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A stomatin dimer modulates the activity of acid-sensing ion channels

Janko Brand, Ewan St. J. Smith, David Schwefel, Liudmila Lapatsina, Kate Poole, Damir Omerbašić, Alexey Kozlenkov, Joachim Behlke, Gary R. Lewin and Oliver Daumke

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(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 07 February 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. As you will see, referees 1 and 3 would support publication here after appropriate revision. Referee 2 is more critical and is concerned that the study provides too little deeper insight into how stomatins actually act at the molecular level to regulate ASICs channel activity. On balance, and given the positive vote by the other two referees, we would be happy to consider a revised version of the manuscript that addresses the referees' concerns in an adequate manner and to their satisfaction. It would be good if you could at least speculate on possible models for mode of action of stomatins based on your structural data and the literature in the discussion section.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as

soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In this ms Brand et al. present the core structure of the mouse stomatin domain. They show that the basic building block is a banana-shaped dimer and disruption of the dimerization interface results in loss of ASICs regulation. Moreover, they have identified a hydrophobic pocket that is important for the modulation of the ASIC function. However, closure of this pocket affects neither stomatin dimerization nor physical interaction with the channel. This pocket seems to play a functional and not structural role in this interaction. Finally, they have highlighted two interphases that play an important role in the crystal packing, and showed that they are also important for the function of the protein on ASICs modulation. Although the crystal structure of a stomatin domain protein from another organism (Pycococcus hirokoshii) has already been described, the authors find that in contrast to this homologue, mammalian stomatin forms a banana-shaped dimer and higher- order oligomers. Finding reported here suggest that higher oligomeric structures may be important for the protein function within the membranes. Overall, I believe that this ms is suitable for publication in EMBO J. However, some control experiments are missing and some points need to be addressed before this study is ready to be published.

Specific comments

Some of the key experiments, such as the demonstration that V197 and T182W are required for ASIC3 activity modulation should be repeated in a stomatin-null cellular background for example using MEFs isolated from the existing stomatin knockout mice (Zhu et al, 1999).

Stomatin localization to membranes has been performed simply by looking at the distribution of wt or mutated stomatin fused with GFP. Based on what is presented in the Supplementary material, it is not possible to deduce whether the different fusions are localized on the plasma membrane or not. However, this is of crucial importance and at the very core of the aim of this study that is to link structure with function. Moreover, stomatin belongs to a family characterized by their specific association with detergent resistant, cholesterol-rich membrane fractions (also known as lipid rafts). Therefore, it is necessary to provide convincing biochemical data on the localization of the different constructs. Sucrose gradient fractionation should additionally be performed to determine whether the different mutations impact the localization on lipid rafts, using a lipid-raft specific protein, such as flotillin-1 as a control for identifying lipid raft fractions.

To test whether stomatin might alter ASIC function, the authors performed experiments at pH 4.0 and 6.0 for ASIC3 and pH 5.0 for ASIC2a. It is known that ASICs show different pH sensitivities when analyzed in heterologous systems. The pH of half maximal activation (pH0.5) for ASIC3 is in the range of 5.9-6.0 and for ASIC2 , which is less sensitive, is about 4.4. Therefore, ASIC2a activity should be tested at pH lower than 5 (as in other studies , for example Price et. al, 2004).

Mutation of residues Leu91 and Ile92 to alaline did not affect the ability of mutant stomatin to modulate ASIC2a and ASIC3 currents. Therefore, the authors conclude that " Leu91 and Ile92 in the loop preceding the stomatin domain do not appear to be the physiological targets of the hydrophobic pocket for inhibition of ASIC function". However, the effects of a change to alaline, which is a small non-polar amino acid, are often unremarkable. The authors should also test whether mutation

of Leu91 and Ile92 to tryptophan, the largest of amino acids, inhibits modulation of ASIC activity by stomatin.

Minor points:
Figure 3C: In order to be consistent with fig 3A, the authors could choose representative cells whose peak current density is close to the median peak current density for each condition (ASIC3 alone or ASIC3 + wt stomatin).

In Figure 4G, shouldn't ASIC3 be replaced by ASIC2a in the x axis?

Referee #2

Brand and colleagues present the crystal structure of the stomatin domain together with detailed structure function analyses of relevant dimerization and oligomerizaton interfaces present in the crystal packing. Stomatin domain containing proteins are part of an important small protein family that can modulate the activity of some acid-sensing ion channels (ASIC).

