THE EMBO JOURNAL

Manuscript EMBO-2017-96685

The organic anion transporter SLCO2A1 constitutes the core component of the Maxi-Cl channel

Ravshan Z. Sabirov, Petr G. Merzlyak, Toshiaki Okada, Md. Rafiqul Islam, Hiromi Uramoto, Tomoko Mori, Yumiko Makino, Hiroshi Matsuura, Yu Xie & Yasunobu Okada

Corresponding author: Yasunobu Okada, SOKENDAI The Graduate University for Advanced Studies

Review timeline:

Submission date:	07 February 2017
Editorial Decision:	09 March 2017
Revision received:	12 August 2017
Editorial Decision:	01 September 2017
Revision received:	08 September 2017
Accepted:	12 September 2017

Editor: Anne Nielsen

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 09 March 2017

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 from the reports, all referees express high interest in the findings reported in your manuscript, but they also raise a number of points that will have to be clarified before they can support publication in The EMBO Journal. In addition, we had to notice that these are rather extensive concerns that could potentially question the overall conclusion in your study. Furthermore, the outcome of the requested additional experiments cannot be predicted at this point.

Should you be able to address these criticisms in full, we could consider a revised manuscript. I should 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 in this revised version. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

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

REFEREE REPORTS

Referee #1:

This is an interesting study reporting the identification of the prostaglandin transporter SLCO2A1 as the protein that likely mediates the Maxi Cl- channel activated by cell swelling and other modalities that deformed the plasma membrane. The authors used an interesting approach of isolating membrane blebs that contain the channel and proteomics to identify SLCO2A1 as a protein in blebs that mediates the current. The authors proceeded by testing modifying expression of SLCO2A1 on the current. The most convincing evidence for SLCO2A1 to function as the maxi Cl- channel was then obtained by purifying and reconstituting SLCO2A1 and obtaining a current resembling the maxi Cl- current.

Together, the results provide significant evidence for SLCO2A1 to be part of or directly mediate the maxi Cl- current. However, several observations are not as clear, evidence to strengthen channel function of SLCO2A1 should be further attempted and several additional controls are needed to exclude misinterpretation.

1. Ion channels are typified by distinctive pores that are formed by transmembrane domains (TMD). Crystal structure and structural models are available for several members of the organic anion transporting polypeptides that point to the transport pathway. A significant strengthening of the conclusions can be obtained by developing a structural model for SLCO2A1 to identify potential pore residue and show that their mutation change channel selectivity. Modification of channel selectivity is a most basic requirement to claim a protein to be a channel.

2. Figure 3: the appearance of a new current in the knockout cells is somewhat disturbing. The authors should at least test if the current is sensitive to Gd3+, BSP and perhaps PGE2 to exclude the possibility that the new current is a subunit of SLCO2A1.

3. Figure 4: Are the flickers in C truly mediated by SLCO2A1 or are they mediated by the channel seen in the knockout cells? The knockout cells should be used to test if they have similar flickers of flickers are induced by BSP in these cells.

4. Figure 6: It is surprising that the effect of BSP on the current of the reconstituted protein is not tested and reported. It should. Identifying a pore mutant and reconstituting such a mutant should be very strong experiment.

5. Figure 7: Testing the prostaglandin binding mutants in the C127 is not satisfactory, especially since the reduction in current observed is less than that in the reconstitution experiments. These experiments should be performed in HEK cells that offer a clean background without contaminating native protein.

6. Figure 9: There is no evidence to support the model and it should not be included. If something, the results with the mutants in Figures 6 and 7 speaks against the model by suggesting that PGE2 and Cl- share the same pathway/structural motifs rather than different conformations.

7. Figure legends are exceedingly and unnecessarily long mixing sections that belong or are restatements of Methods and result sections. The legends should be extensively edited.

Referee #2:

This is a very interesting manuscript in which the authors detail experiments aimed at identifying the molecular basis of the Maxi-Cl anion channel, a large conductance, ATP sensitive anion channel that is found in many cell types and whose molecular identity has remained unknown. The authors use a combination of functional studies, mass spec proteomics, siRNA and CRISPR

knockdown/knockout, to probe whether manipulation of the candidate gene SLCO2A1, a 12 transmembrane prostaglandin transporter, affects Maxi-Cl function. The approach is extensive. Although the aggregate of the results appears to support the conclusion that SLCO2A1, a prostaglandin transporter, is important for Maxi-Cl function many of the individual pieces of evidence are less than convincing and have important deficiencies that conspire to create reasonable doubt about the roles of SLCO2A1.

Controls for reconstitution. There is no explicit removal of DDM in the reconstitutions, leaving concerns about whether the resultant channels are artefacts of the left over detergent. Additionally, there is no control using cells that lack Maxi-Cl activity as a source for membrane fractions. One would like to know how much 'Maxi-Cl like' activity could be found from cells that lack the Maxi-Cl activity. These factors together with the fact that the 'activity' is smeared out over so many different fractions raises the concern that the measured activity arises from some non-specific effect.

The siRNA and CRISPR experiment both indicate a loss of Maxi-Cl activity. The main concern from is that neither manipulation, particularly the CRISPR knockout, completely kills the function. In fact the CRISPR knockout induces some alternative Chloride channel,that while different in properties from Maxi-Cl, still leaves the impression that the loss of function is not really complete. For example, does this remaining activity still have the pharmacological profile of the Maxi-Cl channel as noted in the experiments in Fig. 4? Or sensitivity to gadolinium? Such evidence would test the idea that SLCO2A1 is part of the channel.

The reconstitution experiments are done with exceptionally dirty material (Fig. 6A). This fact, given with the modest results of the mutations, leaves one wondering if the activity is the result of something other than SLOC2A1. As with the work in Fig. 1, a control experiment using a different FLAG-tagged protein, subjected to the same purification and reconstitution procedure, would serve as a critical control to ensure that the activity measured actually comes from SLOC2A1.

