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## Piezo1-dependent stretch-activated channels are inhibited by Polycystin-2 in renal tubular epithelial cells

Remi Peyronnet, Joana R. Martins, Fabrice Duprat, Sophie Demolombe, Malika Arhatte, Martine Jodar, Michel Tauc, Christophe Duranton, Marc Paulais, Jacques Teulon, Eric Honore and Amanda Patel

*Corresponding author: Eric Honore, CNRS*

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### Review timeline:

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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.)

Editor: Barbara Pauly

1st Editorial Decision

24 May 2013

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Thank you very much for the submission of your research manuscript to our editorial office. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. You will see that while referee #1 is rather negative, both referees 2 and 3 support publication of the study in EMBO reports.

Upon further discussing the concerns of referee 1 with the other two reviewers, we have decided to give you the opportunity to revise your manuscript along the lines of the reports.

We would not insist on further elucidating the mechanism by which PC2 inhibits Piezo1, even though you might want to discuss possible scenarios. We would also not require you to show that the Piezo1-mediated currents occur at the apical site of the cells as well and that endogenous PC2 and Piezo1 interact. Nevertheless, upon further discussions with the editors, referee 3 agrees that the conclusion that Piezo1 and PC2 physically interact under native conditions is not supported by direct experimental evidence. Referee 3 states that knockdown of PC2, causing a dis-inhibition of

native Piezo1-like SAC may partially circumvent this weakness by supporting the conclusion that endogenous proteins functionally interact. Some other concerns of reviewer 1 should be addressed as well. For example, the expression levels of Pkd1, Pkd2 and TRP channels in Piezo1-depleted cells should be analyzed at the protein level, at the minimum for PC2. It should also be further discussed whether the fact that the mutant form of PC2 is expressed at a higher level than the wild-type channel could account for the stronger inhibition of Piezo1 by mutant PC2. Please also discuss whether binding between PC2 and Piezo1 is expected to be direct or indirect and, if possible, analyze the effects of interaction mutants on Piezo1 activity. Reviewer 1 also states that additional controls with another transmembrane protein are needed for the co-IPs (comment #7). Referee 3 suggests indicating whether other disease-related PC2 mutations are known to exhibit similar effects on Piezo1 and reviewer 1 also feels that the disease relevance of the proposed regulation of Piezo1 by PC2 should be discussed further. Finally, referee 3 states that the current findings should be considered in the light of PC2's effects on stretch-activated potassium channels.

Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 29,000 characters (including spaces). Currently your manuscript exceeds this limit and I would kindly ask you to shorten the text slightly, also to make room for the additional discussions requested by the reviewers. I would recommend combining the results and discussion section, as this avoids unnecessary redundancies. We can also only display 5 figures in the main body of the manuscript, so please identify one figure that can be moved into the supplementary section or combine to figures if appropriate. Materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

I should also point out that we recently changed our reference style to a number-based one (which also reduces the character count). I am sorry for having to ask you to do this, but could you please change the style before submitting your revised manuscript? Details and the relevant end-note file can be found here:

<http://www.nature.com/embor/about/authors.html#reformat>

We have also started encouraging authors to submit the raw data for microscopical images and western blots to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know ([emboreports@embo.org](mailto:emboreports@embo.org)). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

## REFeree REPORTS:

Referee #1:

The authors show that the Piezo1 channel is inhibited by wild type PC2 or its truncation mutant, PC2-740X. They also show that Piezo1 and PC2 co-immunoprecipitate in transiently transfected cells. The same group has shown before that PC2 or PC2-740X suppressed currents mediated by the native stretch-activated channel (SAC), through an interaction involving Filamin A, in arterial myocytes. At that time the identity of the SAC was unknown. It was subsequently discovered that Piezo1 mediates SAC. The authors now confirm that PC2 inhibits Piezo1 in a different cell type. Manuscript is clear and well written. However, it lacks novelty and fails to provide mechanistic information of how binding of PC2 to Piezo1 leads to inhibition of Piezo1-mediated currents. Another major limitation is lack of evidence that the two endogenous proteins interact under physiological conditions. Finally, gain-of-function mutations in Piezo1 lead to xerocytosis, a hematologic disease, but not a phenotype even remotely associated with phenotypes associated with the loss of PC2, that would be expected to mirror gain-of-function mutations of Piezo1, if authors are correct. The observation that the truncation mutant PC2-740X, which represents a mutation seen in Autosomal Dominant Polycystic Kidney Disease patients inhibits Piezo1 as effectively as wild type PC2, rules out the possibility that this interaction is relevant to Polycystic Kidney Disease. Therefore the paper falls short in 1) demonstrating physiological significance of the purported interaction, 2) providing mechanistic leads of the inhibition, and 3) providing a sufficient level of novelty beyond of what is already known. The work presented is at a very preliminary stage. Other major issues are noted:

- 1) SAC current was recorded from the basolateral surface of proximal convoluted kidney tubules. No currents were recorded from the apical surface. Therefore direct evidence that Piezo1-mediated currents induced by intraluminal pressure is lacking. Piezo1-mediated currents in the apical surface should be shown.
- 2) Expression levels of Pkd1, Pkd2 or TRP channels upon Piezo1 knockdown were determined by qPCR (Fig. Supp. 1). However, this analysis has to be determined at the protein level. Mechanisms of compensation are not exclusive to gene transcription. Furthermore, protein-protein interactions can stabilize the proteins mediating the interaction. Therefore, knockdown of one protein can cause downregulation of the other without affecting mRNA levels. Also, it is important to determine the subcellular localization of endogenous PC2 in cells depleted of Piezo1.
- 3) It appears that Myc-PC2-740X expression is increased when it is co-expressed with Piezo1 and also is expressed at higher level compared to PC2-WT (Fig. 5a and b, lanes 3 and 6, input panels). How can the authors rule out the trivial possibility that PC2-740X is expressed at higher levels than PC2-WT, when co-transfected with Piezo1, and thus the inhibition is more effective?
- 4) The domains and ideally, the residues in PC2 and Piezo1 mediating the interaction should be identified and mutant constructs lacking binding should be used as negative controls for functional studies. This is imperative to rule out non-specific effects. Is the binding direct or indirect? Does it involve Filamin A, as was shown earlier (Cell 2009, 139(3):587-96; PLoS One. 2012;7(7):e40448)? Does Piezo1 interact with Filamin A? TRPC1 was used as a negative control in functional assays (Fig. 4d). Does TRPC1 bind to Piezo1 in transiently transfected cells? This needs to be shown side-by-side (same blot) with PC2 and PC2-740X using a TRPC1 construct tagged with the same tag as PC2 and PC2-740X.
- 5) Fig. 4b. Exogenous Piezo1 increased SACs by 3-fold, which is very strange, as I'd expect that much more protein should be made upon transfection. So, it is confusing as to why only such a small increase in currents was seen. Previous work has shown that Piezo1 forms a homomultimeric channel (Nature 2012, 483(7388):176-81) and therefore, interacting proteins should not be a limiting factor. Can the author explain?
- 6) Interaction of endogenous Piezo1 and PC2 should be shown. The authors claim that there is no good antibody to Piezo1. This is unfortunate, because such a reagent is absolutely necessary to support the main point of the paper that is the claim that the two proteins interact (see also below in point 7).
- 7) There are no appropriate controls in the co-immunoprecipitation experiments in Fig. 5. EGFP is not an appropriate control for a protein-protein interaction of proteins bearing transmembrane segments. Again, a detailed and thorough structure-function analysis is needed to substantiate the claim that these proteins interact. Fig. 5b, lane 6 (IP Probe HA) showing the interaction of Piezo1 and PC2-740X is not convincing, given the interaction is done in transfected cells, where high levels

of exogenous proteins are achieved. Fig. 5b lacks appropriate control. Since there is no transfected HA-Piezo1 in lanes 1-3, of course nothing would be detected in pulldowns of PC2 or PC2-740X (panel labeled: IP Probe HA, lanes 1-3).

8) I was very surprised to see nice staining of Piezo1 in the plasma membrane, which has not been seen before (J Cell Sci. 2010 Jan 1;123(Pt 1):51-61). Can the authors comment on this discrepancy? There is no obvious expression of PC2 in the plasma membrane, which is not surprising (Fig. 6). In fact, very little if any co-localization of Piezo1 and PC2 is detected in transfected cells. How do the authors think that PC2-WT modulates Piezo1? Is it that PC2 is localized in the endoplasmic reticulum (ER) and Piezo1 in the plasma membrane? If so, PC2-WT should only affect a tiny fraction of Piezo1 at the plasma membrane, perhaps where the ER and plasma membranes come in close proximity. I cannot see how PC2, which is localized almost entirely in the ER can suppress Piezo1 currents originating at the plasma membrane to more than 70-90%, without affecting the amount of Piezo1 in the plasma membrane.

Minor points

1. Page 2, line 7, 'among them stretch-activated ion channels' should be changed to 'among them are stretch-activated ion channels'.
2. Page 3, Para 4, line 5 from bottom. There is no Fig. 2c.
3. Page 7, Para 2, line 11, '50  $\mu\text{m}$ ' should be changed to '50  $\mu\text{M}$ '.
4. Page 7, Para 2, line 15, '50  $\mu\text{m}$ ' should be changed to '50  $\mu\text{M}$ '.
5. Page 7, Para 4, line 3, delete blank after a).
6. Page 7, Para 4, line 8, '{plus minus} 1.7, mm Hg' should be changed to '{plus minus} 1.7 mm Hg'.
7. Page 7, Para 5, line 3, delete blank after a).
8. Page 7, Para 5, line 2 from bottom, '{plus minus} 2.0, mm Hg' should be changed to '{plus minus} 2.0 mm Hg'.
9. Page 8, Para 1, line 7, '{plus minus} 1.5, mm Hg' should be changed to '{plus minus} 1.5 mm Hg'.
10. Page 8, Para 1, line 8, '{plus minus} 1.5, mm Hg' change to '{plus minus} 1.5 mm Hg'.
11. Page 8, Para 1, line 9, '{plus minus} 2.1, mm Hg' change to '{plus minus} 2.1 mm Hg'.
12. Page 8, References, line 4 from bottom, volume and pages should be added.
13. Page 10, line 4, volume and pages should be added.
14. Page 12, line 4, volume and pages should be added.