The description and the technical quality of the crystal structures is sound and presented in a balanced way. Based on the structure and the crystal packing the authors designed a number of mutants to prove the importance of dimerization and oligomerization for controlling ASIC3 activity. First they show that the mode of dimerization revealed in the crystal is relevant due to the observation of the same dimerization in vitro and its role in ASIC control. Next they identified a hydrophobic pocket that is important for stomatin function, because a mutant of the pocket failed to modulate proton-gated ASIC3 and 2 currents in whole cell recordings. The last part of the manuscript describes the potential role of the stomatin oligomerization into tubular structures. The relevant interfaces were again tested with regard to the effect of mutations on the modulation of proton-gated ASIC3 and 2, indicating that interfaces of the tubular structures are important for function.

Overall the manuscript is well written and describes a balanced view of the structural analyses. However, no new mechanistic insight how stomatins might modulate channel function are provided. The manuscript lacks data on stomatin dimerization and possible oligomerization in vivo. This is important because others have described stomatin trimerization. It is thus possible that the interfaces observed in the structures are important for function, however independent of some sort of oligomerization. The same is true for the tube-like structure observed in the crystal. It is difficult to image how such a structure is linked to the channels in the membrane.

Page 13 Do the EM images of cyanobacterial stomatin and yeast prohibitin rings have the same dimensions as mstomatin?

Where are the N-terminal and C-terminal domains positioned in the ring/tube structure?

The authors compare stomatin to BAR domains, which is a bit farfetched from all we know about stomatins at the moment. Does the stomatin core structure presented here contribute to membrane interaction in vitro and in vivo? If the banana-shaped dimer is functionally related to BAR domains one would expect a role of the structure in membrane interaction/bending/stabilization...

The IF data in Figure S2 is poorly presented; it is difficult to see the points described in the results section.

Referee #3

This manuscript describes the core structure of the mouse stomatin domain and analyze through a structure-function approach the key regions of the stomatin molecule involved in modulation of acid-sensing ion channels (ASICs). The structure nicely completes the crystal structure of a homologue from Pyrococcus hirokoshii that was previously determined. Stomatins are interesting molecules that have been described to have important roles in mechanotransduction and to modulate

ASICs or related channels in both nematodes and mice. The link between channel modulation and mechanotransduction is well established in C. elegans for the MEC-4/MEC-10 mechanosensitive channel, but remains less clear for ASIC channels in mammals. There is no doubt that a better knowledge of the structure of stomatin and of its interaction with ASIC channels will be important to better understand this link, and more generally to better understand how stomatin and related proteins operate at the plasma membrane.
The manuscript is clear and well written, and the findings are novel and interesting. However, a few points need to be improved or clarified.

1- The effects of stomatin on ASIC channels have been used as an assay of stomatin function. However, the physiological relevance of these effects is not clear in mammals. The manuscript could definitely be reinforced by showing in addition the effect of WT and mutant stomatin (e.g., V197P, T182W and R97D) on mechanosensitive currents in transfected neurons isolated from stomatin mutant mice. These neurons may not show a complete loss of sensory neuron mechanosensitivity, which could make the experiments difficult to analyze. However, experiments could alternatively be done with the related protein SLP3/Stoml3 that similarly interacts with ASIC channels and displays a complete loss of mechanosensitivity in a third of the sensory neurons isolated from knockout mice (Wetzel et al. 2007).

2- The C-terminal domain of stomatin is lacking in the protein used to get the crystal, and it is therefore not clear how this domain could interfere with dimerization. The use of stomatin 85-255 brings some arguments against an artifact due to the shorter construct, but the last ~30 aa are still missing in this extended form.

1st Revision - authors' response 07 June 2012

We would like to thank all three referees for their constructive feedback which has helped to sharpen our manuscript. We experimentally approached a number of your suggestions and clarified others in the revised manuscript. We hope that with the introduced changes, the manuscript can now be accepted for publication in the EMBO Journal. A detailed point-by-point response is shown below.

Referee #1

In this ms Brand et al. present the core structure of the mouse stomatin domain. They show that the basic building block is a banana-shaped dimer and disruption of the dimerization interface results in loss of ASICs regulation. Moreover, they have identified a hydrophobic pocket that is important for the modulation of the ASIC function. However, closure of this pocket affects neither stomatin dimerization nor physical interaction with the channel. This pocket seems to play a functional and not structural role in this interaction. Finally, they have highlighted two interphases that play an important role in the crystal packing, and showed that they are also important for the function of the protein on ASICs modulation. Although the crystal structure of a stomatin domain protein from another organism (Pyrococcus hirokoshii) has already been described, the authors find that in contrast to this homologue, mammalian stomatin forms a banana-shaped dimer and higher-order oligomers.