Overall, the authors seem to have begun to build an interesting case for the involvement of SLOC2A1 in the Maxi-Cl activity. However, the deficiencies in controls leave for an unconvincing manuscript that could be greatly improved by the pursuit of further control experiments.

Referee #3:

Sabirov et al. have isolated and fractionated membrane protein from C127 cell blebs, reconstituted isolated protein into proteoliposomes and subsequently measured on patches Maxi-Cl channel activity. This elegant approach resulted in the identification of SLCO2A1 protein as a constituent of Maxi-Cl activity. This observation is quite remarkable since SLCO2A1 is a member of the 12-membrane spanning superfamily of transporters and has previouslly been shown to encode a prostaglandin transporter. The ms of Sabirov certainly merits publication in EMBO journal. However, the ms requires a major revision as detailed below.
Introduction:
SLCO2A1 is a well known member of a superfamily of transporter proteins. Earlier the SCLO2A1 gene has been shown to encode a prostaglandin transporter suggesting that the protein may be involved in mediating uptake and clearance of prostaglandins, which is of high physiological significance. The should provide in their introduction this important information about SLCO2A1 activity.
Results:
Throughout the ms data on current amplitudes are reported as pA (or % of control), e.g. Fig. 1B. Most likely, channel densities vary from patch to patch. To take these variations into account, I recommend to replace current amplitude values by current density (pA/pF). Anion-selectivity should always be documented as PCl/PNa. This is the case in later sections of Results, but not in the first para.
Fig.1E It is recommended to replace the % data by pA/pF. This will provide the reader with a better set of data to appreciate of what was measured. To set a control 27 times to 100% is uninformative and may even be quite trivial.
The legend to Fig. 2C may be revised for explaining better the current traces shown.

In Fig. 3B controls were set to 130%. 130% of what?
The K613G mutation affects SLCO2A1 transporter activity, whereas the effect on Maxi-Cl activity was apparently not as pronounced. It may be informative to have both data side by side (at least in Discussion) as transporter and channel activity seem to require different parts/states of SLCO2A1 protein. For the general reader, it is not evident why the authors used the R560N mutant. Concerning mutant SLCO2A1 proteins, it is recommended to include one of disease related SLCO2A1 mutants like Pro219Leu and to investigate transporter in comparison to channel properties.
The authors claim that PGE2 blocks significantly SCLO2A1 related ATP-release as well as Maxi-Cl activity. I am not sure whether one may call a reduction of at most 10% really significant. In general, the ATP-release part of Results is the least convincing. Most effects, that are shown, are quite small or do not fit well with current data. For example, HEK cells have no endogenous Maxi-Cl activity, but show a substantial ATP-release. SLCO2A1 transfected HEK cells show a substantial swelling-induced Maxi-Cl activity, but in comparison the swelling-induced ATP release is small. These differences should be addressed.
Discussion
Based on their results the authors propose that SLCO2A1 comes in two flavors or modes, one corresponds to its transporter activity, the other to its Maxi-Cl activity. This is very nice idea, but they should put more juice to there hypothesis. One could imagine that SCLO2A1 acts as a prostaglandin transporter in a monomeric state and as a Maxi-Cl channel in a dimeric state. Since Sabirov et al. have succeded in the purification of the SCLO2A1 protein, it should not be to difficult to investigate a potential effect of swelling on the oligomerization status of SLCO2A1. A corresponding data set would constitute a considerable improvement.

1st Revision - authors' response 12 August 2017

Referee Comments (Qs) and *Authors Responses (Rs)*:

Referee #1:

Q1: Ion channels are typified by distinctive pores that are formed by transmembrane domains (TMD). Crystal structure and structural models are available for several members of the organic anion transporting polypeptides that point to the transport pathway. A significant strengthening of the conclusions can be obtained by developing a structural model for SLCO2A1 to identify potential pore residue and show that their mutation change channel selectivity. Modification of channel selectivity is a most basic requirement to claim a protein to be a channel.

R: According to the Referee comment, we built a homology model of SLCO2A1 protein (Fig EV5) using I-TASSER algorithm (Yang et al, 2015). The overall shape of this structure is similar to that obtained previously using the SWISS-MODEL engine (Zhang et al, 2012). In the model, the residue R560 is located right on the central axis of the protein. This fact may be related to the present result that voltage sensitivity of the inactivation kinetics was affected by the charge-neutralized mutation of this residue, R560N (Fig 5E,F). Two residues, G222 and P219, which are mutated in pachydermoperiostosis (Zhang et al, 2012; Zhang et al, 2014), are also close to the axis. This fact may explain why no channel activity was observed in HEK293T cells transfected with these disease-causing mutants despite their successful expression in the periphery region including the plasma membrane of the cells (Fig EV4). Since the model was built using the crystal structure of the glycerol-3-phosphate transporter as a template, it most likely represents the "inward-open"-like transporter conformation state of SLCO2A1 as PGT. The K613 location is more distant from the central axis, but the following results suggest that in the open-channel conformation of SLCO2A1 as Maxi-Cl, the K613 residue may move closer towards the pore axis and thereby participating in the selectivity filter: its neutralization (K613G) led to a change in anion-to-cation selectivity (Fig 6G: blue and green triangles; and Fig 7D: red circles) with inducing a decrease in the single-channel conductance (Fig 6G: blue circles; and Fig 7D: red circles). Before making more precise structural discussion about the pore construction, however, it must be determined whether the Cl^--conducting pathway is located within the single SLCO2A1 protein or between plural SLCO2A1 proteins in an oligomeric structure. Overexpression of the K613G mutant in C127 cells produced channels with the single-channel amplitude less than that of the native Maxi-Cl (Fig 5B-D) but higher than that observed upon overexpression of the same mutant in HEK293T cells lacking the endogenous