Referee #2:

This is a concise, novel finding demonstrating the importance of Piezo1 in mechanotransduction of renal tubular epithelial cells. Mechanotransduction is not understood well at the molecular level. One important family of pressure sensors are ion channels. Piezo family of mechanically activated ion channels has recently described; however, if Piezos are required for mechanosensitivity of various tissues and biological processes is still completely unknown. This manuscript clearly shows: (1) stretch-activated ion channels (SAC) present in renal tubular epithelial cells are dependent on Piezo1: this is the first demonstration that Piezo1 accounts for SAC activity in a relevant primary cell type in any tissue

(2) Piezo1 can be slowly adapting. This is a surprising finding as Piezos were originally described as rapidly-adapting (mechanosensitive ion channels are often defined according to their inactivation properties). This opens up the possibility that adaptation can be modulated, and that this property is not necessarily inherent to the channel itself.

(3) These authors had shown previously that shear-stress sensing PC1/2 actually inhibit SAC activity (different form of mechanotransduction). Now, the authors show that the target that they had described is Piezo1, as they can reproduce these results in heterologous system.

The only criticism that I can think of is the fact there is no detailed mechanistic investigation of how the inhibition of PC2 on Piezo1 works. However, "EMBO reports" is about describing a focused and exciting new finding, and I believe this is a good match for this format.

Referee #3:

In their manuscript (,Piezo1-dependent stretch-activated channels are inhibited by Polycystin-2 in

renal tubular epithelial cells"), Peyronnet et al detected Piezo1 as a key component of a non-selective cation conductance in primary cultures of tubular epithelial cells, and an inhibitory impact of polycystin PC2 on ionic currents through mechanically gated Piezo1 channels. The authors further highlight that a disease-related PC2 mutant (PC2-740X) exerts an even stronger inhibitory effect and physically interacts with Piezo1 more efficiently than wild-type PC2. The manuscript and the figures are well presented.

#### Major points:

The authors pay caution by noting that the interaction between Piezo1 and PC2 may be indirect. A bridging between the reticular higher-order-network of Piezo1 channel complexes and PC2 e.g. via cytoskeletal elements may indeed provide one possible explanation. Notably, the wild-type PC2, which is mostly retained in the endoplasmic reticulum (ER), is neither targeted to the plasma membrane by co-expressed Piezo1 (that is also being synthesized at the ER) nor is Piezo1 co-retained in the ER in cells that overexpress wt-PC2. This lack of co-targeting or co-retention actually precludes a direct interaction between wtPC2 and Piezo1. Please discuss.

Only the truncated PC2-740X construct strongly interacts with and functionally inhibits Piezo1 and its conductivity. These findings may either limit the physiological relevance of the interaction or they may hint to an important role of a dysregulated PC2:Piezo1 interaction in pathophysiological processes leading to polycystic (kidney) disease. The latter possibility would only hold true if a strengthened and functionally relevant interaction was a more general phenomenon of various disease-causing PC2 mutations. Otherwise, the finding of PC2-740X:Piezo1 interaction may be a specific effect of this protein, but unrelated to physiological or pathophysiological functions of either protein.

Since a strongly overlapping group of authors has recently published a somewhat similar looking inhibition of potassium-selective stretch-activated K<sup>+</sup>-selective cation channels by the PC2-740X construct (Peyronnet et al. 2012 Cell Rep. 1:241), I feel concerned about the selectivity of the observed effects, which are now re-appearing with a molecularly unrelated second interaction partner. Why do TREK-2 K2P currents not re-appear e.g. in experiments such as shown in Fig. 1C? In the 2012 manuscript, SAC currents reversed at -80 mV, whereas now they reverse at 0 mV although the method appears unchanged. Please comment.

If PC2-740X can interact with both channel entities, is there any preference of interaction and does one exclude the other? Does functional inhibition of Piezo1 rely on modulation of TREK-2 K2P currents or vice versa?

1st Revision - authors' response

19 September 2013

#### Referee #1

The authors show that the Piezo1 channel is inhibited by wild type PC2 or its truncation mutant, PC2-740X. They also show that Piezo1 and PC2 co-immunoprecipitate in transiently transfected cells. The same group has shown before that PC2 or PC2-740X suppressed currents mediated by the native stretch-activated channel (SAC), through an interaction involving Filamin A, in arterial myocytes. At that time the identity of the SAC was unknown. It was subsequently discovered that Piezo1 mediates SAC. The authors now confirm that PC2 inhibits Piezo1 in a different cell type. Manuscript is clear and well written. However, it lacks novelty and fails to provide mechanistic information of how binding of PC2 to Piezo1 leads to inhibition of Piezo1-mediated currents.