Finding reported here suggest that higher oligomeric structures may be important for the protein function within the membranes. Overall, I believe that this ms is suitable for publication in EMBO J. However, some control experiments are missing and some points need to be addressed before this study is ready to be published.

Specific comments

Some of the key experiments, such as the demonstration that V197 and T182W are required for ASIC3 activity modulation should be repeated in a stomatin-null cellular background for example using MEFs isolated from the existing stomatin knockout mice (Zhu et al, 1999).

To address this point, we prepared primary mouse adult fibroblast (MAF) cultures from stomatin knock-out and wild type littermates. However, a protocol to efficiently transfect MAFs was hard to establish and it was very difficult to reliably measure currents in transfected cells using patch clamp experiments.
We obtained most consistent results when transfecting high levels of ASIC3 DNA into MAFs. Thus, fibroblasts transfected with what might normally be considered adequate concentrations of plasmid DNA, most often did not show any ASIC-mediated currents, even though they expressed GFP from a second expression plasmid included in the transfection. We could prove in principle that MAFs can be transfected with ASIC3 and that ASIC3-like currents can be recorded from MAFs. Furthermore, ASIC3 currents from stomatin knockout MAFs were larger on average but current amplitudes were more variable than those measured in wild-type cells, but this difference was not statistically significant. The difficulty in getting ASIC3 channels expressed and the high DNA concentrations necessary makes this experiment unfeasible to complete within the revision constraints of this study. The data we have obtained and its limitations are further handled in the Supplementary material (Suppl. Fig. 3).

Stomatin localization to membranes has been performed simply by looking at the distribution of wt or mutated stomatin fused with GFP. Based on what is presented in the Supplementary material, it is not possible to deduce whether the different fusions are localized on the plasma membrane or not. However, this is of crucial importance and at the very core of the aim of this study that is to link structure with function. Moreover, stomatin belongs to a family characterized by their specific association with detergent resistant, cholesterol-rich membrane fractions (also known as lipid rafts). Therefore, it is necessary to provide convincing biochemical data on the localization of the different constructs.

Since the majority of stomatin and ASICs is present in intracellular structures, plasma membrane localization is often covered by the bulk fluorescence in the cell. We repeated these experiments and provide new images in Suppl. Fig. 4 where plasma membrane localization of the stomatin mutants can be more clearly appreciated. We also conducted a bimolecular fluorescence complementation assay which shows more directly that stomatin and ASIC3 are in close proximity to each other at the plasma membrane (Suppl. Fig. 5).

Sucrose gradient fractionation should additionally be performed to determine whether the different mutations impact the localization on lipid rafts, using a lipid-raft specific protein, such as flotillin-1 as a control for identifying lipid raft fractions.

The absence and presence of lipid rafts in the membranes and the methods to explore lipid raft association are discussed highly controversially amongst experts in this field. We agree that this experiment should be performed but the experimental work involved with proper controls makes this undertaking go well beyond what is possible to do in a reasonable revision time. We also have no special expertise in this area and therefore feel it would be better for others to tackle this issue once our paper has been published. The structural data shown here provides a solid basis for such a study.

To test whether stomatin might alter ASIC function, the authors performed experiments at pH 4.0 and 6.0 for ASIC3 and pH 5.0 for ASIC2a. It is known that ASICs show different pH sensitivities when analyzed in heterologous systems. The pH of half maximal activation (pH0.5) for ASIC3 is in the range of 5.9-6.0 and for ASIC2a, which is less sensitive, is about 4.4. Therefore, ASIC2a activity should be tested at pH lower than 5 (as in other studies, for example Price et. al, 2004).

We included these experiments in Suppl. Fig. 6. For most stomatin mutants, data at pH 4 were not statistically significant compared to pH 5. However, the T182W mutant showed a highly significant effect on the inactivation times of ASIC2a currents indicating that mutations in stomatin also interfere at pH 4 with ASIC2a currents.