SLCO2A1 (Fig 6G: blue circles). This fact suggests that the mutant protein may have combined with the endogenous WT SLCO2A1 in C127 cells, yielding channels with intermediate amplitudes, as evidenced by the broad distribution shown in Fig 5D (middle panel). Protein oligomerization could be actually detected on the non-reducing SDS-PAGE gel as faint protein bands with a molecular mass approximately twice and thrice of the monomer (see at arrowheads on Fig 7A). However, more elaborate structure-functional analysis will be necessary to clarify the true Maxi-Cl channel construction. We have such discussed the model in the Discussion section of the revised manuscript on page 12 (line 29) to page 13 (line 24).
When charge-neutralized mutants, K613G and R560N, of SLCO2A1 were overexpressed in C127 cells, the single-channel conductance was decreased compared with that of the native Maxi-Cl (Fig 5B-D). However, the cation-to-anion selectivity was not significantly affected by these mutations implying that K613G or R560N transfected in C127 cells form the channel by oligomerization with the native wild-type SLCO2A1 proteins. In our additional experiments, we attempted to perform similar experiments with HEK293T cells clean with no endogenous SLCO2A1 and no Maxi-Cl channel background. Actually, K613G channels were found to become relatively more selective to cations with P_{Na}/P_{Cl} of 1.9 (Fig 6G: blue and green triangles) and to exhibit a markedly smaller single-channel conductance (Fig 6G: blue circles). Similar reversal of anion-to-cation selectivity and marked reduction of the single channel conductance were also found in the K613G channels when reconstituted into proteoliposomes (Fig 7D: red circles). These results have been described on page 8 (lines 6-18) and from page 8 (line 31) to page 9 (line 2) and discussed on page 11 (lines 9-12) and page 13 (lines 6-12) in the revised manuscript. These K613G data, especially modification of channel selectivity by charge-neutralized mutation, strongly support the conclusion that SLCO2A1 constitutes the core component or the pore of Maxi-Cl channel.

Q2: Figure 3: the appearance of a new current in the knockout cells is somewhat disturbing. The authors should at least test if the current is sensitive to Gd3+, BSP and perhaps PGE2 to exclude the possibility that the new current is a subunit of SLCO2A1.

R: In our additional experiments, according to the Referee recommendation, we tested the effects of PGE2, BSP and Gd^{3+} on the non-Maxi-Cl channels which newly emerged in SLCO2A1-deficient CRISPR/Cas9 knockout (KO) cells. The results of these experiments are presented in Fig EV2A (center panel) and Fig EV3.
We found that, in contrast to the Maxi-Cl channel, these non-Maxi-Cl channels were insensitive to PGE2 (Fig EV2A: center panel). This new result, in conjunction with the PGE2 sensitivity of the wild-type Maxi-Cl channel (Fig EV2A: left panel) and of the reconstituted SLCO2A1 channel (Fig EV2A: right panel), supports that SLCO2A1 per se constitutes the core component of Maxi-Cl. This new finding has now been noted on page 7 (lines 3-5) and discussed on page 11 (lines 28-31) of the revised manuscript
Non-Maxi-Cl channels in SLCO2A1 KO cells retained sensitivity to BSP (Fig EV3B,C). This new result, in conjunction with the BSP insensitivity of the reconstituted recombinant channel, suggests that the observed inhibitory effect of this drug on the native Maxi-Cl channel is indirect and possibly mediated by an auxiliary protein which is absent in our reconstitution system. In agreement with this inference, we found that an SLCO2A1-unrelated channel, VSOR (also called VRAC) was also sensitive to BSP (at 10 to 50 μM: Sabirov and Okada, unpublished data). Moreover, the inhibitory effect of BSP on Maxi-Cl currents was not voltage-dependent and observed at both positive and negative voltages with very similar IC_{50} values (Fig 3B,C) ruling out the plug-in open-channel block mechanism which should be strongly voltage-dependent. Thus, the flickery events shown in Fig 3C possibly reflect the drug binding/unbinding events on an auxiliary regulatory subunit of Maxi-Cl. This new finding has been described on page 7 (lines 4-5) and page 9 (lines 7-11), and the interpretation for them has been discussed in the Discussion section on page 11 (line 31) to page 12 (line 6) of the revised manuscript.
Our additional experiments also showed that the non-Maxi-Cl-type MAC-like channel in SLCO2A1 KO cells is sensitive to Gd^{3+} (Fig EV3A). This new finding has been noted on page 7 (lines 4-5) in the revised manuscript. This new result looks contradictory to the assumption that Gd^{3+} is a specific blocker for Maxi-Cl. However, it must be noted that MAC activity in mouse B cells, which is distinct from Maxi-Cl in lacking voltage-dependent inactivation kinetics and with exhibiting smaller unitary conductance (270 pS), was found to be sensitive to Gd^{3+} (Nam et al, 2006). On the other hand, the VSOR, CFTR and ASOR anion channels are known to be Gd^{3+}-insensitive (Hazama et al, 2000; Sabirov et al, 2001; Sato-Numata et al, 2016). Taken together, it is conceivable that Gd^{3+} is specific for the MACs among anion channels but cannot discriminate

between different subtypes of MACs. Such discussion has been given on page 12 (lines 6-14) in the revised manuscript.

Q3: Figure 4: Are the flickers in C truly mediated by SLCO2A1 or are they mediated by the channel seen in the knockout cells? The knockout cells should be used to test if they have similar flickers of flickers are induced by BSP in these cells.