**These findings are novel and significant for a better understanding of renal mechanotransduction. Novelty of the work: 1) Renal SACs lack inactivation and show slow deactivation, unlike exogenous Piezo1; 2) SACs in renal tubular cells are Piezo1-dependent; 3) PC2 and pathogenic mutants inhibit exogenous Piezo1, as well as native**

renal SACs; 4) Piezo1 and PC2 are present in a molecular complex; 5) The N terminal region of PC2 is critically required for co-immunoprecipitation with and for regulation of Piezo1.

Another major limitation is lack of evidence that the two endogenous proteins interact under physiological conditions.

There is no antibody available to detect native Piezo1. None of the commercially antibodies have been shown to be specific.

Finally, gain-of-function mutations in Piezo1 lead to xerocytosis, a hematologic disease, but not a phenotype even remotely associated with phenotypes associated with the loss of PC2, that would be expected to mirror gain-of-function mutations of Piezo1, if authors are correct.

Gain of function mutations may only affect a specific cell type. For instance, although TRPC6 is widely expressed, gain of function mutations cause focal segmental glomerulosclerosis, but do not affect other organs {Reiser et al., 2005; Winn et al., 2005}.

The observation that the truncation mutant PC2-

740X, which represents a mutation seen in Autosomal Dominant Polycystic Kidney Disease patients inhibits Piezo1 as effectively as wild type PC2, rules out the possibility that this interaction is relevant to Polycystic Kidney Disease.

PC-742X produces a stronger inhibition on SACs, as compared to PC2 WT. As recognized by reviewer 3, this finding may illustrate the possible role of a dysregulated PC2/Piezo1 functional interaction in some aspects of PKD (as now stated).

Therefore the paper falls short in 1) demonstrating physiological significance of the purported interaction, (this mechanism may be involved in some aspects of PKD) 2) providing mechanistic leads of the inhibition (we now show the evidence for a molecular complex involving Piezo1 and PC2), and 3) providing a sufficient level of novelty beyond of what is already known (see above comments about novelty). The work presented is at a very preliminary stage. We believe that we provide an in depth and very careful characterization of the role of Piezo1 in renal cells and its regulation by PC2. Other major issues are noted:

1) SAC current was recorded from the basolateral surface of proximal convoluted kidney tubules. No currents were recorded from the apical surface. Therefore direct evidence that Piezo1-mediated currents induced by intraluminal pressure is lacking. Piezo1-mediated currents in the apical surface should be shown. There are only very few reports in which the authors have been able to perform single channel recordings at the apical side of isolated renal tubular cells. Those experiments were performed mainly on rat or rabbit isolated tubules. We tried very hard to perform such recordings on isolated tubules from mouse kidney, but failed to obtain quality recordings. The apical surface is covered with the brush border which probably prevents gigaseal formation. Moreover, these tubules are typically less than 50 micrometer in diameter and it is extremely difficult to microdissect and be sure that you are really patching the apical side. When a renal tubule is under high intraluminal pressure, both apical and basolateral membranes are stretched {Jensen et al., 2007}.

2) Expression levels of Pkd1, Pkd2 or TRP channels upon Piezo1 knockdown were determined by qPCR (Fig. Supp. 1). However, this analysis has to be determined at the protein level. Mechanisms of compensation are not exclusive to gene transcription. Furthermore, protein-protein interactions can stabilize the proteins mediating the interaction. Therefore, knockdown of one protein can cause downregulation of the other without affecting mRNA levels. Also, it is important to determine the subcellular localization of endogenous PC2 in cells depleted of

Piezo1. Not all antibodies are available to study the expression of the various TRPs at the protein level. However, we now show that, at least PC2 protein expression or its subcellular localization is not altered upon Piezo1 knock-down (Fig. Supp 1).

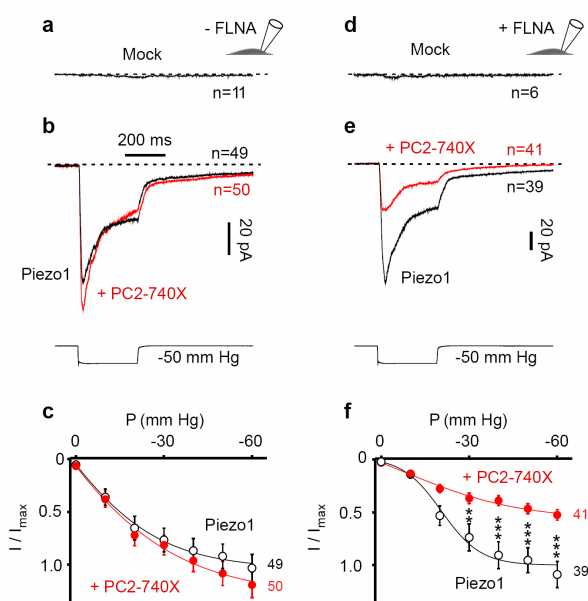
3) It appears that Myc-PC2-740X expression is increased when it is co-expressed with Piezo1 and also is expressed at higher level compared to PC2-WT (Fig. 5a and b, lanes 3 and 6, input panels). How can the authors rule out the trivial possibility that PC2-740X is expressed at higher levels than PC2-WT, when co-transfected with Piezo1, and thus the inhibition is more effective?