Mutations of residues Leu91 and Ile92 to alanine did not affect the ability of mutant stomatin to modulate ASIC2a and ASIC3 currents. Therefore, the authors conclude that "Leu91 and Ile92 in the

loop preceding the stomatin domain do not appear to be the physiological targets of the hydrophobic pocket for inhibition of ASIC function". However, the effects of a change to alaline, which is a small non-polar amino acid, are often unremarkable. The authors should also test whether mutation of Leu91 and Ile92 to tryptophan, the largest of amino acids, inhibits modulation of ASIC activity by stomatin.

The interaction of Leu91 and Ile92 with the hydrophobic pocket is exclusively mediated by the hydrophobic side chains and not by other parts of the protein. This is mentioned now in the text. By removing these side chains, as in our double alanine mutant, we therefore completely remove any prospective interaction sites with the hydrophobic pocket which is exactly the scope of these mutations. It is unpredictable whether two tryptophans at this position would or would not bind into the pocket; furthermore, they might have other effects on stomatin function, e.g. influence the membrane interaction since these residues are in close proximity to the membrane anchoring loop. Because of this, we decided not to carry out the suggested experiments.

However, to obtain more functional insights into the interaction of stomatin with ASIC3, we analyzed the sequence in the C-terminus of ASIC3 and found a similar hydrophobic motif consisting of two aliphatic amino acids. Interestingly, mutations of these two residues in ASIC3 did not disturb the interaction with stomatin but led to massively increased proton gated ion currents which were only weakly suppressed by stomatin co-expression (see new Fig. 5). Thus, our data point to a mechanism where stomatin interacts with the C-terminus of ASCI3 to modify ion currents. We can speculate that stomatin may in part suppress ASIC3 currents by binding the two aliphatic amino acids in the C-terminus of the ASIC3 channel.

Minor points:

Figure 3C: In order to be consistent with fig 3A, the authors could choose representative cells whose peak current density is close to the median peak current density for each condition (ASIC3 alone or ASIC3 + wt stomatin).

This is a misunderstanding; Figure 3C shows ASIC2a currents, not ASIC3 currents. The two traces for ASIC2a in the absence and presence of stomatin are representative examples for these measurements.

In Figure 4G, shouldn't ASIC3 be replaced by ASIC2a in the x axis?

Thanks a lot, this has been changed.

Referee #2

Brand and colleagues present the crystal structure of the stomatin domain together with detailed structure function analyses of relevant dimerization and oligomerizaton interfaces present in the crystal packing. Stomatin domain containing proteins are part of an important small protein family that can modulate the activity of some acid-sensing ion channels (ASIC).

The description and the technical quality of the crystal structures is sound and presented in a balanced way. Based on the structure and the crystal packing the authors designed a number of mutants to prove the importance of dimerization and oligomerization for controlling ASIC3 activity. First they show that the mode of dimerization revealed in the crystal is relevant due to the observation of the same dimerization in vitro and its role in ASIC control. Next they identified a hydrophobic pocket that is important for stomatin function, because a mutant of the pocket failed to modulate proton-gated ASIC3 and 2 currents in whole cell recordings. The last part of the manuscript describes the potential role of the stomatin oligomerization into tubular structures. The relevant interfaces were again tested with regard to the effect of mutations on the modulation of proton-gated ASIC3 and 2, indicating that interfaces of the tubular structures are important for function.

Overall the manuscript is well written and describes a balanced view of the structural analyses.

Thank you for the positive comments.

However, no new mechanistic insight how stomatins might modulate channel function are provided.

We now show data where we identify a hydrophobic motif in the C-terminus of ASIC3. Mutations in this motif do not affect the physical interaction with stomatin but induce massively increased ASIC3 currents which are only partially inhibited by stomatin co-expression (see new Fig. 5). Based on these data, we suggest a mechanism where stomatin controls ASIC function by association with the C-terminus of ASIC3.

The manuscript lacks data on stomatin dimerization and possible oligomerization in vivo.

In Suppl. Fig. 2, we now provide bimolecular fluorescence-based data where we show association of full-length stomatin in vivo. We also show that the stomatin V197P mutant assembles with reduced efficacy in vivo.

This is important because others have described stomatin trimerization. It is thus possible that the interfaces observed in the structures are important for function, however independent of some sort of oligomerization. The same is true for the tube-like structure observed in the crystal. It is difficult to image how such a structure is linked to the channels in the membrane.

Oligomerization of full-length stomatin has been described previously, and it was also shown that a mutation at position W184A in interface-1 interferes with oligomerization of full-length human stomatin (Umlauf et al. 2006). Thus, we are confident that a stomatin oligomer covering at least interface-1 exists in vivo.