R: In the presence of BSP, we observed flickers not only in native C127 cells (Fig 3C) but also in the SLCO2A1-deficient cells (Fig EV3C, Inset). We initially deemed that this frickery block by BSP is due to its open-channel blocking action. However, this inhibitory effect was equally observed at positive and negative voltages (Fig 3B). Such voltage-independent inhibition of Maxi-Cl channels by BSP may rule out the plug-in, open-channel block mechanism which should be strongly voltage-dependent. Moreover, taken together with BSP sensitivity of the Maxi-Cl-unrelated anion channel, VSOR, it is likely that the two types of channels coded by different genes share a BSP-sensitive component independent of SLCO2A1. Consistent with this idea, the Maxi-Cl activity reconstituted with recombinant SLCO2A1 into the proteoliposomes was totally insensitive to BSP. Thus, we now interpret that the voltage-independent flickery events observed with native Maxi-Cl channels as well as with non-Maxi-Cl channels in SLCO2A1-deficient cells, probably, reflect the binding/unbinding process on a BSP-sensitive regulatory component rather than an open-channel block which must be strongly voltage-dependent. These results have been noted on page 6 (lines 20-25), page 7 (line 5) and discussed on page 11 (line 31) to page 12 (line 6) of the revised manuscript.

Q4: Figure 6: It is surprising that the effect of BSP on the current of the reconstituted protein is not tested and reported. It should. Identifying a pore mutant and reconstituting such a mutant should be very strong experiment.

R: When we tested the effect of BSP, according to the Referee comment, we found that this drug at 30–100 μM did not affect the reconstituted channel (n = 9). Thus, it is likely that the inhibitory effect of BSP on the native channel is indirect and possibly mediated by an auxiliary protein which is absent in the reconstitution system. In agreement with this inference, we found that the non-Maxi-Cl channels newly emerged in SLCO2A1-deficient C127 cells were also sensitive to BSP (Fig EV3B,C). Furthermore, it must be noted that the SLCO2A1-unrelated anion channel, VSOR, was found to be sensitive to BSP (at 10 to 50 μM: Sabirov and Okada, unpublished data). In addition, the Maxi-Cl inhibition by BSP was observed both at positive and negative voltages (Fig 3A,B) with very similar IC_{50} values (Fig 3C) ruling out the plug-in open-channel block mechanism which should be strongly voltage-dependent. Thus, the flickery events shown in Fig 3C and also observed in SLCO2A1-deficient cells (Fig EV3C, Inset) possibly reflect the drug binding/unbinding events on an auxiliary regulatory subunit of Maxi-Cl. These results are described on page 9 (lines 7-11) and discussed on page 11 (line 31) to page 12 (line 6) in the revised manuscript.

Q5: Figure 7: Testing the prostaglandin binding mutants in the C127 is not satisfactory, especially since the reduction in current observed is less than that in the reconstitution experiments. These experiments should be performed in HEK cells that offer a clean background without contaminating native protein.

R: We have performed additional experiments with the K613G mutant expressed in HEK293T cells, according to the Referee Comment, and found that this mutant generated markedly reduced macro-patch currents (Fig 6C: blue column) with a markedly reduced single-channel conductance (Fig 6G: blue circles), reflecting a decrease in the total positive charge inside the lumen of the channel. Moreover, K613G channels expressed in HEK293T cells became relatively more selective to cations with P_{Na}/P_{Cl} of 1.9 (Fig 6G: blue triangles). We have revised the Figures and Legends accordingly, described the results on page 8 (lines 6-18) and discussed on page 11 (lines 9-12) and page 13 (lines 6-18) in the revised manuscript.

Q6: Figure 9: There is no evidence to support the model and it should not be included. If something, the results with the mutants in Figures 6 and 7 speaks against the model by suggesting that PGE2 and Cl- share the same pathway/structural motifs rather than different conformations.

R: We have removed the previous Figure 9 and revised the text accordingly.

Q7: Figure legends are exceedingly and unnecessarily long mixing sections that belong or are restatements of Methods and result sections. The legends should be extensively edited.

R: We have revised the Figure Legends to make them shorter and to remove restatements of Methods and Results sections. However, we would like to keep the comprehensiveness of the Legends and retain some details which are necessary to fully understand the figures.

Referee #2:

Q1: Controls for reconstitution. There is no explicit removal of DDM in the reconstitutions, leaving concerns about whether the resultant channels are artefacts of the left over detergent. Additionally, there is no control using cells that lack Maxi-Cl activity as a source for membrane fractions. One would like to know how much 'Maxi-Cl like' activity could be found from cells that lack the Maxi-Cl activity. These factors together with the fact that the 'activity' is smeared out over so many different fractions raises the concern that the measured activity arises from some non-specific effect.

R: At the stage of passing through the Sephadex G-10 column, most of the DDM was removed and the residual detergent did not affect the proteoliposome formation and reconstituted channel activity. This has now been noted in the Results section (page 5, lines 6-7) and in Appendix Supplementary Methods (page 2, lines 20-22) of the revised manuscript.
The recombinant SLCO2A1 and K613G proteins were prepared using HEK293T cells which lack the endogenous Maxi-Cl activity and exhibited the channel events. In contrast, the Maxi-Cl events and even any Maxi-Cl-like activity were never detected in Control experiments where the same purification-reconstitution procedures were performed with mock-transfected control HEK293T cells (n = 25), with the cells transfected with FLAG-tagged LRRC8A protein (n = 25) which is known to be the necessary but not sufficient component of the VSOR anion channel or VRAC (Voss et al, 2014; Qiu et al, 2014; Okada et al, 2017), or with the cells transfected with FLAG-tagged SLC3A2 protein (n = 15) which is a beta-subunit of the large neutral amino acid transporter (Wagner et al, 2001). These additional control experiments clearly proved that the observed channel activity is specific for the SLCO2A1 protein. These new control experimental results have now been included in the revised version on page 9 (lines 12-21). It should be noted that the same trace of amount of DDM, if any, was present in all the control experiments thus proving that the channel activity was not due to an artifact of the left-over detergent.