We agree that PC2-740X expression is consistently higher than that of PC2 in whatever is the cell line used (for example see {Peyronnet et al., 2012}). This is now clearly stated in the manuscript. However, enhanced plasma membrane localization {Chen et al., 2001; Peyronnet et al., 2012} might also contribute to the stronger inhibition of Piezo1 observed with PC2-740X (Fig. 6). Moreover, lack of interaction with PC1 (due to the deletion of the interacting coiled-coil domain) may also be at play {Sharif Naeini et al., 2009; Tsiokas et al., 1997}, as now discussed.

4) The domains and ideally, the residues in PC2 and Piezo1 mediating the interaction should be identified and mutant constructs lacking binding should be used as negative controls for functional studies. We have now demonstrated that the N terminal domain of PC2 is required for co-immunoprecipitation with Piezo1 (Fig. 5a). Importantly, the N terminal deleted PC2 mutant fails to affect Piezo1 SAC activity (Fig. 5b). This is imperative to rule out non-specific effects. We have now provided in Fig. 5a additional negative controls (Kv2.1 and Kv9.3). Is the binding direct or indirect? We demonstrate that Piezo1 and PC2 are present in a molecular complex using co-IP experiments. At this stage, we cannot answer whether this interaction is direct or indirect. Does it involve Filamin A, as was shown earlier (Cell 2009, 139(3):587-96; PLoS One. 2012;7(7):e40448)?

FLNA is required for Piezo1 inhibition by PC2-740X in M2 cells (see our preliminary data below). In future experiments, we will determine whether FLNA is required for the interaction of PC2 and Piezo1. However, we feel that the role of FLNA deserves to be addressed in a separate study and we have decided not to include those data in the revision.

Does Piezo1 interact with Filamin A? This will be addressed in a future study. TRPC1 was used as a negative control in functional assays (Fig. 4d). Does TRPC1 bind to Piezo1 in transiently transfected cells?



Legend: a) Native SAC activity in mock transfected M2 cells (-FLNA). b) Piezo1 was expressed together with PC2-740X in M2 cells. c) Co-expression with PC2-740X failed to significantly affect Piezo1 SAC activity (measured at -80 mV). d) Native SAC activity in A7 cells (+FLNA). e) Co-expression of PC2-740X with Piezo1 in A7 cells. f) In the presence of FLNA, PC2-740X significantly inhibited Piezo1 SAC activity.

Since TRPC1 is known to interact with PC2 {Bai et al., 2008}, we chose not to use TRPC1 as a negative control. Instead, we used Kv2.1 (same topology



and comparable size with PC2) which has previously been shown not to be influenced by PC-740X (Sharif Naeini et al., 2009). This needs to be shown side-by-side (same blot) with PC2 and PC2-740X using a TRPC1 construct tagged with the same tag as PC2 and PC2-740X. We have used Kv2.1 and Kv9.3 as negative controls for the co-immunoprecipitation with Piezo1 (Fig. 5a).

5) Fig. 4b. Exogenous Piezo1 increased SACs by 3-fold, which is very strange, as I'd expect that much more protein should be made upon transfection. So, it is confusing as to why only such a small increase in currents was seen. Previous work has shown that Piezo1 forms a homomultimeric channel (Nature 2012, 483(7388):176-81) and therefore, interacting proteins should not be a limiting factor. Can the author explain?

Whatever the cell lines used (PCT, COS or M2), Piezo1 current amplitude never exceeded (at maximum) 200 pA at -80 mV for a pulse pressure of -60 mm Hg (mean pipette resistance: 1.4 Mohm), either using a Piezo1 ires EGFP construct, or a Piezo1-EGFP fusion. In comparison, as a positive control, we routinely express TREK-1 and obtain stretch-activated potassium currents as large as 50 nA per patch. Since Piezo1 is a very large protein (the tetramer is predicted to be 1.2 Md (Coste et al., 2012)), we think that it is a key limitation for its heterologous expression. However, the amplitude and kinetics of the exogenous currents were significantly higher and different from the native currents. Thus, we feel confident that the exogenous currents shown in the present study are due to Piezo1 expression (as already shown by others).

6) Interaction of endogenous Piezo1 and PC2 should be shown. The authors claim that there is no good antibody to Piezo1. This is unfortunate, because such a reagent is absolutely necessary to support the main point of the paper that is the claim that the two proteins interact (see also below in point 7).

Those experiments will be performed in the future when reagents will become available and data will be presented in a separate study.

7) There are no appropriate controls in the co-immunoprecipitation experiments in Fig. 5. EGFP is not an appropriate control for a protein-protein interaction of proteins bearing transmembrane segments. Again, a detailed and thorough structure-function analysis is needed to substantiate the claim that these proteins interact. Fig. 5b, lane 6 (IP Probe HA) showing the interaction of Piezo1 and PC2-740X is not convincing, given the interaction is done in transfected cells, where high levels of exogenous proteins are achieved. Fig. 5b lacks appropriate control. Since there is no transfected HA-Piezo1 in lanes 1-3, of course nothing would be detected in pulldowns of PC2 or PC2-740X (panel labeled: IP Probe HA, lanes 1-3).