In a recently accepted manuscript which is attached to this submission, we provide further evidence that the membrane environment is crucial for stomatin's function in modulating ion currents (see Lapatsina et al., 2012). We envisage a mechanism where a tubular stomatin oligomer assembles at the plasma membrane to create a microenvironment controlling ion channel function. This has now been added to the discussion.

Page 13 Do the EM images of cyanobacterial stomatin and yeast prohibitin rings have the same dimensions as mstomatin?

The outer diameter for cyanobacterial stomatin is 16 nm and of prohibitin 20 nm. The mouse stomatin oligomer in the crystal has an outer diameter of 8 nm which is indicated in Fig. 6 and Suppl. Fig. 7. However, we do not know the diameter of the stomatin oligomer *in vivo*. Slight adjustments in the oligomerization interfaces can potentially lead to oligomers of different diameters. This is now mentioned in the discussion.

Where are the N-terminal and C-terminal domains positioned in the ring/tube structure?

We have added a new figure of the oligomer in Supp. Fig. 3C where the positions of the N- and C-termini relative to the tubular ring are indicated. Both N- and C-termini localize to a gap in the oligomer, and an inspection of our 3D pdf figure further helps to clarify these positions within the oligomer. It is not possible to predict from our structure whether N- and C-termini are extending to the interior or exterior of the tubule. This is now mentioned in the figure legend of Suppl. Fig 7.

The authors compare stomatin to BAR domains, which is a bit farfetched from all we know about stomatins at the moment. Does the stomatin core structure presented here contribute to membrane interaction in vitro and in vivo? If the banana-shaped dimer is functionally related to BAR domains one would expect a role of the structure in membrane interaction/bending/stabilization.

As pointed out in our discussion, stomatins are stably incorporated into the membrane via an amino-terminal hydrophobic hairpin loop. In the absence of this hairpin, the stomatin domain does not bind with high affinity to membranes, as assayed by liposome binding assays (see discussion). We were not able to prepare a stomatin construct containing the hydrophobic loop so we could not carry out

liposome binding and deformation studies to determine whether the stomatin domain directly interacts with membranes. However, almost all SPFH domain containing proteins are found at membranes and some of them were shown to remodel membranes in vivo (e.g. flotillin). A tubular arrangement, as seen in our crystal structure, is consistent with a role of the SPFH domain in membrane remodeling, and we at least would like to mention this in the discussion. We have recently obtained some evidence that over-expression of a closely related stomatin protein (STOML3) appears to affect plasma membrane dynamics (see Figure 9 in Lapatsina et al 2012). Again the structure/function data shown in this paper will allow a much more focused exploration of this issue in the future.

The IF data in Figure S2 is poorly presented; it is difficult to see the points described in the results section.

We repeated the experiments and provide better images where membrane localization of the various stomatin mutants can be recognized more clearly (Suppl. Fig. 4). We also provide new BiFC data to monitor the ASIC3 stomatin interaction at the plasma membrane (Suppl. Fig. 5). The majority of stomatin and ASIC3, as already mentioned, is found in intracellular regions of the cell.

Referee #3

This manuscript describes the core structure of the mouse stomatin domain and analyze through a structure-function approach the key regions of the stomatin molecule involved in modulation of acid-sensing ion channels (ASICs). The structure nicely completes the crystal structure of a homologue from Pyrococcus hirokoshii that was previously determined. Stomatins are interesting molecules that have been described to have important roles in mechanotransduction and to modulate ASICs or related channels in both nematodes and mice. The link between channel modulation and mechanotransduction is well established in C. elegans for the MEC-4/MEC-10 mechanosensitive channel, but remains less clear for ASIC channels in mammals. There is no doubt that a better knowledge of the structure of stomatin and of its interaction with ASIC channels will be important to better understand this link, and more generally to better understand how stomatin and related proteins operate at the plasma membrane. The manuscript is clear and well written, and the findings are novel and interesting. However, a few points need to be improved or clarified.

Thanks a lot for the positive comments.

1- The effects of stomatin on ASIC channels have been used as an assay of stomatin function. However, the physiological relevance of these effects is not clear in mammals. The manuscript could definitely be reinforced by showing in addition the effect of WT and mutant stomatin (e.g., V197P, T182W and R97D) on mechanosensitive currents in transfected neurons isolated from stomatin mutant mice. These neurons may not show a complete loss of sensory neuron mechanosensitivity, which could make the experiments difficult to analyze.