Q2: The siRNA and CRISPR experiment both indicate a loss of Maxi-Cl activity. The main concern from is that neither manipulation, particularly the CRISPR knockout, completely kills the function. In fact the CRISPR knockout induces some alternative Chloride channel, that while different in properties from Maxi-Cl, still leaves the impression that the loss of function is not really complete. For example, does this remaining activity still have the pharmacological profile of the Maxi-Cl channel as noted in the experiments in Fig. 4? Or sensitivity to gadolinium? Such evidence would test the idea that SLCO2A1 is part of the channel.

R: In our additional experiments, according to the Referee recommendation, we tested the effects of PGE2, BSP and Gd^{3+} on the non-Maxi-Cl channels which newly emerged in SLCO2A1-deficient CRISPR/Cas9 knockout (KO) cells. The results of these experiments are presented in Fig EV2A (center panel) and Fig EV3.
We found that, in contrast to the Maxi-Cl channel, these newly emerged non-Maxi-Cl channels were insensitive to PGE2 (Fig EV2A: center panel). This new result, in conjunction with the PGE2 sensitivity of the wild-type Maxi-Cl channel (Fig EV2A: left panel) and of the reconstituted SLCO2A1 channel (Fig EV2A: right panel), strongly supports that SLCO2A1 per se constitutes the core component of Maxi-Cl. This new finding has now been noted on page 7 (lines 3-5) and discussed on page 11 (lines 28-31) of the revised manuscript
Non-Maxi-Cl channels in SLCO2A1 KO cells retained sensitivity to BSP (Fig EV3B,C). This new result, in conjunction with the BSP insensitivity of the reconstituted recombinant channel, suggests that the observed inhibitory effect of this drug on the native Maxi-Cl channel is indirect and possibly mediated by an auxiliary protein which is absent in our reconstitution system. In agreement with this inference, we found that an SLCO2A1-unrelated channel, VSOR (also called VRAC) was also sensitive to BSP (at 10 to 50 μM: Sabirov and Okada, unpublished data). Moreover, the inhibitory

effect of BSP on Maxi-Cl currents was not voltage-dependent and observed at both positive and negative voltages with very similar IC_{50} values (Fig 3B,C) ruling out the plug-in open-channel block mechanism which should be strongly voltage-dependent. Thus, the flickery events shown in Fig 3C possibly reflect the drug binding/unbinding events on an auxiliary regulatory subunit of Maxi-Cl. This new finding has been described on page 7 (lines 4-5) and page 9 (lines 7-11), and the interpretation for them has been discussed in the Discussion section on page 11 (line 31) to page 12 (line 6) of the revised manuscript.

Our additional experiments also showed that the non-Maxi-Cl-type MAC-like channel in SLCO2A1 KO cells is sensitive to Gd^{3+} (Fig EV3A). This new finding has been noted on page 7 (lines 4-5) in the revised manuscript. This new result looks contradictory to the assumption that Gd^{3+} is a specific blocker for Maxi-Cl. However, it must be noted that MAC activity in mouse B cells, which is distinct from Maxi-Cl in lacking voltage-dependent inactivation kinetics and with exhibiting smaller unitary conductance (270 pS), was found to be sensitive to Gd^{3+} (Nam et al, 2006). On the other hand, the VSOR, CFTR and ASOR anion channels are known to be Gd^{3+}-insensitive (Hazama et al, 2000; Sabirov et al, 2001; Sato-Numata et al, 2016). Taken together, it is conceivable that Gd^{3+} is specific for the MACs among anion channels but cannot discriminate between different subtypes of MACs. Such discussion has been given on page 12 (lines 6-14) in the revised manuscript.

Q3: The reconstitution experiments are done with exceptionally dirty material (Fig. 6A). This fact, given with the modest results of the mutations, leaves one wondering if the activity is the result of something other than SLOC2A1. As with the work in Fig. 1, a control experiment using a different FLAG-tagged protein, subjected to the same purification and reconstitution procedure, would serve as a critical control to ensure that the activity measured actually comes from SLOC2A1.

R: As shown in Fig. 7A (FLAG eluate), the recombinant material used in the reconstitution experiments showed almost only single bands (at around 70 kD) under SDS-PAGE electrophoresis, indicating that the recombinant preparation is not dirty. Minor faint bands observed above the main 70 kD band in Fig 7A (at arrowheads) would represent oligomeric ones because their molecular mass correspond to approximately twice and thrice of the main band. Such has been noted in the Discussion section (page 13, lines 20-22) of the revised manuscript.
The Maxi-Cl events and even any Maxi-Cl-like activity were never detected in Control experiments where the same purification-reconstitution procedures were performed with mock-transfected control HEK293T cells (n = 25), with the cells transfected with FLAG-tagged LRRC8A protein (n = 25), or with the cells transfected with FLAG-tagged SLC3A2 protein (n = 15). These additional control experiments clearly proved that the observed channel activity is specific for the SLCO2A1 protein. These new control experimental results have now been included in the revised version on page 9 (lines 12-21).

Referee #3:

Q1: Introduction: SLCO2A1 is a well known member of a superfamily of transporter proteins. Earlier the SCLO2A1 gene has been shown to encode a prostaglandin transporter suggesting that the protein may be involved in mediating uptake and clearance of prostaglandins, which is of high physiological significance. The should provide in their introduction this important information about SLCO2A1 activity.

R: We have revised the Introduction (page 4, lines 11-17) with noting the following sentences: "The Slco2a1 gene is known to encode the prostaglandin transporter PGT (Kanai et al, 1995) involved in a physiologically highly significant process of uptake and clearance of prostaglandins (Schuster, 1998, 2002; Schuster et al, 2015); its deficiency in mice leads to a failure of the ductus arteriosus to close after birth due to increased levels of extracellular prostaglandin E2 (PGE2) (Chang et al, 2010); and in humans, mutations in SLCO2A1 gene are associated with pachydermoperiostosis (Sasaki et al, 2012; Seifert et al, 2012; Zhang et al, 2012; Zhang et al, 2014) and enteropathy (Umeno et al, 2015; Hosoe et al, 2017)."