See above comments. We have now provided two additional negative controls with Kv2.1 and Kv9.3 (Fig. 5a). Moreover, we show that the N terminal deleted PC2 mutant fails to immunoprecipitate with Piezo1.

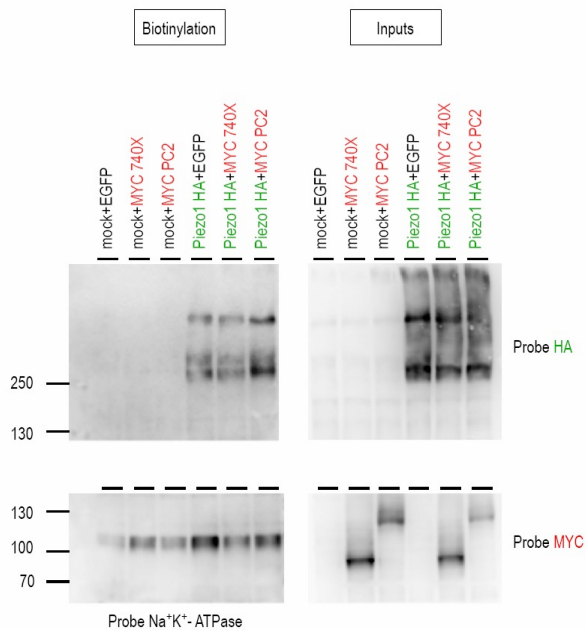
8) I was very surprised to see nice staining of Piezo1 in the plasma membrane, which has not been seen before (J Cell Sci. 2010 Jan 1;123(Pt 1):51-61). Can the authors comment on this discrepancy?

We have expressed a Piezo1-EGFP fusion (active as demonstrated electrophysiologically; Fig. 5b) construct in PCT cells. Fluorescence was observed by confocal microscopy in living cells. Using this strategy, membrane staining of Piezo1 is particularly evident, as illustrated



in this report (and of course plasma membrane expression was absent when Piezo1-EGFP was omitted or when EGFP was expressed alone). When using tagged Piezo1 constructs expressed in transfected cells which have been subsequently fixed by PFA (as shown by others), cellular sublocalization is much more difficult to see accurately because cellular structures are poorly preserved due to fixation.

Using biotinylation experiments (see figure on the left), we confirmed that PC2 or PC2-740X expression does not decrease the amount of Piezo1-HA at the plasma membrane. If necessary, we will illustrate those data in an additional figure.



Legend: Biotinylation of PCT cells expressing Piezo1-HA together with either MYC-PC2 or MYC-PC2-740X. Biontilyated proteins are shown on the left panels (revealed by an anti HA antibody or an anti Na<sup>+</sup>K<sup>+</sup> ATPase antibody for a positive control).

The biotinylation blots were negative for calnexin indicating that the samples were not contaminated with biotinylated proteins in the ER (not shown).

There is no obvious expression of PC2 in the plasma membrane, which is not surprising (Fig. 6). Most of PC2 WT is seen in the ER {Koulen et al., 2002}. However, one cannot rule out that a small fraction of PC2 may

still be present at the plasma membrane (as demonstrated by others and confirmed by our own group {Bai et al., 2008; Ma et al., 2005; Peyronnet et al., 2012}), but difficult to visualize by confocal microscopy. In fact, very little if any co-localization of Piezo1 and PC2 is detected in transfected cells. How do the authors think that PC2-WT modulates Piezo1? Another possibility, as now discussed, is that ER located PC2 may interact with plasma membrane Piezo1 when both membranes are in close contact. Such interaction may be direct or indirect and could involve an intermediate component (for instance filamin A). Is it that PC2 is localized in the endoplasmic reticulum (ER) and Piezo1 in the plasma membrane? If so, PC2-WT should only affect a tiny fraction of Piezo1 at the plasma membrane, perhaps where the ER and plasma membranes come in close proximity. This is a possibility, explaining why PC2 WT has a reduced effect, as compared to PC2-740X which is more abundant at the plasma membrane. I cannot see how PC2, which is localized almost entirely in the ER can suppress Piezo1 currents originating at the plasma membrane to more than 70-90%, without affecting the amount of Piezo1 in the plasma membrane. Our measurements provided in Supp 6, clearly show that Piezo1 localization at the plasma membrane is unaltered in the presence of PC2-740X. We confirmed those data with biotinylation experiments (see above). We believe that we have done a very careful quantitative analysis of the fluorescence data and we are very confident about those results.

Minor points (All those have been corrected).

1. Page 2, line 7, 'among them stretch-activated ion channels' should be changed to 'among them are stretch-activated ion channels'.
2. Page 3, Para 4, line 5 from bottom. There is no Fig. 2c.
3. Page 7, Para 2, line 11, '50  $\mu\text{m}$ ' should be changed to '50  $\mu\text{M}$ '.
4. Page 7, Para 2, line 15, '50  $\mu\text{m}$ ' should be changed to '50  $\mu\text{M}$ '.
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14. Page 12, line 4, volume and pages should be added.

