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## Evidence for a protein tether involved in somatic touch

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### Review timeline:

Submission date:	15 September 2009
Editorial Decision:	21 September 2009
Rebuttal:	22 September 2009
Additional Correspondence:	22 September 2009
Editorial Decision:	28 October 2009
Revision received:	02 December 2009
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### Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

21 September 2009

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Thank you very much for submitting your manuscript to the EMBO Journal. I have now had the opportunity to read the manuscript carefully and to discuss it with the other members of our editorial team and I am very sorry to say that we cannot offer to publish it.

The analysis reports on RA-mechanosensitive currents in isolated mouse sensory neurons in response to mechanical stimuli. TEM analysis shows that tether-like links are formed between fibroblast/laminin and neurites. Protease treatment, subtilisin or blisterin, leads to loss of RA-mechanosensitive currents and to reduced length of the tether-like filaments as seen by TEM analysis. Using an in vitro skin nerve preparation, the findings also provide support for that subtilisin can affect mechanoreceptors in this setting. We appreciate the link between the observed tether-like filaments and mechanosensitive currents and recognize that this provides some support for that the tether-like filaments might connect mechanosensitive channels with the extracellular matrix. However, the analysis also remains rather correlative and it is not clear what the target(s) of the proteases is and if they specifically target the tether-like filaments. Overall, I am afraid that we are unfortunately not persuaded that the present analysis is well suited for publication in the EMBO Journal.

Please note that we publish only a small percentage of the many manuscripts that we receive at the EMBO Journal, and that the editors have been instructed to only subject those manuscripts to external review which are likely to receive enthusiastic responses from our reviewers and readers. As in our carefully considered opinion, this is not the case for the present submission, I am afraid our conclusion regarding its publication here cannot be a positive one. I am sorry to have to disappoint you on this occasion.

Yours sincerely,

Editor  
The EMBO Journal

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Rebuttal

22 September 2009

The authors appealed the initial editorial decision and after discussion with the Executive Editor it was decided to send the manuscript out for full review.

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Additional Correspondence

22 September 2009

Thank you for your email. In response to your rebuttal, I have discussed the manuscript further with our executive editor and we have no objections to sending the study out for full review. I will get back to you as soon as I hear back from the referees.

Best wishes

Editor  
The EMBO journal

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2nd Editorial Decision

28 October 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed.

As you can see below, while referees #1 and 2 are very supportive of the analysis, referee #3 finds the analysis too preliminary and correlative. In addition to these reports, I have also sought further editorial advice on the study and the advisor raises similar issues as referee #3. I have also discussed the study, reports and advice received further with our executive editor and while the limitations of the study are clear, there is also a great interest in this analysis as is evident by the comments made by both referees # 1 and 2. Given all the available input received, I would like to ask you to submit a suitable revised manuscript should you be able to address the concerns raised in full. This would involve experimentally addressing all of the concerns raised by referees #2 and #3 and to resolve the issues concerning figure 2A (see below). Finally, we also feel that the present title is misleading and should be changed to better reflect the actual data provided. Possible examples could be "A protein tether associated with mechano-transduction (or touch sensitivity) by sensory neurons" or "Evidence for a protein tether involved in mechano-transduction (or touch sensitivity) by sensory neurons", but I will leave it up to you to come up with a good title.

I should also remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:  
<http://www.nature.com/emboj/about/process.html>

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor  
The EMBO Journal

## REFEREE REPORTS

### Referee #1 (Remarks to the Author):

The authors developed an *in vitro* model for mechanosensitive cells of the skin, showing that culturing sensory neurons on top of fibroblasts leads to a coupled pair of cells: mechanical stimulation of either the sensory neuron or the fibroblast led to mechanotransduction currents in the neuron. This result suggests strongly that an interaction between these cells permits mechanotransduction. TEM imaging on the neuron/fibroblast co-cultures indeed revealed a candidate for this interaction, a 100 nm tether connecting the two cell types. The structure of these tethers recalls tip links of inner ear hair cells, although the tethers are not sensitive to the same biochemical agents as are tip links. However, the tethers are sensitive to proteases, including the relatively selective protease blisterase. The protease effect seemed selective for RA mechanotransduction currents, as SA currents and other voltage gated currents were unaffected. This is an important result as it suggests the proteases are not having a general effect on channel function. In addition, TEM shows that the proteases eliminate the 100 nm tether. Finally, RA currents are lost in skin-nerve preparations that have been treated with subtilisin.

The authors suggest the obvious, that the tether is responsible for mechanotransduction. Of course, this is a difficult assertion to prove, but the evidence presented here strongly suggests that conclusion. The authors suggest characteristics of the tether that could allow its identification, which will be necessary in order to prove its role in transduction. In some ways, the situation here is similar to that in hair cells after identification of the tip link; even there, exactly how the tip link functions to gate transduction channels is not clear, despite the tip link having been identified.

### Comments

Figure 4, an absolutely critical figure, is very confusing. What are the differences between panels B & C, D & E, etc.? The legend doesn't help. This figure needs each of the panels to be explained very clearly.

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

This interesting manuscript describes the role of a protein tether in mammalian touch sensitivity. Disruption of this tether (viewed by electron microscopy) by subtilisin completely suppresses the rapidly activating mechano-sensitive current in touch-sensitive DRG neurons. Moreover, the latency of the slowly activating current is drastically enhanced.

This is a very original and important study that establishes for the first time in mammals the role of a tether in touch sensitivity. Similar findings were previously reported in the nematode *C. elegans* by Chalfie. All together these results demonstrate that this conserved function plays a key role in touch sensitivity.

The experiments are very well controlled and the manuscript is beautifully written and presented. There are only two points which could be improved:

- 1) The authors mention that MscL and MscS are directly activated by tension in the bilayer. The important work on mechano-gated K2P channels should also be cited. Please cite a recent Nature review about TREK-1 in this paragraph.
- 2) The authors previously demonstrated that inactivation of SLP3 leads to a complete loss of mechanoreceptor function. Is it possible that SLP3 function may be required for the formation of the tether? Could you please check this point by measuring the tether length on cultured DRG neurons isolated from the SLP3 KO mice.

In conclusion, this is a beautiful study which is important for the field of mechanosensory transduction. I would like to congratulate the authors for the quality of their work.

Referee #3 (Remarks to the Author):

This paper investigates the mechanosensitivity of DRG neurons and suggests that a protein link, possibly located on neurites, is necessary for the activation of rapidly-adapting currents in sensory neurons. It is suggested that low-threshold mechanoreceptors but not most nociceptors do express this protein. Disruption of this link with non specific endopeptidases abolishes mechanical currents. The enzyme also alters the response of mechanosensitive fibers in vivo.

Overall, the results reported in this manuscript are too preliminary and speculative. There is no clear causal link between the presence of this enigmatic protein and the gating of rapid mechanosensitive currents, given that acutely dissociated DRG neurons without neurites have been previously shown to express mechanosensitive currents.

I have also major concerns with the reported experiments:

- 1) What is the nature of the rapidly-activated current? Is it the RA cationic current described by Wood and Levine groups or the RA sodium current described previously by the authors? A more detailed characterization of the current (reversal potential, selectivity and so on) is clearly needed here given the conflicting data in the literature.
- 2) It is argued that the protein link may transfer force from the fibroblast to mechanosensitive channels present in the sensory neuron, but no direct evidence is presented; the link may simply serve to attach the neuron to the fibroblast or to the laminin substrate and may have nothing to do with direct channel gating. Moreover, it is not clear if the protein link is only present on neurites. EM data of cell bodies should be provided. To strengthen the authors' theory it must be shown that acutely dissociated DRG neurons (2-4 hr) are not mechanosensitive.
- 3) Figure 2A: I found unethical to illustrate data with current traces that have been previously published. Records shown in figure 2A have been published twice in Hu and Lewin (2006) and in Wetzel et al., (2006). In general, this manuscript lacks raw data. Fig.2B: the series resistance in this current clamp recording is far too high to be presented.
- 4) The effects of the enzyme on inward (?) and outward currents should be illustrated.
- 5) The comparison of latency is pertinent if speed clamp and space clamp are identical in all experiments, which is very much unlikely. Were they estimated and how?

1st Revision - authors' response

02 December 2009

Referee #1 (Remarks to the Author):

The authors developed an in vitro model for mechanosensitive cells of the skin, showing that culturing sensory neurons on top of fibroblasts leads to a coupled pair of cells: mechanical stimulation of either the sensory neuron or the fibroblast led to mechanotransduction currents in the neuron. This result suggests strongly that an interaction between these cells permits mechanotransduction. TEM imaging on the neuron/fibroblast co-cultures indeed revealed a candidate for this interaction, a 100 nm tether connecting the two cell types. The structure of these tethers recalls tip links of inner ear hair cells, although the tethers are not sensitive to the same biochemical agents as are tip links. However, the tethers are sensitive to proteases, including the relatively selective protease blisterase. The protease effect seemed selective for RA mechanotransduction currents, as SA currents and other voltage gated currents were unaffected. This is an important result as it suggests the proteases are not having a general effect on channel function. In addition, TEM shows that the proteases eliminate the 100 nm tether. Finally, RA currents are lost in skin-nerve preparations that have been treated with subtilisin.

The authors suggest the obvious, that the tether is responsible for mechanotransduction. Of course,

this is a difficult assertion to prove, but the evidence presented here strongly suggests that conclusion. The authors suggest characteristics of the tether that could allow its identification, which will be necessary in order to prove its role in transduction. In some ways, the situation here is similar to that in hair cells after identification of the tip link; even there, exactly how the tip link functions to gate transduction channels is not clear, despite the tip link having been identified.

*We are very happy that this reviewer appreciates the paper and has summarized very clearly the main results above. The reviewer is of course right in asserting that final proof of the tether can only follow its molecular identification. We have nevertheless carried out further control experiments that lend further weight to our conclusion that the presence of the tether is necessary for the activation of the RA current. We showed in the original submission that treatment of cultured sensory neurons with PIPLC an enzyme that cleaves all GPI anchored proteins from the membrane does not affect any mechanotransduction currents. We know from our own experiments that an identical enzyme treatment is effective at stripping GPI-anchored proteins from the membrane (Sturzebecher at al. in revision, MS attached) and it is known that some GPI-anchored proteins can be quite large. It was reasonable to then examine such PIPLC treated cultures with TEM as of course the absence of long tethers in such an experimental situation, without any effect on mechanotransduction, would effectively quash the conclusion that the tether is always associated with RA-currents. We have now carried out this experiment in addition and the results are included in the revised MS (revised Figure 4). We find as predicted that the tether protein of ~100 nm is quantitatively unaffected by such enzyme treatment which lends further rigorous support for our conclusions.*

#### Comments

Figure 4, an absolutely critical figure, is very confusing. What are the differences between panels B & C, D & E, etc.? The legend doesn't help. This figure needs each of the panels to be explained very clearly.

*We have added new data to this figure (see above) and have also rearranged the figure and rewrote the legend to make the figure, hopefully, much easier to read.*

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

This interesting manuscript describes the role of a protein tether in mammalian touch sensitivity. Disruption of this tether (viewed by electron microscopy) by subtilisin completely suppresses the rapidly activating mechano-sensitive current in touch-sensitive DRG neurons. Moreover, the latency of the slowly activating current is drastically enhanced.

This is a very original and important study that establishes for the first time in mammals the role of a tether in touch sensitivity. Similar findings were previously reported in the nematode *C. elegans* by Chalfie. All together these results demonstrate that this conserved function plays a key role in touch sensitivity.

*We are again delighted that this reviewer found our paper to be highly original and of general interest. We have answered the specific minor criticisms below.*

The experiments are very well controlled and the manuscript is beautifully written and presented. There are only two points which could be improved:

1) The authors mention that MscL and MscS are directly activated by tension in the bilayer. The important work on mechano-gated K2P channels should also be cited. Please cite a recent Nature review about TREK-1 in this paragraph.

*We have added new references as suggested.*

2) The authors previously demonstrated that inactivation of SLP3 leads to a complete loss of mechanoreceptor function. Is it possible that SLP3 function may be required for the formation of the tether? Could you please check this point by measuring the tether length on cultured DRG neurons

isolated from the SLP3 KO mice.

*This is an interesting suggestion and we had indeed considered doing this experiment. However, our own data on mechanotransduction currents in SLP3 mutants suggest that the number of cells with an RA current would be reduced but not abolished (Wetzel et al. 2007). Thus, for example, if the number of tethers was reduced by 50% in SLP3 mutant mice it is not clear whether a statistically significant change could be detected by measuring objects in random TEM sections. At the moment we measure around 1000 objects in each experiment and we have made a statistical estimate that this number would have to be increased by 3 fold in order to detect a significant reduction in tether number by 50%. The need to sample even more ultra-thin sections would make this experiment very labour intensive so that it is unlikely that we could carry out this experiment in a reasonable time. To sample and measure around 1000 objects takes about two months. As pointed out above, we have added an additional control experiment using TEM that strongly increases the strength of our conclusions.*

In conclusion, this is a beautiful study which is important for the field of mechanosensory transduction. I would like to congratulate the authors for the quality of their work.

Referee #3 (Remarks to the Author):

This paper investigates the mechanosensitivity of DRG neurons and suggests that a protein link, possibly located on neurites, is necessary for the activation of rapidly-adapting currents in sensory neurons. It is suggested that low-threshold mechanoreceptors but not most nociceptors do express this protein. Disruption of this link with non specific endopeptidases abolishes mechanical currents. The enzyme also alters the response of mechanosensitive fibers in vivo.

Overall, the results reported in this manuscript are too preliminary and speculative. There is no clear causal link between the presence of this enigmatic protein and the gating of rapid mechanosensitive currents, given that acutely dissociated DRG neurons without neurites have been previously shown to express mechanosensitive currents.

*This reviewer is obviously more skeptical about the conclusions that we have made on the basis of the experimental findings, but, it is rather unclear to us why this is the case. One point that this reviewer makes is that acutely dissociated DRG neurons without neurites also display mechanosensitive currents. We have never disputed this fact, indeed we have shown that mechanosensitive currents can be measured in adult sensory cell bodies as well as in acutely dissociated embryonic and early post-natal neurons (Hu and Lewin, 2006; Lechner et al. 2008). Just because RA-mechanosensitive currents can be measured after mechanical stimulation of the soma does not mean that a tether protein is not involved. We have indeed carefully re-examined our TEM sections, where cell somas are naturally relatively rare (see Figure 4a for a sense of the scale). We do in fact observe tether-like proteins at the interface between soma and the laminin containing matrix (see new supplementary Figure S2). We thus fail to see why this should be a killer argument against the idea that the tether molecules is necessary for the RA-mechanosensitive current.*

I have also major concerns with the reported experiments:

1) What is the nature of the rapidly-activated current? Is it the RA cationic current described by Wood and Levine groups or the RA sodium current described previously by the authors? A more detailed characterization of the current (reversal potential, selectivity and so on) is clearly needed here given the conflicting data in the literature.

*The current that we measure has been characterized by us in detail in three published reports. Every time that we have looked at the reversal potential of the RA-current it reverses at very positive potentials and we have also repeatedly documented the fact that the current is almost completely blocked when Na<sup>+</sup> ions are replaced with the non-permeant cation NMDG (Hu and Lewin, 2006 Figure 5C; Lechner et al. 2009 Supplementary Figure 4). We cannot account for the results of other groups but would like to point out that in all the studies published from the Wood group it is stated that the mechanosensitive current is non-selective but no Figures or data are shown to substantiate this claim (see Drew et al. 2004 quote iMA currents are non-selective cation currents with a*

reversal potential of around 0 mV in quasi-physiological solutions (L.J. Drew & Cesare P, unpublished data). In the paper from the same group on rat neurons (Drew et al. 2001, no information on the selectivity of the current is given. One paper from Jon Levine's group has measured the reversal potential of the current in rat neurons and show in their paper a non-selective RA-current (McCarter and Levine, Mol Pain 2006). We have never seen such a thing and it is possible that the discrepancy is because of the method of mechanical stimulation or even the species (rat versus mouse). Nevertheless, we regularly measure RA-currents and also the reversal potential of the current in this study was occasionally measured and as we have reported and documented before the results showed a positive reversal potential indicative of a high Na<sup>+</sup> ion selectivity.

2) It is argued that the protein link may transfer force from the fibroblast to mechanosensitive channels present in the sensory neuron, but no direct evidence is presented; the link may simply serve to attach the neuron to the fibroblast or to the laminin substrate and may have nothing to do with direct channel gating. Moreover, it is not clear if the protein link is only present on neurites. EM data of cell bodies should be provided. To strengthen the authors' theory it must be shown that acutely dissociated DRG neurons (2-4 hr) are not mechanosensitive.

*We have now shown an example of tether-like molecule under the cell soma of the sensory neurons (see above). The reviewer suggests that we show that sensory neurons acutely isolated in culture do not have an RA-current as it is presumably assumed that tethers will not be available to attach to laminin in that time. We do not agree that this experiment is really equivalent to treating attached sensory neurons with protease and then waiting for recovery of the current and the tether (which took around 18 hours). First, dissociation of sensory neurons is done with a combination of enzymatic digestion (collagenase/trypsin) and mechanical dissociation with a fire polished Pasteur pipette. As a rule, the less the enzymatic digestion the better the neurons. Thus, one cannot assume that the tether is completely ablated in these acutely dissociated cells. We have published experiments in which we showed that RA currents are present in acutely isolated neurons (4-6 hours after plating) (Lechner et al. 2009). Nevertheless, in these experiments not all cells were found to possess a mechanosensitive current in the soma. In order to answer the thrust of this reviewer's criticism we have now included additional data on neurons recorded after plating on a laminin-free substrate consisting of just poly-L-lysine. Neurons on PLL do attach, but fail, in most cases, to grow neurites, nevertheless in many cases we did observe mechanosensitive currents in these cells (9/17 tested), but noticed that sensory neurons only thrived when a fibroblast was nearby. We conclude that mechanosensitive currents were only present as the fibroblasts laid down an ECM capable of supporting the attachment of tethers (see revised MS Page 7 paragraph 1).*

3) Figure 2A: I found unethical to illustrate data with current traces that have been previously published. Records shown in figure 2A have been published twice in Hu and Lewin (2006) and in Wetzel et al., (2006).

*We agree with this comment although the traces are purely there to illustrate the types of current seen. We have replaced all the examples shown with traces obtained from the same types of current measured in the present study.*

In general, this manuscript lacks raw data. Fig.2B: the series resistance in this current clamp recording is far too high to be presented.

*As far as we are aware it is in fact impossible to tell from the presented trace precisely how high the series resistance is from the example recording. The example trace is from a large sensory neuron, which of course has a relatively low input resistance, this means that you need to inject quite a bit of current into the soma to produce enough depolarization to evoke a spike in such large diameter cells. The series resistance  $R_s$  in this particular example is 15 mOhm with a pipette resistance of 8 mOhm these values are in the normal range for a cell with a large soma size.*

*The reviewer asks for more raw data to be included by which we think he means current and/or voltage traces. We are happy to oblige and included example traces showing voltage gated inward and outward currents in control and subtilisin treated cells as well as proton gated inward currents in both groups. These traces have been added to Figure 3 (part A). In the revised Figure example traces of inward and outward currents to voltage steps are shown before and after subtilisin*

*treatment. In addition, we show some examples of pH gated currents observed in subtilisin treated cells (Figure 3 C,D).*

4) The effects of the enzyme on inward (?) and outward currents should be illustrated.

*There was little or no effect on such currents after enzyme treatment and this is illustrated in the revised Figure 3.*

5) The comparison of latency is pertinent if speed clamp and space clamp are identical in all experiments, which is very much unlikely. Were they estimated and how?

*The speed of the clamp was in fact likely to be very similar in all experiments and we have actually measured this in most cases. The space clamp is for the most part not relevant as we have previously demonstrated that the latency of the current is normally NOT dependent on the distance between the site of stimulation and the soma (Hu and Lewin, 2006). Thus the latency of the current evoked 70  $\mu\text{m}$  away from the soma or directly adjacent to the soma is essentially the same (see Hu and Lewin, 2006 Table 1). Indeed, we do not claim that we are clamping the voltage of the membrane at the site of the neurite stimulation. However, in all cases the cells are superfused with TTX to prevent regenerative responses in the neurites. The effects we observed on the latency of the SA current following treatment with proteases was in fact also observed when the cell soma was directly stimulated.*

3rd Editorial Decision

08 December 2009

Thank you for submitting your revised manuscript to the EMBO Journal. I have now had the opportunity to carefully evaluate the revised manuscript and the introduced changes. I have also discussed the revision further with our executive editor and we both appreciate the introduced changes. I am therefore pleased to inform you that we will accept the manuscript for publication here. You will receive the formal acceptance letter shortly.

Best wishes

Editor  
The EMBO Journal