It was previously shown that stomatin only plays a minor role in mechanosensation (Martinez-Salgado et al 2008). The examination of each mutant examined here in the context of STOML3 (strongly involved in mechanosensation) on endogenous mechanosensitive currents in sensory neurons would be a massive undertaking and clearly not feasible in a short period of time. Instead, we explored stomatin function in mouse adult fibroblasts derived from stomatin knockout mice. Our new data in Suppl. Fig. 3 point to a trend that ASIC3 channels are not so tightly controlled in stomatin knockout fibroblasts compared to wild type fibroblasts. Cleary, more work needs to be spent to characterize the expression of ion channels and stomatin in these cells, but we feel that this would constitute an entire new research project on its own.

However, experiments could alternatively be done with the related protein SLP3/Stoml3 that similarly interacts with ASIC channels and displays a complete loss of mechanosensitivity in a third of the sensory neurons isolated from knockout mice (Wetzel et al. 2007).

These experiments would be very interesting, although the ion channels regulated by SLP3 are not known so far. However, isolation of mouse neurons from SLP3 knockout mice, and rescue experiments with wild type GFP-tagged SLP3 took almost a year of work. In our case, we would

need to test all mutants, and obtain many measurements from transfected neurons for each mutant to obtain reliable statistics. These experiments would take much more than a year and were therefore not included in the current manuscript.

2- The C-terminal domain of stomatin is lacking in the protein used to get the crystal, and it is therefore not clear how this domain could interfere with dimerization. The use of stomatin 85-255 brings some arguments against an artifact due to the shorter construct, but the last ~30 aa are still missing in this extended form.

The stomatin 85-255 constructs corresponds in length to the crystallized trimeric Pyrococcus construct, so we are confident that the comparison between our dimeric structure and the trimeric stomatin 85-255 construct is valid. It is, however, clear that the C-terminal residues of stomatin are also involved in oligomerization (see Umlauf et al, 2006) which is mentioned now in the discussion. Unfortunately, a construct of stomatin extending until the C-terminus is insoluble so we could not characterize its oligomerization properties.

To still address this point, we provide new bimolecular fluorescence complementation data in Suppl. Fig. 2 which show that mutations in the stomatin dimer interface lead to decreased assembly rates of full-length stomatin in vivo.

2nd Editorial Decision 25 June 2012

Thank you for sending us your revised manuscript. In the meantime, the referees have seen it again and are supportive. Still, referees 1 and 3 are not fully satisfied and I would like to ask you to look into the points raised and to address or respond to them.

In addition, there are a few editorial issues that need further attention:

* Please add the details for the PDB coordinates to this final version of the manuscript.

* Please add the statistical details for figure 2C to the legend.

* As a new initiative, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you for your cooperation. I am looking forward to your amended manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The authors have addressed most of the comments from the previous round of review. The manuscript is now significantly improved. Some issues still remain however. For example, membrane localization of stomatin and its association with ASIC is inferred indirectly, rather than unequivocally demonstrated. Also the impact of various stomatin mutations on the localization of

the protein (and its potential association with membrane microdomains - lipid rafts) is only superficially addressed. I appreciate the authors' argument about time constrains, although, these points are important for strengthening their study and conclusions. Regardless of this, at least for the association between stomatin and ASIC, the bimolecular fluorescence complementation assay now offered by the authors provides compelling evidence in support of their model.

Referee #2

The authors addressed the questions raised adequately and improved the manuscript considerably, which is now suitable for publication in the EMBO J.

Referee #3

The authors have addressed most of the initial comments. The data shown in new Suppl. Fig. 3 are however not fully convincing in the present form and need to be further improved or removed. In addition, the authors suggest in the legend of Suppl. Fig. 3 that the endogenous levels of stomatin in fibroblasts is too low to efficiently block proton gated currents carried by overexpressed ASIC3 channels, which is not in agreement with their response to point #1 suggesting, based on the same data, that ASIC3 channels are not so tightly controlled in stomatin knockout fibroblasts compared to wild type fibroblasts.

2nd Revision - authors' response 03 July 2012

Please find below our responses to the requests of the editor and the three referees.