Q2: Results: Throughout the ms data on current amplitudes are reported as pA (or % of control), e.g. Fig. 1B. Most likely, channel densities vary from patch to patch. To take these variations into account, I recommend to replace current amplitude values by current density (pA/pF). Anion-

selectivity should always be documented as PCl/PNa. This is the case in later sections of Results, but not in the first para.

R: Although the whole-cell currents can easily and routinely be expressed as the current density (pA/pF), it is not the case for the currents recorded from tiny patch membranes, because the fast capacitative transients in excised patches is composed of two main components: the capacitance of the patch pipette and the capacitance of the excised membrane patch which is proportional to the patch membrane area. We did not measure these components separately and, therefore, we cannot express the patch currents as currents densities as it is routinely done for whole-cell current amplitudes. However, it must be noted that the patch membrane area is not much different from each other, because the tip size of patch pipette, which can be monitored by its electric resistance, was controlled to be nearly constant at around ~2 MΩ.

Since we did not apply any NaCl gradient in this particular on-bleb and on-proteoliposome experiments for Fig. 1, the P_{Cl}/P_{Na} value cannot be evaluated. In Fig 1B and G, the anion selectivity of the channel was demonstrated by replacing all NaCl with equimolar Na-glutamate. We expressed the selectivity as the permeability ratio $P_{glutamate}/P_{Cl}$ of 0.11 to 0.14 which is close to the values hitherto reported for Maxi-Cl in many cell types (Sabirov & Okada, 2009). These values have now been given in the main text on page 5 (lines 16-18) of the revised manuscript. A high anion-to-cation selectivity was further confirmed by the fact that even when the patch pipettes were filled with NMDG-Cl (instead of NaCl), the I-V curve was essentially identical to that observed with patch pipettes filled with NaCl solution in Fig. 1B (red circles). This new result has now been shown in Fig 1B (green triangles) and mentioned in the text (page 5, line 1) of the revised manuscript.

Q3: Fig.1E It is recommended to replace the % data by pA/pF. This will provide the reader with a better set of data to appreciate of what was measured. To set a control 27 times to 100% is uninformative and may even be quite trivial.

R: We have replaced the % data with the pA data in Fig 1E. We cannot use the unit of pA/pF for currents recorded from excised patch membranes for the reasons explained in the response to Q2.

Q4: The legend to Fig. 2C may be revised for explaining better the current traces shown.

R: We have added the following sentence to the Legend of Fig 2C to make it more understandable: "The currents were elicited by step pulses from +50 mV to −50 mV in 10-mV increments (pulse protocol shown at the top)."

Q5: In Fig. 3B controls were set to 130%. 130% of what?

R: Fig 3B (Fig 4B in the revised version) shows the mean patch currents expressed in pA, not %, at +25 mV in WT and KO cells.

Q6: The K613G mutation affects SLCO2A1 transporter activity, whereas the effect on Maxi-Cl activity was apparently not as pronounced. It may be informative to have both data side by side (at least in Discussion) as transporter and channel activity seem to require different parts/states of SLCO2A1 protein. For the general reader, it is not evident why the authors used the R560N mutant.

R: In revised Results section (page 8, lines 6-13), we have written that in the HEK293T expression system, the charge-neutralized and PGT function-impairing mutant, K613G, generated markedly reduced macro-patch currents (Fig 6C: blue column), with a markedly reduced single-channel conductance (Fig 6G: blue circles), reflecting a decrease in the total positive charge inside the lumen of the channel, and also that the K613G channels became relatively more selective to cations, because under a NaCl gradient, the reversal potential shifted, in a rightward direction, by 8.9 ± 1.5 mV, corresponding to P_{Na}/P_{Cl} = 1.9 ± 0.2 (Fig 6G: blue triangles). Also, in revised Discussion section (page 10, line 34 to page 11, line 2), we have noted that overexpression of SLCO2A1 charge-neutralized and PGT function-imparing mutants, K613G and R560N, considerable decreased the unitary current amplitude of Maxi-Cl in C127 cells.
Also, in the Discussion section of the revised manuscript (page 11, lines 9-11), we have noted that when transfected into HEK293T cells or reconstituted into proteoliposomes, the charge-neutralized and PGT function-impairing mutant, K613G, reversed anion-to-cation selectivity with exhibiting a markedly smaller single-channel conductance.

In addition, we have discussed the location of K613 residue by using the homology model of Fig EV5 on page 13 (lines 6-14) in the revised manuscript. Since the model was built using the crystal structure of the glycerol-3-phosphate transporter as a template, it most likely represents the "inward-open"-like transporter conformation state of SLCO2A1 as PGT. The K613 location is more distant from the central axis, but the following results suggest that in the open-channel conformation of SLCO2A1 as Maxi-Cl, the K613 residue may move closer towards the pore axis and thereby participating in the selectivity filter. Its neutralization (K613G) led to a change in anion-to-cation selectivity (Fig 6G: blue and green triangles; and Fig 7D: red circles) with inducing a decrease in the single-channel conductance (Fig 6G: blue circles; and Fig 7D: red circles). Before making more precise structural discussion about the pore construction, however, it must be determined whether the Cl^--conducting pathway is located within the single SLCO2A1 protein or between plural SLCO2A1 proteins in an oligomeric structure.

We used K613G and R560N mutants because these mutants are known to impair the transporter function of SLCO2A1 (Chan et al, 2002), as noted in the revised Results section (page 7, lines 13-15), and also because these mutations neutralize a positively charged residue which may interact with anions.