#### Referee #2

This is a concise, novel finding demonstrating the importance of Piezo1 in mechanotransduction of renal tubular epithelial cells. Mechanotransduction is not understood well at the molecular level. One important family of pressure sensors are ion channels. Piezo family of mechanically activated ion channels has recently described; however, if Piezos are required for mechanosensitivity of various tissues and biological processes is still completely unknown. This manuscript clearly shows:

- (1) stretch-activated ion channels (SAC) present in renal tubular epithelial cells are dependent on Piezo1: this is the first demonstration that Piezo1 accounts for SAC activity in a relevant primary cell type in any tissue
- (2) Piezo1 can be slowly adapting. This is a surprising finding as Piezos were originally described as rapidly-adapting (mechanosensitive ion channels are often defined according to their inactivation properties). This opens up the possibility that adaptation can be modulated, and that this property is not necessarily inherent to the channel itself.
- (3) These authors had shown previously that shear-stress sensing PC1/2 actually inhibit SAC activity (different form of mechanotransduction). Now, the authors show that the target that they had described is Piezo1, as they can reproduce these results in heterologous system.

The only criticism that I can think of is the fact there is no detailed mechanistic investigation of how the inhibition of PC2 on Piezo1 works. **Using co-immunoprecipitation experiments, we now provide evidence that Piezo1 and PC2 are present in a molecular complex. We demonstrate that the N terminal domain of PC2 is critically required for the association (direct or indirect) with Piezo1 and inhibition of its SAC activity (Fig. 5).**

**We thank you for your positive comments.**

However, "EMBO reports" is about describing a focused and exciting new finding, and I believe this is a good match for this format.

Referee #3

In their manuscript („Piezo1-dependent stretch-activated channels are inhibited by Polycystin-2 in renal tubular epithelial cells"), Peyronnet et al detected Piezo1 as a key component of a non-selective cation conductance in primary cultures of tubular epithelial cells, and an inhibitory impact of polycystin PC2 on ionic currents through mechanically gated Piezo1 channels. The authors further highlight that a disease-related PC2 mutant (PC2-740X) exerts an even stronger inhibitory effect and physically interacts with Piezo1 more efficiently than wild-type PC2. The manuscript and the figures are well presented.

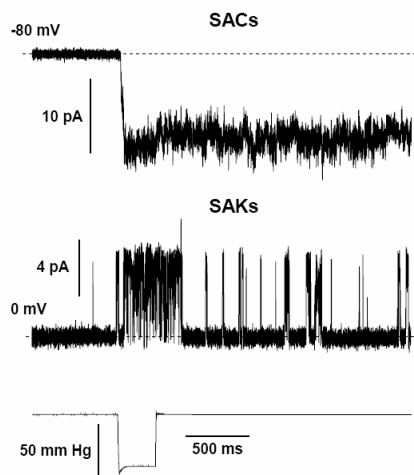
Major points:

The authors pay caution by noting that the interaction between Piezo1 and PC2 may be indirect. A bridging between the reticular higher-order-network of Piezo1 channel complexes and PC2 e.g. via cytoskeletal elements may indeed provide one possible explanation. Notably, the wild-type PC2, which is mostly retained in the endoplasmic reticulum (ER), is neither targeted to the plasma membrane by co-expressed Piezo1 (that is also being synthesized at the ER) nor is Piezo1 co-retained in the ER in cells that overexpress wt-PC2. This lack of co-targeting or co-retention actually precludes a direct interaction between wtPC2 and Piezo1. Please discuss.

**We now discuss the possibility that a small fraction of PC2 at the plasma membrane may inhibit Piezo1. Another possible mechanism may involve PC2 at the ER membrane which would influence Piezo1 at the cell surface when both membranes become in close contact. Such functional interaction may possibly involve an intermediate component, as now discussed.**

Only the truncated PC2-740X construct strongly interacts with and functionally inhibits Piezo1 and its conductivity. These findings may either limit the physiological relevance of the interaction or they may hint to an important role of a dysregulated PC2:Piezo1 interaction in pathophysiological processes leading to polycystic (kidney) disease. The latter possibility would only hold true if a strengthened and functionally relevant interaction was a more general phenomenon of various disease-causing PC2 mutations. Otherwise, the finding of PC2-740X:Piezo1 interaction may be a specific effect of this protein, but unrelated to physiological or pathophysiological functions of either protein. **We have now demonstrated that another pathogenic point mutant (PC2D509V) similarly inhibits SACs in PCT cells (page 3, last lines).**

Since a strongly overlapping group of authors has recently published a somewhat similar looking inhibition of potassium-selective stretch-activated K<sup>+</sup>-selective cation channels by the PC2-740X construct (Peyronnet et al. 2012 Cell Rep. 1:241), I feel concerned about the selectivity of the observed effects, which are now re-appearing with a molecularly unrelated second interaction partner. Why do TREK-2 K<sub>2</sub>P currents not re-appear e.g. in experiments such as shown in Fig. 1C? **In the experiment illustrated in Fig. 1C, the holding potential was -80 mV at which potassium channel currents reverse direction.** In the 2012 manuscript, SAC currents reversed at -80 mV, whereas now they reverse at 0 mV although the method appears unchanged. **We are recording on one hand, K<sup>+</sup>-selective SAK currents (i.e. TREK-2) reversing at -80 mV (see figure below), and on the other hand cationic non-selective SAC currents (i.e. Piezo1) reversing at 0 mV.**



Legend: cell-attached patch recording of SACs and SAK in a same patch from a cultured PCT cell. The holding potential was held at -80 mV to record non-selective SACs (lack of inactivation and ultra slow deactivation are evident) and subsequently at 0 mV to visualize K<sup>+</sup>-selective SAKs (presumably TREK-2 {Peyronnet et al., 2012}).

Density of SAKs is lower than SACs (although conductance is higher). In about 50 % of the active patches we recorded both channel types at the same time (difference in single channel conductance, kinetics and reversal potential), as illustrated. We have now indicated that both channels can be found in the same patch. For

clarity and to avoid confusion, in the present paper we have only illustrated patches in Fig. 1a and 1b, where SAKs are absent at 0 mV.

Pease comment.

If PC2-740X can interact with both channel entities, is there any preference of interaction and does one exclude the other?

Using co-IP experiments we could show in preliminary experiments that TREK-1 (also inhibited by PC2 {Peyronnet et al., 2012}), unlike TASK-2, is present in a molecular complex together with PC2. However, since the present manuscript only concerns Piezo1, and for clarity we chose not to include those data. Does functional inhibition of Piezo1 rely on modulation of TREK-2 K<sub>2</sub>P currents or vice versa? The idea that stretch activation of TREKs may interfere with the activity of Piezo1 or vice-versa is an interesting and provocative idea. Whether channels are found in the same microdomains is likely, as we can detect their simultaneous activity in the same patches (see attached recording). We detected SAC activity in TREK-2 PCT KO cells. Moreover, we recorded SAKs in PCT cells transfected with siRNA Piezo1. Thus, at least, it seems that the mechanosensitivity of these channels does not require the presence of the other type of mechano-gated channel.

We have evidence that the actin cross-linking protein filamin A is critically required for the inhibition of Piezo1 by PC2-740X in M2 cells (see comments to reviewer 1). Similar findings were obtained for the inhibition of the K<sub>2</sub>P channel TREK-2 by PC2-740X {Peyronnet et al., 2012}. We will address this specific issue in a future study.

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2nd Editorial Decision

30 September 2013

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. I am happy to report that both reviewers who were asked to assess the revised version now support publication of your study in EMBO reports.

I would, however, ask to you pay attention to some formal points that would need to be corrected before we can proceed with the official acceptance of your manuscript:

1. Your study currently has 6 main figures. Per journal policy, we can only accept 5 figures in the main manuscript and I would therefore kindly ask you to identify one figure that can be moved to the supplementary section.
2. Similarly, with almost 34,000 characters, the text exceeds our limits and I would kindly ask you to shorten it so that it does not exceed roughly 28,000 characters (including spaces and references). Having said this, I also noticed that the current materials and methods section is very short and I would kindly ask you to expand this section so that the main experimental procedures are displayed in the main body of the text. Shortening of the text may be achieved by combining the results and discussion section, which are, at the moment, separate.
3. Throughout the manuscript, please also clearly state which statistical test have been used and how many independent times each experiment has been repeated (please not that 'independent' refers to biological, not technical replicates).

Once these issues have been addressed, please submit the final version of your manuscript through our website again.

I look forward to seeing the final version as soon as possible.

## REFeree REPORTS:

Referee #2:

The authors have addressed all my concerns.

Referee #3:

I agree with reviewer #1 that parts of the manuscript make the reader suspicious of the exact mechanism, the relevance for regulation of SAC in native systems and the physiological significance of the proposed mechanism. Nonetheless, the functional impact is shown not only in a heterologous system, but also in renal tubular cells. The mode of interaction (direct or indirect) remains to be demonstrated and may then explain the mechanism of the functional modulation.

A couple of issues that remain include the concern that the stronger expression of the 740X mutant rather than the chances of the protein structure per se may bear responsible for the differences to wild-type PC2. However, if the mechanism holds true, this finding may open new avenues for the understanding of mechanosensation in ciliated cells in physiological and diseased states.

2nd Revision - authors' response

07 October 2013

Please find attached the revised version of our manuscript entitled: “ **Piezo1-dependent stretch-activated channels are inhibited by Polycystin-2 in renal tubular epithelial cells**”.

We have made the following corrections:

- 1) One figure has been moved to the supplementary information (Fig. Supp 9). The total number of figures is now of 5.
- 2) We have shortened the manuscript to 27960 characters, including spaces and references. We have extended the method section in the main text. We have now merged results and discussion.
- 3) We have added one section about statistical analysis in the Supplementary information. We have indicated that (n) means independent biological experiments.

I hope that the manuscript will be now acceptable for publication.

3rd Editorial Decision

07 October 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: [emboreports@embo.org](mailto:emboreports@embo.org)]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.