** Please add the details for the PDB coordinates to this final version of the manuscript.*

We performed a last round of refinement and subsequently submitted the pdb coordinates of the three crystal forms to the pdb database. The pdb codes are included in the new manuscript. Slight changes were introduced to the refinement statistics.
In response to your previous request, we also added one paragraph to the discussion dealing with the regulation of ASICs.

** Please add the statistical details for figure 2C to the legend.*

We included the required statistical details.

** As a new initiative, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.*

We submitted the required source data.

Referee #1

The authors have addressed most of the comments from the previous round of review. The manuscript is now significantly improved. Some issues still remain however. For example, membrane localization of stomatin and its association with ASIC is inferred indirectly, rather than unequivocally demonstrated. Also the impact of various stomatin mutations on the localization of

the protein (and its potential association with membrane microdomains - lipid rafts) is only superficially addressed. I appreciate the authors' argument about time constrains, although, these points are important for strengthening their study and conclusions. Regardless of this, at least for the association between stomatin and ASIC, the bimolecular fluorescence complementation assay now offered by the authors provides compelling evidence in support of their model.

We agree with this reviewer's assessments that our manuscript offers many new insights to further analyze membrane/raft localization of stomatin and its interaction with ASICs. We are also very happy that this reviewer agrees with us that the new BiFC assays address this issue and show unequivocally that ASICs, stomatin and stomatin mutants can interact with each other at the plasma membrane.

Referee #2

The authors addressed the questions raised adequately and improved the manuscript considerably, which is now suitable for publication in the EMBO J.

We are delighted that this reviewer is happy to see our manuscript published in the EMBO Journal.

Referee #3

The authors have addressed most of the initial comments. The data shown in new Suppl. Fig. 3 are however not fully convincing in the present form and need to be further improved or removed. In addition, the authors suggest in the legend of Suppl. Fig. 3 that the endogenous levels of stomatin in fibroblasts is too low to efficiently block proton gated currents carried by overexpressed ASIC3 channels, which is not in agreement with their response to point #1 suggesting, based on the same data, that ASIC3 channels are not so tightly controlled in stomatin knockout fibroblasts compared to wild type fibroblasts.

We are also delighted that this reviewer feels that we have addressed most of the initial comments. The reviewer makes one further important point which we need to address. He comments on the experiments that we have carried out with primary fibroblasts isolated from stomatin -/- mice and wild type mice. These experiments turned out to be more difficult than we had originally anticipated. First, it took some time to establish the transfection conditions necessary to obtain primary cells that express ASIC3 channels, the activity of which could be recorded at the plasma membrane. In our CHO cells experiments, two expression plasmids were routinely transfected, one containing eGFP at a final concentration 4 times lower than the second plasmid encoding ASIC3. Under these conditions, 99% of the cells showing green fluorescence exhibited ASIC-like membrane currents. However, the same experimental conditions using primary fibroblasts required about four times higher DNA concentrations (eGFP to ASIC plasmid ratio remained the same), but only 50% of the cells with green fluorescence exhibited ASIC3-like membrane currents. Thus, in primary fibroblasts there may be an intrinsic deficit in the trafficking/targeting of ASIC protein to the plasma membrane. This observation naturally complicates the comparison between stomatin-/- and wild type fibroblasts. The reviewer is thus quite correct to point out that we were contradicting ourselves when we speculated that endogenous levels of stomatin in fibroblasts may be too low to block ASIC3 channels. We have reformulated the text of the offending supplementary figure legend to correct this impression. Because we used non-immortalized primary fibroblasts, we have no guarantee that cells which are isolated from different mice represent a uniform cell population. Thus, some cells from wild type mice may have originally expressed high stomatin levels and others maybe none. Also this makes the comparison between the two genotypes problematic. Nevertheless, the fact that we observed a very homogenous amplitude distribution of ASIC-like currents in wild type cells and a much more diverse population of currents including very large amplitude currents in stomatin-/- cells was encouraging and supportive of the hypothesis that endogenous stomatin can indeed inhibit/regulate ASIC currents. Although we would have liked to add to this dataset, the time available was not sufficient; the data shown in Supplementary Figure 3 is from a total of four independent primary fibroblast cultures obtained from 2 wild type and 2 stomatin-/- mice. Each new fibroblast culture needs time to establish and even then, the very low transfection efficiency means that the sample of cells in which pH gated currents can be recorded is always low. The effort involved in obtaining the data for Supplementary Figure 3 was therefore quite considerable and we

believe our description of the experiment will provide useful information for future experimenters who may wish to try out similar experiments.

