, Karamanidis et al. 2007). This corresponds to an increase of 5.9% in 16 days. In comparison, in our *in vitro* experiment we found an increase of 4.1% in 16 days. Thus, our data suggest that the increase found *in vitro* represents a meaningful tissue adaptation comparable to *in vivo* exercise effects.

To address this point, we have now added the following sentence to the revised manuscript (page 15, lines 12-16):

> *Yoda1-stimulated fascicles showed a 4.1% average stiffness increase in the 16 days of culture. Interestingly, in humans, a 14-weeks exercise intervention (four-times a week) with high-magnitude tendon strain induced a 36% increase in Achilles tendon stiffness (Arampatzis, Karamanidis et al. 2007), corresponding to a 5.9% increase in 16 days. Thus, our data suggest that the tissue stiffness adaptation found in vitro is comparable to in vivo exercise effects.*

• Page 13; lines 1-2: the investigators suggested that the mechanical differences could be due to denser collagen crosslinking. Why not measure this to check this hypothesis? Various previous studies have quantified mature and immature crosslinks in tendons and other tissues; this type of evaluation could provide significant insight here.

Please find this point addressed above, in the section “Points raised by more than one reviewer”.

• Fig 5f: it appears that the max jump height values might be larger for the non-carriers compared to the carriers. Is this a significant difference? If so, what does this mean? Why is the CMJ-DCMJ internal comparison within groups more important than the comparison of raw values across groups?

Also for this point, we kindly refer to the beginning section “Points raised by more than one reviewer”.

• The heterozygotes and homozygotes were grouped together for the human E765del carriers. Was there any difference in measured properties between these two genotypes?

Of note, these data are now in Figure 7 in the revised manuscript.

Thank you for this comment. We identified 20 heterozygous and two homozygous E756del carriers, and 43 non-carrier controls from genotyping of the saliva samples. Because of the limited number of homozygous E756del carriers, we grouped the data of heterozygous and homozygous E756del carriers and performed statistical analysis between non-carriers and carriers. Nonetheless, further investigations between heterozygous and homozygous E756del carriers would certainly be interesting, particularly because our data suggest that homozygous carriers might potentially have a stronger phenotype than heterozygous carriers.

By normalizing the DCMJ-height to the CMJ-height, we found an average jumping performance of 97.7% in non-carriers, 109.1% in heterozygous E756del carriers and 128.7% in the two homozygous E756del carriers (**Figure R1**, meant solely for this point-by-point response letter). When converting the jump height into potential energy, we found an average of -1.7 J for non-carriers, +6.5 J for heterozygous E756del carriers and +23.7 J for the two homozygous E756del carriers (**Figure R1**). Nonetheless, a greater number of participants is needed to statistically analyze differences between heterozygous and homozygous carriers. In this regard, a significantly larger study would need to be conducted, given the low frequency of homozygous carriers.

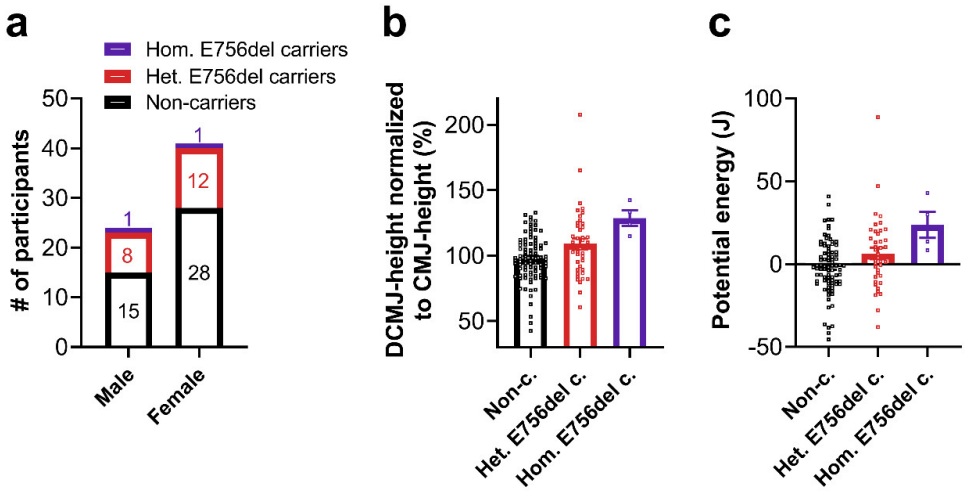


Fig. R1. Human jumping performance of heterozygous and homozygous E756del carriers and non-carrier controls. (**a**) n=20 heterozygous E756del carriers, n=2 homozygous E756del carriers and n=43 non-carriers identified in 65 African American participants. (**b**) Normalized jumping performance. (**c**) Conversion of jump height difference (between DCMJ and CMJ) into potential energy. Data are means±SEM.

• The authors mention that the E756del mutation protects against malarial infection; is this mechanism related at all to the mechanism at play in tendon mechanobiology? Or is this believed to be due to a separate/distinct action of this mutation?

Ma et at. (*Cell*, 2018) demonstrate that human red blood cells (RBCs) from E756del carriers are dehydrated and show a reduced *Plasmodium* infection *in vitro (Ma, Cahalan et al. 2018)*. *Plasmodium* is the parasite causing malaria. Additionally, they developed a mouse model of hereditary xerocytosis (a blood disorder characterized by RBC dehydration) and show that PIEZO1 overactivity in RBCs and T cells prevents experimental cerebral malaria induced by *Plasmodium* infection. These evidences and the fact that malaria-endemic regions report a high frequency of the E756del mutation indicate an association of the E756del mutation with malaria resistance (Ma, Cahalan et al. 2018).

Hence, the potential malaria resistance associated with the *PIEZO1* gain-of-function mutation E756del is more likely due to a distinct action of PIEZO1 in RBCs and in T cells, independent from the mechanism observed in tenocytes in this study.

• On page 20, the authors suggest that pharmacological activation of PIEZO1 could potentially help in tissue reinforcement and tendon healing. However, it's not clear how this might happen. Perhaps the authors could elaborate on this potential mechanism and expand their thoughts on this topic.