Q7: Concerning mutant SLCO2A1 proteins, it is recommended to include one of disease related SLCO2A1 mutants like Pro219Leu and to investigate transporter in comparison to channel properties.

R: In our additional experiments, according to the Referee comment, we tested the disease-causing mutants, G222R and P219L, which are mutated in pachydermoperiostosis (Zhang et al, 2012; Zhang et al, 2014), and found that they were successfully expressed in the periphery region including the plasma membrane of the cells (Fig EV4) but produced no evident channel activity (n=14 for each mutant). These results strongly suggest that the recombinant SLCO2A1 protein serves as the core component or the pore of Maxi-cl channel. These new results have been described on page 8 (lines 18-23) and discussed on page 11 (lines 6-7) of the revised manuscript.

In the revised Discussion section (page 12, line 35 to page 13, line 4), we have noted that the two residues, G222 and P219, are also close to the central axis, and this fact may therefore account for why no channel activity was observed with disease-causing mutants. We have illustrated the location of these residues on the homology model in Fig EV5 in the revised manuscript.

Q8: The authors claim that PGE2 blocks significantly SCLO2A1 related ATP-release as well as Maxi-Cl activity. I am not sure whether one may call a reduction of at most 10% really significant. In general, the ATP-release part of Results is the least convincing. Most effects, that are shown, are quite small or do not fit well with current data. For example, HEK cells have no endogenous Maxi-Cl activity, but show a substantial ATP-release. SLCO2A1 transfected HEK cells show a substantial swelling-induced Maxi-Cl activity, but in comparison the swelling-induced ATP release is small. These differences should be addressed.

R: The PGE2-induced reduction in ATP release from C127 cells at the level of around 10% was statistically significant (Fig EV2B) and just in good agreement with a reduction in the activity in native and reconstituted recombinant Maxi-Cl channels (Fig EV2A: left and right panels). This fact has been noted in the revised manuscript on page 9 (lines 31-35). The PGE2 effect is relatively small, probably, because of less efficient substrate binding to the SLCO2A1 conformation as the Maxi-Cl channel compared to the SLCO2A1 conformation as the PGT transporter. Such has been noted in the revised manuscript on page 6 (lines 14-17).

In the revised Results section (page 9, line 35 to page 10, line 12), we have noted that the HEK293T cells have no endogenous Maxi-Cl activity and displayed much less ATP release in response to the hypoosmotic stress compared to the C127 cells with presenting the absolute level of ATP release from HEK293T cells together with that from C127 cells (Fig EV2C). A trace of ATP release from swollen mock-transfected HEK293T cells may be mediated via some pathways other than Maxi-Cl, such as pannexins, connexins and exocytosis (Dubyak, 2012). Heterologous expression of SLCO2A1 and the K613G mutant significantly augmented the swelling-induced release of ATP from HEK293T cells, as expressed as % of Control in Fig EV2D. The relative effects of the gene expression on the swelling-induced ATP release (Fig EV2D) were less compared to the effects on the channel activity (Fig 6C), because 10 times less amount of the plasmid was transfected for ATP release experiments to avoid any deteriorating effect of SLCO2A1 overexpression observed with higher DNA doses (see Appendix Supplementary Methods). Also, it must be noted that ATP

release assay was performed with cell monolayers which contained not only GFP-positive but also GFP-negative cells, whereas patch-clamp recordings were made only from GFP-positive or well gene-transfected cells.

Q9: Discussion
Based on their results the authors propose that SLCO2A1 comes in two flavors or modes, one corresponds to its transporter activity, the other to its Maxi-Cl activity. This is very nice idea, but they should put more juice to their hypothesis. One could imagine that SCLO2A1 acts as a prostaglandin transporter in a monomeric state and as a Maxi-Cl channel in a dimeric state. Since Sabirov et al. have succeeded in the purification of the SCLO2A1 protein, it should not be too difficult to investigate a potential effect of swelling on the oligomerization status of SLCO2A1. A corresponding data set would constitute a considerable improvement.

R: We built a homology model of SLCO2A1 and discussed it in the revised Discussion section (page 12, line 29 to page 13, line 12) implying that PGE2, ATP and chloride share the same permeation or conductive pathway. We have also written (page 13, lines 12-24) as follows: "Before making more precise structural discussion about the pore construction, however, it must be determined whether the Cl^--conducting pathway is located within the single SLCO2A1 protein or between plural SLCO2A1 proteins in an oligomeric structure. Overexpression of the K613G mutant in C127 cells produced channels with the single-channel amplitude less than that of the native Maxi-Cl (Fig 5B-D) but higher than that observed upon overexpression of the same mutant in HEK293T cells lacking the endogenous SLCO2A1 (Fig 6G: blue circles). This fact suggests that the mutant protein may have combined with the endogenous WT SLCO2A1 in C127 cells, yielding channels with intermediate amplitudes, as evidenced by the broad distribution shown in Fig 5D (middle panel). Protein oligomerization could be actually detected on the non-reducing SDS-PAGE gel as faint protein bands with a molecular mass approximately twice and thrice of the monomer (see at arrowheads on Fig 7A). However, more elaborate structure-functional analysis will be necessary to clarify the true Maxi-Cl channel construction."
We agree with the Referee that the issue of swelling-induced oligomerization will be important to understand the true construction of the Maxi-Cl channel. We preliminarily attempted to monitor the swelling-induced oligomerization using native-blue electrophoresis, which, however, turns out to be a technically demanding method, and this attempt is to be left for a future study.

2nd Editorial Decision 01 September 2017

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by all three original referees and their comments are shown below. As you will see, the referees find that all criticisms have been sufficiently addressed and recommend the manuscript for publication, pending very minor text changes.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to receiving your final revision.