We are grateful to the reviewer for this comment. Tendon diseases are typically characterized by decreased tissue stiffness and impaired performance (Nourissat, Berenbaum et al. 2015). The gold standard treatment for such diseases is (still) physical therapy (Vicenzino, de Vos et al. 2020). This treatment strategy applies physical exercises in a recurrent fashion and can thereby induce beneficial tissue responses (Riley 2008, Vicenzino, de Vos et al. 2020). Although an

extensive repertoire of exercises are being used, they commonly share the fact that mechanical forces are being applied onto the tendon.

Based on our study, it appears plausible that physical therapy is designing and applying exercises that target the activation of PIEZO1 in tendons through mechanical stimulations. Our *in vitro* experiment with recurrent pharmacological PIEZO1 activation showed tissue adaptations (Fig. 5ab) that are comparable to those triggered by exercise interventions (Arampatzis, Karamanidis et al. 2007).

Hence, a therapeutic approach targeting the activation of PIEZO1 with pharmacology could plausibly (in theory) restore the stiffness and improve the tissue function of a diseased tendon. However, it remains unknown if a PIEZO1 activation in a diseased human tendon triggers a beneficial effect. We view this to be interesting for future preclinical study.

To clarify this better, we have now added the following sentences in the revised manuscript (page 27, lines 22 and page 28, lines 1-5):

> *Our data suggest that pharmacological activation of PIEZO1 stimulates tissue reinforcement mechanisms that may be relevant to treatment of diseased and/or mechanically-inferior tendons, perhaps mimicking the effects triggered by exercise-based physical therapy. While this is speculative and would require substantial preclinical investigation, the identification of PIEZO1 as a druggable target to drive tissue adaptation could open new paths for clinical treatment of tendon disorders.*

• What is OGB-1? Should be defined.

Thank you for spotting the missing explanation for this acronym. OGB-1 stands for Oregon Green BAPTA-1. We have now included this in line 1 of page 6.

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

This study by Passini et al., is focused on understanding how tendons sense and adapt to mechanical loads, thereby facilitating tendon function and skeletal movement. This work identifies shear stress, via collagen fascicle sliding, as a critical component of mechanosensing, with Piezo1 identified as a molecule regulator of this process. A combination of in vitro and in vivo studies using Piezo1 gain of function approaches suggests Piezo1 regulates tendon strength and stiffness, while natural Piezo1 gain-of-function mutations in humans are associated with increased jumping height. **The data demonstrating strain-regimen calcium responsiveness are very interesting, and the human components adds a novel application of these data**.

The **primary weaknesses of this manuscript are that the data provided only partially support the authors conclusion that Piezo is the tendon mechanosensory**, there is insufficient mechanistic characterization of key phenotypes, and there is some concern as to the rigor/ consistency of characterization of key experimental models. As such, the advance to the field is seen as moderate.

We thank the reviewer for his/her positive and constructive feedback. We have revised our manuscript with respect to the referees' critical comments. We have performed several additional experiments and analyses to address their points and strengthen the conclusions of the study.

Major issues:

1. While there is a clear mechanical phenotype in the Piezo1 GOF mice, the **characterization of the Piezo1 KO is lacking.** More specifically, the Piezo1 KO data in Figure 3 only demonstrates changes in Ca2+ signaling- it is unclear if Piezo1 KO/ LOF also alters tendon mechanics and does not support Piezo1 as the shear stress sensor.

We are thankful for this comment. Fascicle stretching causes shear stress on tissue-resident tenocytes (Figure 2). And we have demonstrated using a microfluidic flow chamber that indeed shear stress triggers a robust calcium response in isolated tenocytes (Figure 2). This shear stress-induced response was mediated by PIEZO1 as *in vitro* tenocytes depleted from PIEZO1 show clearly reduced shear stress-induced calcium signals (Figure 3). These results support the conclusion that PIEZO1 is the shear stress sensor in tenocytes.

As suggested by the reviewer, we have now performed additional experiments and added new data to showcase that indeed loss of PIEZO1 in tenocytes alters tendon mechanics by reducing the stiffness (**NEW Figure 4e**, page 13). To test this, we have generated tendon-targeted *Piezo1* knockout mice by crossing *Scx-CreERT2* mice with *Piezo1-flox* mice. *Scx-creERT2;Piezo1*$^{fl/fl}$ mice and littermate controls (*Piezo1*$^{fl/fl}$) were treated with tamoxifen at postnatal day P1 to P3 and studied at a young adult age. Despite the fact that we were only able to deplete *Piezo1* expression in tendons by about 40% in conditional knockouts (**NEW Figure 4a and b**, page 13), we could reveal that not only the stretch-induced calcium response in tendons was reduced (**NEW Figure 4c and d**) but that also tendon stiffness was decreased (**NEW Figure 4e**).

Altogether, our *in vitro* and *in vivo* loss-of-function and gain-of-function *Piezo1* experiments highlight that PIEZO1 is a mechanosensor crucial for shear sensation in tenocytes and that its signaling is critical in adjusting the tendon mechanical properties.

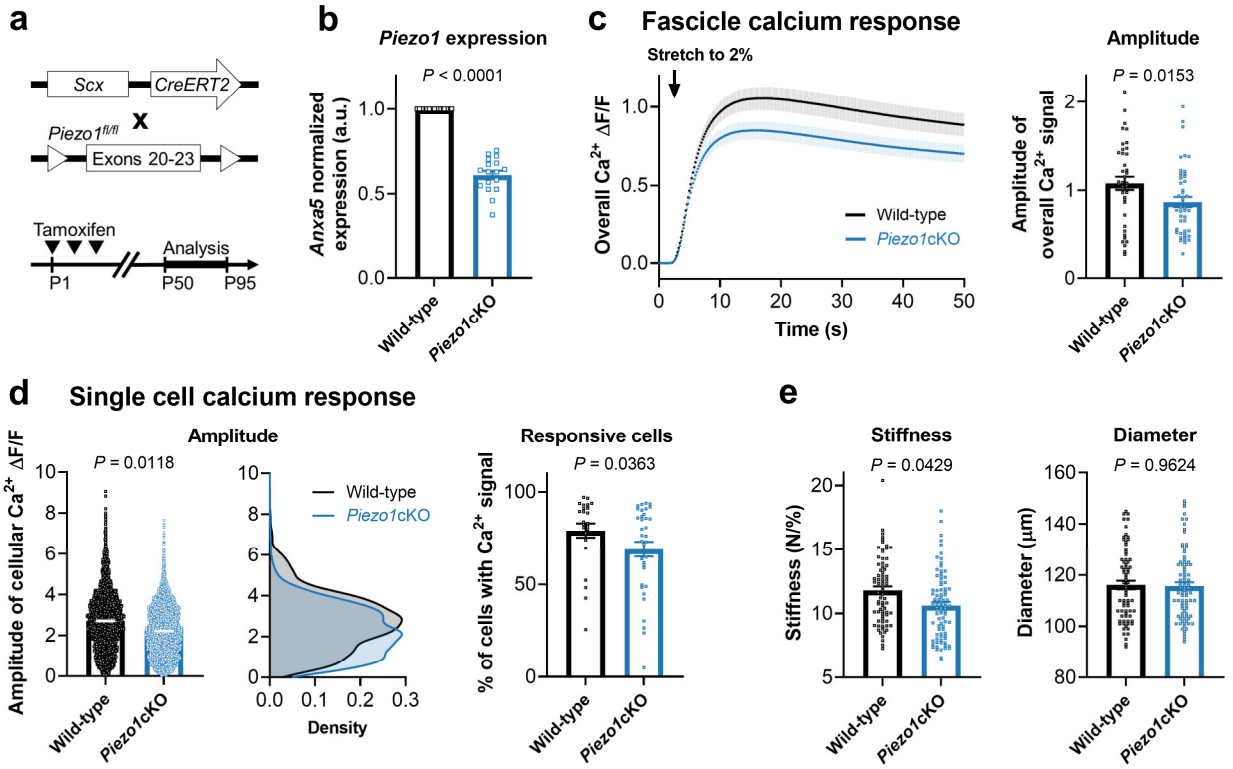


Fig. 4. Decreased stretch-induced calcium response and stiffness in fascicles from tenocyte-targeted Piezo1 knockout mice. *(**a**) Generation of Scx-creERT2;Piezo1*$^{fl/fl}$ *mice. Tamoxifen injections were performed at P1-P3 and the analysis was carried out between P50 and P95. (**b**) Reduced Piezo1 expression in tail tendon fascicles from Piezo1cKO mice (Scx-creERT2;Piezo1*$^{fl/fl}$*, n=17 mice) compared to their wild-type littermates (Piezo1*$^{fl/fl}$ *, n=15 mice),*

*unpaired Student's t-test. (**c**) The overall stretch-induced calcium response is reduced in fascicles from 7-11-week-old Piezo1cKO mice (n=9 mice) compared to wild-type littermate controls (n=6 mice), 6 fascicles were tested per mouse. (**d**) Corresponding single cell analysis shows that tenocytes in fascicles from Piezo1cKO mice exhibit a reduced amplitude of the stretch-induced calcium signals and a reduced % of responsive cells. (**e**) Ramp-to-failure tests show a decreased stiffness of fascicles from 10-13-week-old Piezo1cKO mice (n=14 mice) compared to wild-type littermate controls (n=12 mice, 6 fascicles tested per mouse). Unless indicated otherwise, statistics was performed with linear mixed effects models (mouse ID as random effect and litter as fixed effect). Data are means±SEM.*

These data are now reported in the results section of the revised manuscript (page 13, lines 1-10), as follows:

> *Next, to further assess if PIEZO1 also mediates mechano-sensation in tissue-resident tenocytes, we generated tenocyte-targeted, conditional Piezo1 knockout mice (Piezo1cKO) by crossing mice expressing the tamoxifen-sensitive Cre-recombinase CreERT2 under the Scleraxis promoter (Scx-CreERT2, (Howell, Chien et al. 2017)) with mice carrying the loxP-flanked Piezo1 alleles. Scx-creERT2;Piezo1$^{fl/fl}$ pups were injected at postnatal days P1-P3 with tamoxifen (Fig. 4a), which led to a 40% reduction in Piezo1 mRNA expression in tail tendon fascicles from 7-13-week-old Piezo1cKO mice compared to their Piezo1$^{fl/fl}$ littermate controls (Fig. 4b). Even with only a partial reduction in Piezo1 expression, fascicles from Piezo1cKO mice and their resident tenocytes showed a reduced stretch-induced Ca^{2+} response compared to littermate controls (Fig. 4c, d). This loss of function experiment provides further support that PIEZO1 is a tendon mechanosensor.*

And the result section on the stiffness changes (**NEW Figure 4e)** is now on page 14 (lines 16-21) as follows:

> *First, with ramp-to-failure tests, we characterized the biomechanical properties of the tail tendon fascicles from Piezo1cKO mice and littermate controls. Interestingly, we found on average a 10% reduction in stiffness in fascicles from Piezo1cKO mice (Fig. 4e). This effect is not caused by differences in the diameter of the fascicles (Fig. 4e) and suggests that PIEZO1 regulates the tendon tissue stiffness.*

We note that while knockout efficiency (40% on average) was sufficient to allow a reasonable evaluation of loss-of-function in tail tendon fascicles, we could achieve only very low knockout efficiency in the load bearing foot flexor tendons from *Piezo1*cKO (10-15%), which hindered further analysis with these tendons.

2. As all LOF experiments were conducted in vitro rather than in vivo, direct comparisons to the GOF are not possible. In addition, the Yoda1 data only indicate that pharmacological activation of Piezo1 increases tendon stiffness, but there is no mechanical component to these assays (e.g. comparisons between Yoda1 treatment +/- mechanical stimulation). As such, these data simply demonstrate that activation of Piezo1 contributes to enhanced mechanical properties, but do not define Piezo1 as the mechanosensor.

As mentioned above, we have now performed additional experiments in tendon-specific *Piezo1* knockout mice. Piezo1 loss-of-function *in vivo* revealed a reduction in tendon stiffness (**NEW Figure 4e**, page 13). We previously showed that tendons from *Piezo1*GOF were stiffer (Figure 5). Together, those experiments emphasize the role of PIEZO1 in regulating the tendon stiffness.

As suggested, we have now performed additional experiments and compared pharmacological versus mechanical stimulation of fascicles. More specifically, we performed a gene expression analysis of fascicles stimulated with Yoda1 or with mechanical stretch of 2%, which corresponds to the mechanical threshold identified in our calcium imaging experiments. Importantly, Yoda1 and mechanical stimulations evoke similar gene expression profiles, with an upregulation of crosslinking enzymes but not of type I collagen (**NEW Figure 5c**, page 17). This suggests that PIEZO1 activity regulates collagen crosslinking, and likely explains how Yoda1-treatment increases the stiffness of *ex vivo* fascicles (Figure 5b).

As mentioned above, our *in vitro* experiments show that PIEZO1 is the crucial shear sensor in tenocytes. And we now also provide additional evidence that *in vivo* reduction of *Piezo1* specifically in tenocytes reduces the stretch-induced calcium response in tail fascicles (**NEW Figure 4c and d,** page 13).

NEW Figure 4 and the corresponding results section are reported on page 13-14 of the revised manuscript. **NEW Figure 5c** is on page 17 and results section are on page 15 (lines 17-22).

3. Given that this is a global gain of function, the phenotype cannot necessarily be attributed to a tendon-specific effect of Piezo1GOF. For example, Piezo1 activation via short-term Yoda1 treatment increases bone mass, which would likely increase tendon mechanical properties simply by virtue of having to transmit heavier forces- this should be discussed in greater detail.

We thank the reviewer for this comment. The phenotypes observed in *Piezo1*GOF mice are likely due to tendon effects, but we cannot rule out other contributing factors as *Piezo1*GOF is global. However, given that we observed a similar stiffness regulation in tendon explants upon pharmacological PIEZO1 stimulation in tenocytes (Figure 5b) and tenocyte-specific, conditional *Piezo1*cKO mice demonstrate a reduced fascicle stiffness (**NEW Figure 4d**), our data strongly indicate that the tendon phenotypes in *Piezo1*GOF mice are primarily caused by a tenocyte-specific effect.

To clarify this better, we have now added the following sentences to the revised manuscript (page 16, lines 12-19):

> *These biomechanical differences are particularly prominent at high tendon strains and can likely be attributed to a tenocyte-specific effect, but we cannot rule out other contributing factors as these mice are constitutive Piezo1GOF mice (GOF mutation in all tissues). However, given that PIEZO1 activation in tissue-resident tenocytes regulates the stiffness of tendon explants (Fig. 5b) and tenocyte-targeted Piezo1cKO mice show tail tendon fascicles with reduced stiffness (Fig. 4e), our data indicate that the tendon phenotypes in Piezo1GOF mice are primarily caused by a tenocyte-specific effect.*

4. Related to mechanism, on pg 19 lines 1-5 the authors state “Instead, we suspect that a denser collagen cross-linking network causes the phenotypes.” Given the importance of this potential contribution to the tendon phenotype, direct assessment of cross-linking (e.g. via LOX etc) should be conducted.

We kindly refer to the beginning section “Points raised by more than one reviewer”.

5. Fig 3- The authors are isolating a heterogeneous population of cells- how does their knockdown (shown by mRNA isolated from the total tendon cell population in 3C) relate to the data shown in 3E at the single cell level? Do all tendon cells express all calcium channels?

The tendon cells used in our *in vitro* experiments were isolated from tendon tissues using collagenase digestion. These cells were not sorted by specific markers, and therefore might represent a heterogeneous population of cells. However, these tendon cells express all of the investigated ion channels and demonstrate a similar gene expression profile between different donors (**Figure R2**, meant solely for this point-by-point response letter), indicating a relatively homogenous population of primary cells.

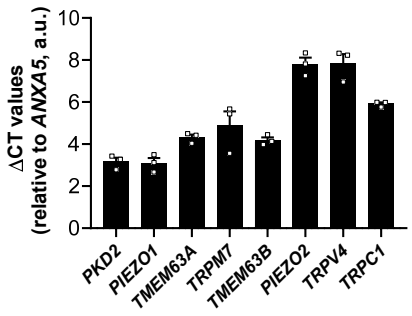
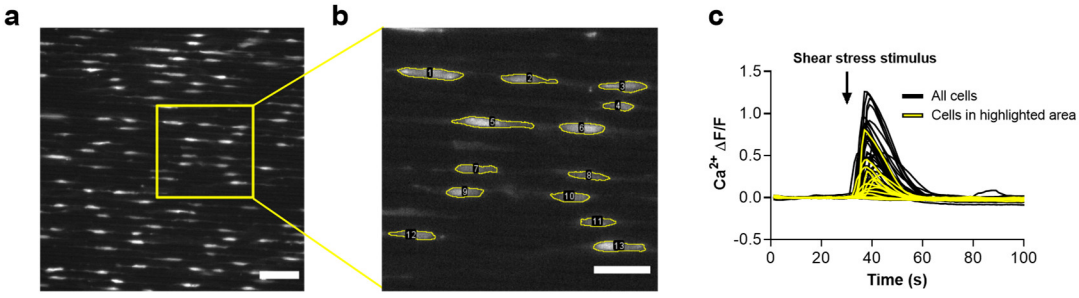


Fig. R2. Gene expression profile of the investigated ion channels in tendon cells (human semitendinosus tendons, n=3 human donors). Data shown as mean ± sem.

To better clarify how we analyzed the shear stress response (Figure 3e-h) of the candidate knockouts (Figure 3c), we now added the **NEW Figure S3** (page 55). For each shear stress chamber, we imaged hundreds of cells over a period of 100 s (**NEW Figure S3a**). After 30 s of baseline, cells were stimulated with 5 Pa for 5 s. To analyze the calcium response, we segmented the cells (**NEW Figure S3b**) and obtained the fluorescent time trace for each cell (**NEW Figure S3c**). From these fluorescent traces, we calculated the % of responsive cells and the amplitude of the calcium signals.



NEW Fig. S3. Analysis of calcium images from shear stress experiments. *(**a**) Entire field of view (scale bar, 100 µm). (**b**) Segmentation of single cells (scale bar, 50 µm). (**c**) Corresponding time traces of the fluorescence signals for all single segmented cells.*

Cells isolated from a tendon tissue were expanded in culture and then separated into different flasks (one flask for each candidate gene). Then, we performed the CRISPR-mediated knockdown for each candidate separately (Figure 3c). Subsequently, cells were seeded in the chambers for assessing their shear stress response (Figure 3e-h). For each candidate, we tested multiple chambers. And this was repeated three times, each time with cells from a different donor.

How can they be sure that the differences that they're showing in 3E are not just an efficiency issue (ie. not all cells express all calcium channels, not all cell types are targeted equally by their CRISPR?

Apart from the *PKD2* knockdown cells (average knockdown of 53%), the other candidates demonstrated a high CRISPR-knockdown efficiency (average of 97% for *PIEZO1*, 92% for *TMEM63A*, 87% for *TRPM7*, 83% for *TMEM63B*, 92% for *PIEZO2*, 99% for *TRPV4* and 97% for *TRPC1*). These efficiencies can be considered as optimal for primary cell cultures.

Although tendon cells express the ion channels TMEM63A, TRPM7, TMEM63B, PIEZO2, TRPV4 and TRPC1 (**Figure R2**) and their respective CRISPR-knockdowns showed high efficiency, no differences were detected in their shear stress response. Hence, the results shown in Figure 3e-h cannot be attributed to issues in knockdown efficiency.

Also, what are the kinetics of the various Ca channels- ie. do they all respond to stimulus on the same time scale (they only measured for 5sec)?

The gating kinetics can vary between different mechanosensitive ion channels and typically ranges from a few ms to ca. 1 s (transition time from closed to open state) (Murthy, Dubin et al. 2017).

The shear stress experiments with the purpose of testing the various knockouts were performed with a 5 s shear stimulus and a total image acquisition period of 100 s. Prior to those experiments, we also tested the shear stress response of tendon cells upon stimuli of different durations. The percentage of responsive cells increases with increasing stimulus duration, and saturates above a duration of 5 s (**Figure R3**, meant solely for this point-by-point response letter). Hence, stimulations of > 5 s seem to trigger a similar response than a 5 s stimulus. Based on this, we investigated the shear stress response with a 5 s stimulus.

Our data suggest that PIEZO1 is the crucial shear stress sensor in tendon cells. Nonetheless, the other mechanosensitive ion channels expressed by tendon cells might be responsible to detect other type of mechanical stimuli. To investigate this, new assays will be needed.

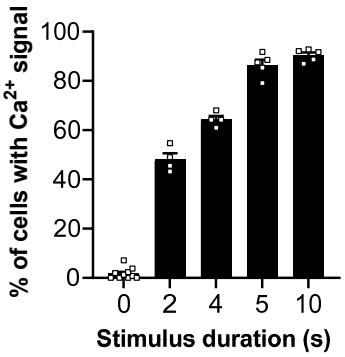
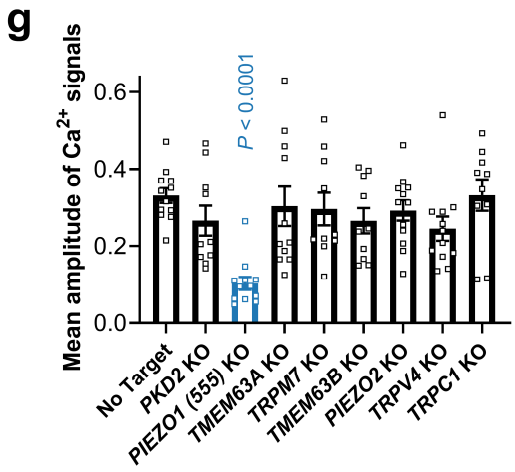


Fig. R3. Response rate of tendon cells (human flexor digitorum tendons) to a shear stress stimulus with a magnitude of 5 Pa and a duration between 2 and 10 seconds (for each condition n≥4 chambers, cells from 2 human donors). Data shown as mean ± sem.

Rather than total # of calcium events as the outcome, could the % change in intracellular calcium levels at baseline compared to stimulated be shown instead? This would overcome the issue with comparing total population properties to single cell properties.

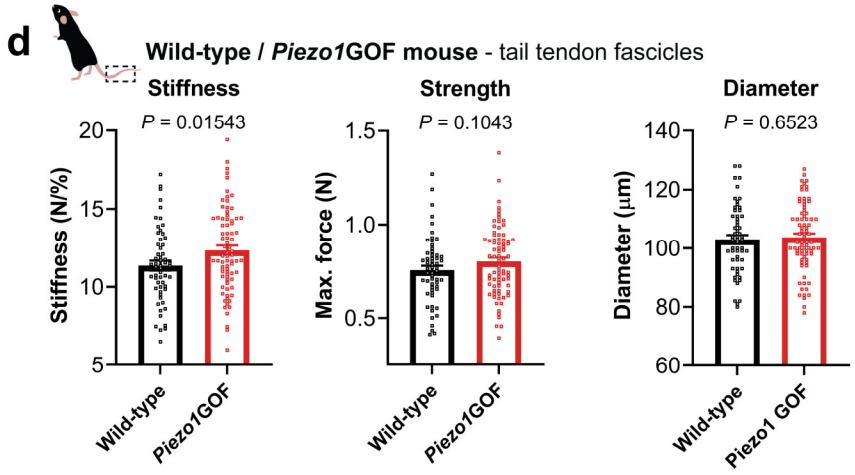
As suggested, in addition to Figure 3e showing the % of responsive cells, we now added a **NEW Figure 3g** (page 11, and figure legend on page 12, lines 13-17) that shows the amplitude of the calcium signals (i.e. change in intracellular calcium levels) for all of the candidate knockouts. In line with Figure 3e, from all the candidates only the *PIEZO1* KO cells show a reduced amplitude (P<0.0001).



***NEW Fig. 3g**: PIEZO1 depleted cells show a reduced amplitude of the Ca2+ signals (averaged over all single segmented cells). For each candidate n≥10 chambers were tested with cells from 3 human donors (semitendinosus tendons), one-way ANOVA with multiple comparisons (Dunnett's test).*

6. Multiple tendons are used throughout this study. Given the clear heterogeneity between tendons this variable could have potentially important effects. Are there differences in the effects of PIEZO1 GOF between types of tendons (positional vs load bearing, tail (in vitro studies) vs. Achilles/flexor for the in vivo)?

We thank the reviewer for this comment. In addition to the plantaris tendon (load-bearing tendon, Figure 5e and f) we additionally tested the tail tendon fascicles (positional tendon) of *Piezo1*GOF mice (**NEW Figure 5d,** page 17). Similar to the plantaris tendon, also tail tendon fascicles of *Piezo1*GOF mice show an increased stiffness (**NEW Figure 5d**). While plantaris tendons exhibit a 19% average increase in stiffness in *Piezo1*GOF mice compared to wild-type littermates, tail tendon fascicles exhibit a 9% average increase. This supports the thesis that PIEZO1 drives tissue adaptation according to the mechanical loads applied to the tissue, as load-bearing tendons experience higher mechanical loads than positional tendons (Screen, Berk et al. 2015).



NEW Fig. 5d: *Positional tendons: ramp-to-failure tests with tail tendon fascicles show increased stiffness but unaffected diameter in constitutive Piezo1GOF mice (n=13 mice, 10 heterozygous and 3 homozygous, 6 fascicles tested per mouse) compared to wild-type littermate controls (n=9 mice, 6 fascicles tested per mouse) from 6 litters in total.*

These data are now reported in the results section of the revised manuscript (page 16, lines 5-7), as follows:

> *Ramp-to-failure experiments with tail tendon fascicles showed on average a 9% increase in stiffness (Fig. 5d), which is in line with the role of PIEZO1 in regulating the mechanical properties observed in vitro and in Piezo1cKO mice.*

Furthermore, the identification of PIEZO1 as a shear sensor is based on *in vitro* experiments performed with load-bearing tendons (human semitendinosus tendons, Figure 3) and positional tendons (rat tail tendon fascicles, Figure S5).

7. Unrelated to the science, this reviewer would urge careful thought as to the description of human subjects on page 20, lines 3-8 and how these statements may be perceived.

We respect the advice urging sensitivity, as well as the rationale for this advice. We consider that a possible link between E756del prevalence and athletic (sprinting, jumping) performance is thought provoking and worthy of explicit mention. We have adapted the paragraph as follows (now on page 27, lines 13-16):

> *The tendon performance phenotype demonstrated by E756del carriers might potentially contribute to the fact that nearly all the top 500 sprint times of the men's 100 m are held by athletes hailing from countries with high E756del prevalence (Olympic Games, 100m men, World Athletics, 100 meters men). However, whether the E756del allele is overrepresented in elite sprinters remains to be clarified.*

Also, on page 21 line 4-5, we have now adapted the sentence to:

> *Interestingly, athletes hailing from countries with high E756del prevalence excel in power sports performance related to sprinting and jumping.*

Minor

1. In Figure 5F, I don't believe this was directly quantified, but the data suggest that E756del carriers may have reduced jumping height as baseline. How this fits in to the data interpretation should also be clarified.

We kindly refer to the beginning section "Points raised by more than one reviewer".

2. Figure 2, while important from a technical development perspective, does not make a substantive contribution to the authors conclusion, and could be considered as supplemental information.

Figure 2 demonstrates the role of shear stress as a key stimulus driving calcium signals in tendon cells. We consider this an important finding for the field of tendon mechanobiology and therefore propose to leave Figure 2 as a main figure.

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

Summary: The following manuscript utilizes in vitro, in situ, and human studies to pursue a detailed multi-hierarchical level hypothesis on how mechano-signaling determines tendon stiffness and human jumping performance. Utilizing calcium imaging in both tendon cell culture and in situ explant culture the authors demonstrated that tenocytes most likely detect mechanical forces through shear stress induced by collagen fiber-sliding. CRISPR/Cas9 screening in human and rat tenocytes identified PIEZO1 as the crucial shear sensor. In rodents, elevated mechano-signaling resulted in increased tendon stiffness and strength both in vitro by Yoda1 activation and in vivo by a Piezo1 gain-of-function mutation (a 16% average increase in normalized jumping height). The results of these multiple studies lead to the conclusion that PIEZO1-mediated mechano-signaling regulates tendon stiffness and impacts human athletic performance.

Degree of Advance: This **study has an amazing depth to it utilizing a hierarchal level of advanced cellular characteristic tools, tissue characterization, and human subject analysis**. This article **highly advances the state of tendon mechanobiology and health**. The conclusions are original.

We thank the reviewer for his/her positive and constructive feedback.

Major Issues: None

Minor Issues / missing statistics / missing citations

1. Page 5, Lines 9 – 11: Regarding the strain rate dependence and multiple versus single calcium signaling responses with higher strain rates. Do the authors believe that the higher strain rates only stimulated the cells to calcium signaling once or did the higher strain rates induce multiple signals, but it just appears as one long calcium signal as the rate was high enough to not allow

the cell to re-establish baseline calcium levels. Did the authors measure the length of calcium signaling?

We thank the reviewer for this comment. As suggested, we now measured the length of the calcium signals, and found a duration of 28.2 ± 16.5 s (mean±SD). This was added to the revised manuscript on page 5, lines 8-9.

The stretching protocol at high strain rate (1% strain/s) takes 10s, which is too fast for tenocytes to re-establish baseline calcium levels during the stretching protocol. Hence, at high strain rate we observed only one calcium signal per cell. However, whether this single signal is caused by a cumulation of multiple smaller signals at the same time remains an open question.

It seems plausible that mechanosensitive ion channels get activated gradually during a stretching protocol and that a population of channels (activated with certain offsets) contributes to the observed signal.

2. Page 5, Line 14: Do you have a reference or more reasoning behind the statement “potential cell-cell communication processes occurring predominately at low strain rate”?

During calcium imaging, we noticed that cells adjacent to an activated cell often show a calcium signal shortly after (qualitative assessment). It appears therefore that the calcium signal propagates to neighboring cells by cell-cell communication possibly via gap junctions and/or purinergic signaling (Lavagnino, Wall et al. 2015). In some cases, we observed that the calcium signal propagated through three or even four cells, causing a calcium wave (**Fig. R4**, meant solely for this point-by-point response letter, **NEW Movie S2**).

This phenomenon seems particularly prominent at low strain rate as the stretching is performed slowly over an extended period of time. This allows sequential calcium signaling likely originating from a propagation of the primary signal induced mechanically. At high strain rate, cells exhibit the calcium signals in a synchronized manner and within a narrow time window. Hence, calcium signals induced by cell-cell communication seem to play a minor role at high strain rate.

Uncoupling calcium signals induced by cell-cell communication from those induced mechanically will be key to understand the contribution of cell-cell communication in tendon mechanobiology. This is a future research area of great interest.

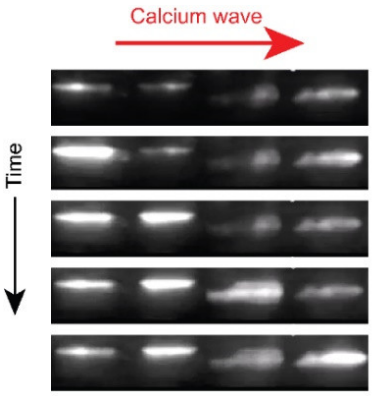


Fig. R4. Calcium wave observed in tendon explants. Fluo-4 stained tissue-resident tenocytes seem to propagate the mechanosensitive response to adjacent cells.

We have now added the movie corresponding to Figure R4 to the supplementary materials and referenced it in the revised manuscript (**NEW Movie S2**, page 5, line 16).

3. Figure 1 d and g: In Figure 1d there are ** and ***, I assume these relate to significant differences, but it is not clear what they mean. In Figure 1g the figure legend states significant differences in calcium although it is not apparent from the figure either through annotation or through the mean and standard deviation levels.

We are thankful for this comment. As required by the journal, we have now reported the exact p-values.

Figure 1 d shows the cumulative sum of the first Ca^{2+} signals for the three different strain rates (low 0.01% strain/s, medium 0.1% strain/s and high 1.0% strain/s). From these data, we defined the mechanical thresholds at 50% of the cumulative curve. Hence, the identified mechanical thresholds correspond to the tissue strain at which 50% of the cells show a first Ca^{2+} signal. Differences in the mechanical thresholds between strain rates were analyzed using a one-way ANOVA with multiple comparisons (Tukey's test). P-values are reported in the figure.

We have now clarified this point in the legend of Figure 1d of the revised manuscript (page 7, lines 3-9), as follows:

> *The cumulative sum of the first Ca^{2+} signals for three different strain rates (low 0.01% strain/s, medium 0.1% strain/s and high 1.0% strain/s) showing a right-shift with increasing strain rate. The mechanical threshold was defined at 50% of the cumulative curve and corresponds to the tissue strain at which 50% of the cells showed a first Ca^{2+} signal. Mechanical thresholds were found at (from low to high*

strain rate): 1.96±0.35% strain (n=7 fascicles), 2.72±0.33% strain (n=7 fascicles) and 3.62±0.38% strain (n=6 fascicles), one-way ANOVA with multiple comparisons (Tukey's test).

In Figure 1 g we quantified the increases in [Ca^{2+}], represented as Δ [Ca^{2+}], between baseline (timepoint preceding the elevation) and active state for stretch-induced and spontaneous Ca^{2+} signals.

We have now clarified this point in the legend of Figure 1g of the revised manuscript (page 7, lines 13-15), as follows:

Quantification of the increases in [Ca^{2+}], represented as Δ [Ca^{2+}], between baseline and active state for stretch-induced Ca^{2+} signals (n=25 cells from 6 fascicles) and spontaneous Ca^{2+} signals (n=43 cells from 13 fascicles).

4. Figure 2: Has the value for p-values associated with ** and ***, but not Figure 1 which has the * symbols, whereas Figure 2 has none.

Thank you for spotting the missing information. We included the exact p-values in the Figures.

5. Page 15, Line 15-17: "These biomechanical differences (higher stiffness and strength w/o differences in geometry with PIEZO1 compared to wild type) are particularly prominent at high tendon loading" It appears that all of these tests in this section are at the same loading rate (1%/second). How is this statement backed by the current study?

The force-strain curves of *Piezo1*GOF tendons (ramp to failure tests, Figure 5c) increasingly diverge from the curves of wild-type tendons with increasing tissue strain. Hence, at high tendon loading (i.e. at high tissue strain and forces) the differences between the two groups are particularly prominent.

With "high tendon loading" we meant high tendon strain and therefore replaced it in the revised manuscript with "high tendon strains" (page 16, line 13).

6. Page 15, Line 2: Is it known that being a E756del heterozygous carrier will lead to the PIEZO1GOF mutation or does it require a homozygous carrier?

A heterozygous E756del carrier has one copy of the *PIEZO1*GOF allele (E756del) and one copy of the wild-type allele. The *PIEZO1*GOF allele (E756del) leads to an overactive PIEZO1, whereas the wild-type allele leads to a wild-type PIEZO1. Hence, heterozygous E756del carriers express both variants of the channel, whereas homozygous E756del carriers express only the overactive channel because they carry two copies of the *PIEZO1*GOF allele (E756del).

Consequently, homozygous E756del carries might show a stronger phenotype than heterozygous E756del carriers.

7. Page 18, Lines 8-10: There is a previous study that found that loaded rat tail tendon under a microscopic device to determine that in-situ tenocytes respond to increased strain with increased calcium signaling. It is quite old, so it may be difficult to locate.

It was originally published as an abstract:

Shirakura, K., Ciarelli, M.J., Arnoczky, S.P., and Whallon, J.H.: Deformation Induced Calcium Signaling, Trans. Combined Ortho. Res. Soc. 1995, p 94.

This was then re-presented in a book chapter: Arnoczky S.P., Lavagnino M., Egerbacher M. (2007) The response of tendon cells to changing loads: Implications in the etiopathogenesis of tendinopathy. In: Tendinopathy in athletes, Encyclopedia of Sports Medicine, (eds S.L.Y. Woo, P. Renstrom, S.P. Arnoczky), Oxford, UK: Blackwell Publishing. pp. 46–59. https://doi.org/10.1002/9780470757987.ch4
This article being 25 years old does not have the technology, breadth, or depth of the current article, but would still be worth a citation. The abstract is cited in your reference number 48.

Thank you for pointing this out. Following the reviewer's suggestion, we have now included the citation of the book chapter in the revised manuscript (Reference 52, page 25, line 3).

8. Page 25, Line 8: How was the fiber length lfiber= 900 µm determined?

The observations of fiber kinematics during tissue stretching were limited to the field of view. Hence, the fiber length we were able to observe and analyze corresponds to the width of the field of view, i.e. 900 µm. This is now specified in line 19-20 (page 34).

References:

(Olympic Games, 100m men). "Olympic Games, 100m men." www.olympic.org/athletics/100m-men.
(World Athletics, 100 meters men). "World Athletics, 100 meters men." www.worldathletics.org/records/all-time-toplists/sprints/100-metres/outdoor/men/senior.
Arampatzis, A., K. Karamanidis and K. Albracht (2007). "Adaptational responses of the human Achilles tendon by modulation of the applied cyclic strain magnitude." J Exp Biol **210**(Pt 15): 2743-2753.
Berridge, M. J., P. Lipp and M. D. Bootman (2000). "The versatility and universality of calcium signalling." Nat Rev Mol Cell Biol **1**(1): 11-21.
Grienberger, C. and A. Konnerth (2012). "Imaging calcium in neurons." Neuron **73**(5): 862-885.
Howell, K., C. Chien, R. Bell, D. Laudier, S. F. Tufa, D. R. Keene, N. Andarawis-Puri and A. H. Huang (2017). "Novel model of tendon regeneration reveals distinct cell mechanisms underlying regenerative and fibrotic tendon healing." Scientific reports **7**(1): 1-14.
Janssen, L. J., S. Mukherjee and K. Ask (2015). "Calcium homeostasis and ionic mechanisms in pulmonary fibroblasts." American journal of respiratory cell and molecular biology **53**(2): 135-148.
Lavagnino, M., M. E. Wall, D. Little, A. J. Banes, F. Guilak and S. P. Arnoczky (2015). "Tendon mechanobiology: Current knowledge and future research opportunities." J Orthop Res **33**(6): 813-822.
Ma, S., S. Cahalan, G. LaMonte, N. D. Grubaugh, W. Zeng, S. E. Murthy, E. Paytas, R. Gamini, V. Lukacs, T. Whitwam, M. Loud, R. Lohia, L. Berry, S. M. Khan, C. J. Janse, M. Bandell, C. Schmedt, K. Wengelnik, A. I. Su, E. Honore, E. A. Winzeler, K. G. Andersen and A. Patapoutian (2018). "Common PIEZO1 Allele in African Populations Causes RBC Dehydration and Attenuates Plasmodium Infection." Cell **173**(2): 443-455 e412.
Marturano, J. E., J. F. Xylas, G. V. Sridharan, I. Georgakoudi and C. K. Kuo (2014). "Lysyl oxidase-mediated collagen crosslinks may be assessed as markers of functional properties of tendon tissue formation." Acta biomaterialia **10**(3): 1370-1379.
Murthy, S. E., A. E. Dubin and A. Patapoutian (2017). "Piezos thrive under pressure: mechanically activated ion channels in health and disease." Nat Rev Mol Cell Biol **18**(12): 771-783.
Nourissat, G., F. Berenbaum and D. Duprez (2015). "Tendon injury: from biology to tendon repair." Nat Rev Rheumatol **11**(4): 223-233.
Riley, G. (2008). "Tendinopathy--from basic science to treatment." Nat Clin Pract Rheumatol **4**(2): 82-89.
Screen, H. R., D. E. Berk, K. E. Kadler, F. Ramirez and M. F. Young (2015). "Tendon functional extracellular matrix." J Orthop Res **33**(6): 793-799.
Snedeker, J. G., A. Ben Arav, Y. Zilberman, G. Pelled and D. Gazit (2009). "Functional fibered confocal microscopy: a promising tool for assessing tendon regeneration." Tissue Eng Part C Methods **15**(3): 485-491.
Stauber, T., U. Blache and J. G. Snedeker (2020). "Tendon tissue microdamage and the limits of intrinsic repair." Matrix Biol **85-86**: 68-79.

Vicenzino, B., R.-J. de Vos, H. Alfredson, R. Bahr, J. L. Cook, B. K. Coombes, S. N. Fu, K. G. Silbernagel, A. Grimaldi and J. S. Lewis (2020). "ICON 2019—International Scientific Tendinopathy Symposium Consensus: There are nine core health-related domains for tendinopathy (CORE DOMAINS): Delphi study of healthcare professionals and patients." British journal of sports medicine **54**(8): 444-451.
Zeeman, R., P. J. Dijkstra, P. B. van Wachem, M. J. van Luyn, M. Hendriks, P. T. Cahalan and J. Feijen (1999). "Successive epoxy and carbodiimide cross-linking of dermal sheep collagen." Biomaterials **20**(10): 921-931.