REFEREE REPORTS

Referee #1:

The authors thoroughly addressed all reviewers comments and in my opinion this highly significant manuscript is ready for publication.

Referee #2:

Sabirov et al. EMBOJ-2017-96685R

This revised manuscript has addressed the concerns raised in the initial review. The new controls

now provide a more convincing case that SLCO2A1 is a key component of the Maxi-Cl channel. Caveats, especially regarding the appearance of 'Maxi-Cl like' activity are clearly explained. Overall, it seems that this manuscript is an important advance for the field.

There is one point of presentation that should be addressed. In the introduction the authors assert on the last line of the first paragraph that Maxi-Cl is formed by a common core component denoted as MAC-1. This is the conclusion they would like to draw from the experiments, and seems out of place in the first paragraph of the introduction. It would seem more appropriate for them to state something like 'we present data that indicates (or suggests) that there a common component to Maxi-Cl that we name 'MAC-1'. It would seem best to leave this sort of statement as a bit more open ended than as presented

Referee #3:

The revised manuscript of Savirov et al. has considerably improved. Inclusion of more pharmacological experiments, additional studies with important disease-related mutants and, importantly, presentation of a strucutral model now provide strong evidence for the conclusions that SLCO2A1 constitutes core component of the Maxi-Cl channel. The detailed point-to-point response deals over all satisfactorily with the criticisms that were raised by the referees. The revised manuscript provides sufficient novelty to the Maxi-Cl channel field.

2nd Revision - authors' response 08 September 2017

Referee Comments (Qs) and *Authors Responses (Rs)*:

Referee #2:

Q1: There is one point of presentation that should be addressed. In the introduction the authors assert on the last line of the first paragraph that Maxi-Cl is formed by a common core component denoted as MAC-1. This is the conclusion they would like to draw from the experiments, and seems out of place in the first paragraph of the introduction. It would seem more appropriate for them to state something like 'we present data that indicates (or suggests) that there a common component to Maxi-Cl that we name 'MAC-1'. It would seem best to leave this sort of statement as a bit more open ended than as presented

R: According to the Referee's suggestion, in the Introduction section, we have replaced the sentence 'Thus, it can be deemed that Maxi-Cl is formed by a common core component, designated here MAC-1' with the following: 'In the present study, thus, we examined a possibility that there is a common core component for Maxi-Cl which we named MAC-1'

3rd Editorial Decision 12 September 2017

Thank you for submitting the final version of your manuscript to The EMBO Journal, I am pleased to inform you that your manuscript has now been officially accepted for publication here.

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Corresponding Author Name: Yasunobu Okada
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2017-96685

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was chosen to ensure the adequate statistical analysis of the obtained experimental results. The number of the data points is explicitly indicated on the figures and/or in the Legends. We have chosen the target confidence interval of 0.05 and used data variance and distribution type as a basis for the sample sizes, which varied from n=5 (minimal for the simple end-point well-reproducible measurements) up to several hundreds observations for the latency analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal studies, the sample size was chosen to ensure the adequate statistical analysis of the obtained experimental results.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Two types of criteria were used: 1) detail inspection for possible artifacts; and 2) 3 x sigma criterion when no obvious experimental error could be traced.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	The animals with same age and weight were randomly allocated to the Control and Treatment groups. In order to reduce any subjective biasis, ATP measurements in coronary effluent samples were performed by assistants who were unaware of the possible positive or negative outcome.
For animal studies, include a statement about randomization even if no randomization was used.	The animals with same age and weight were randomly allocated to the Control and Treatment groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	In order to reduce the subjective biasis, ATP measurements in coronary effluent samples were performed by assistants who were unaware of the possible positive or negative outcome.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was used in animal experiments
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. For channel amplitudes, the normal distribution was verified by histogram analysis. The channel latencies are distributed exponentially, and therefore exponential fits were used to derive the time constants and nonparametric Kolmogorov–Smirnov test to evaluate the differences.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Anti-SLCO2A1 polyclonal antibody was a kind gift from Prof. Ken-ichi Hosoya (Department of Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan). Ref. Tachikawa, M., et al. 2012. A clearance system for prostaglandin D2, a sleep-promoting factor, in cerebrospinal fluid: Role of the blood-cerebrospinal barrier transporters. J Pharmacol Exp Therap 343 (3): 608-616 Anti-Na+/K+ ATPase α1 monoclonal antibody (sc-21712) was purchased from Santa Cruz Biotech. Ref. Naito, D., et al. 2015. The coiled-coil domain of MURC/cavin-4 is involved in membrane trafficking of caveolin-3 in cardiomyocytes. Am J Physiol Heart Circ Physiol 309: H2127-H2136.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The mouse mammary cell line, C127, was obtained from the American Type Culture Collection. Mouse neuroblastoma; and C1300 cells were purchased from RIKEN BRC (Tsukuba, Japan).

* for all hyperlinks, please see the table at the top right of the document

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Wild-type C57BL/6J mice (9–10 weeks old, female) were purchased from Japan SLC Inc. Mice were kept in animal house in the National Institute for Physiological Sciences, and all the experimental procedures were done within 4 to 5 days after arrival.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The experimental protocol was approved by the Ethics Review Committee for Animal Experimentations of the National Institute for Physiological Sciences and by the Animal Care and Use Committee of Shiga University of Medical Science.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	The experimental protocols are compliant with the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We presented the results of the LC-MS/MS study of the bleb membrane proteins as Dataset EV1, which is only 88 kB and is readily available for the peer review process.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We would like to present the results of the LC-MS/MS study of the bleb membrane proteins as Dataset EV1, which is only 88 kB and is readily available for the peer review process.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Although our homology model is not central to our study, we presented it as Fig EV5. Since this model was built using a well-known I-TASSER algorism, ce cited the server name (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) with citing the original paper (Yang et al, 2015), instead of data deposition.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA