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The interaction of ATP11C-b with ezrin contributes to its polarized localization

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MS TITLE: Polarized localization of ATP11C-b requires interaction with ezrin

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Please see attachedThis manuscript aims to understand the mechanism mediating the polarized localization to contractile cell regions of ATP11C-b (P4-ATPase Flippase) that translocates phosphatidylserine (PS) and Phosphatidylethanolamine (PE) from the outer leaflet to the inner leaflet of the plasma membrane (PM).
ATP11C encodes for three splice variants, and only ATP11C-b shows polarized localization in contractile regions of migrating breast cancer cells (MDA-MB-231) and pro B-cells (BaF3), while the other spliceoforms are evenly distributed. The authors show that replacing three amino acids (LLY) of the ATP11C-b C-terminal with alanines causes uniform localization of ATP11C-b but does not influence the enzymatic flippase activity, indicating that the LLxY motif is required for polarized localization. Yeast two-hybrid and pull down assays show that the ATP11C-b LLxY motif specifically interacts with the FERM domain of the cortical actin linker protein ezrin, and weakly with other ERM proteins moesin and radixin. Studies with phospho-ezrin mutants show that ATP11C-b preferentially binds to the active ezrin confirmation (T567D), with no effect on its flippase activity. Ezrin (and ERM) knockdowns show they are required for polarized distribution of ATP11C-b, while ATP11c knockouts show a minor effect on activated ERM protein distribution.

Comments for the author

This paper is a straightforward and well-executed study of the mechanism of localization of a lipid flippase isoform, and reveals an interesting interaction with the cortical actin membrane-linking proteins of the ERM family. The experiments are well-performed and thorough. The paper will make a nice contribution to JCS once the following issues are dealt with.

Major comments-
1) Authors show that Atp11c-knockout can lead to the redistribution of p-ERM in the whole-cell. However, ERM proteins are well-known to bind to PI (3,4) P2, to mediate their localization. Does Atp11c knockout effect PI (3,4) P2 localization? Or is the effect on ERM proteins of Atp11c loss solely through the their interaction with each other. Pharmacological disruption of PI (3,4) P2 levels and/or biosensor localization in the knockout cells would answer this question.

2) ERM protein activity is important to maintain the uropod and back identity of the cells. Do authors see loss of cytoskeletal polarity in the Atp11c-knockout cells? This should be examined and quantified.

3) Discussion section para 6 where authors discuss Atp11c depletion did not cause a dramatic impact on steady-state polarization and migration might be due to only 50% reduction in PE-flipping activity. This hypothesis could be tested by pharmacological inhibition of PE activity in Atp11c depleted cells (as shown in Das et al., 2012), but at the least should be discussed.

Minor comments-
1) Results section para 1, line 11, ATP11C-a distribution in BaF3 cells is shown in figure 1Da, not 1Ca.
2) Results section para 5, mutations of L1108…. in yeast two-hybrid assay is shown in Figure 2E, not 2D.
3) Discussion section para 1, authors cite the ERM proteins contribute to neutrophil polarization by inhibiting Rac signaling (Liu et al., 2015),
this is an over the interpretation of the paper cited. ERM protein knockdown leads to a decrease in overall active Rac, Rho, and Cdc42 levels.
4) Discussion section para 3, statement ‘Therefore, ezrin and MPA linked to plasma membrane….protrusions’ is not very unclear. Also, the authors conclude ‘MPAct is concentrated in

the ATP11C-b localized plasma membrane region, Ezrin links ATP11C-b to the cell region where MPA is enriched', this seems to be an over-interpretation of data because, in 3ala mutants, MPAct localization is not impacted when ATP11c-b is redistributed to the whole cell. How does p-ERM look in this condition?

Reviewer 2

Advance summary and potential significance to field

The authors addressed the regulatory mechanism underlying the ATP11C-b localization to the restricted plasma membrane region in polarized motile cells.
Complementary methodological approaches were used. They identify the ezrin (and other ERM family proteins) as an interaction partner of ABC11C-b. They find that ABC11C-b expression is to a limited extent required for the polarized distribution of phosphorylated ezrin and that the expression of ezrin and/or other ERM family members is required for the polarized distribution of ABC11C-b in 13% / 35% of the cells. The enzymatic activity of ABC11C-b does not appear to be involved and the interaction between ABC11C-b and ezrin/ERM does not appear to affect steady state cell polarity. No functional assays have been performed. The data indicate that the interaction between ABC11C-b and ezrin affects each other's polarized distribution to some extent with unclear functional relevance.

Comments for the author

The conclusions drawn from the presented data are too strong and need adjustment (see comments below). Also, some experiments are performed in one cell type and other experiments are performed in another cell type. If the purpose of using two cell types is to demonstrate that any effect is not cell type-specific but applies to multiple polarized cell types, then all experiments should be performed in both cell types. There is a lack of functional relevance of the ABC11C-b-ezrin interaction.

Specific comments:
1. In figures 1-3 only on cell per conditionis depicted. However, for example Figure 6 shows that the polarized distribution of pERM is seen in only ~30% of all BaF3 cells (why is this?). Is this also the case for the exogenously expressed phospho-mimetic ezrin(TD)? If yes, then the images shown in figure 3 are not representative for the cell population. If no, then the discrepancy must be explained. For clarity, figures 1-3 require quantification and it should be shown to what extent do ABC11C-b and (endogenous) pERM colocalize in those cells where pERM is not confined to the cell body or uropod. In line with the observations in Baf3 cells, what percentage of MDA-MB-231 cells show a polarized distribution of pERM?
2. Figure 3C and 3F should also show the (lack of) co-localization of ABC11C-b and ezrin(TA).
3. Figure 5. Why only show MDA-MB-231 cells? The effect of ezrin/ERM knockdown on ABCB11C-b distribution should also be shown in BaF3 cells.
4. Figure 5. It would be on interest to see whether expression of the (dominant-negative) ezrin(TA) mutant affects the polarized distribution of ABC11C-b.
5. Figure 6. Why only show BaF3 cells? The effect of ABC11C-b knockout on pERM distribution should also be shown in MDA-MB-231 cells.
6. Figure 6. In the majority of cells, pERM is not enriched in the uropod. What is the reason for this? What is the distribution of ABC11C-b in the cells where pERM is not enriched in the uropod?
7. Figures 6 and 7. How is enrichment of pERM and MPAct in the uropod operationally defined? By quantitative analyses of microscopy images? This needs to be explained in the methods section.
8. The suggestion that the limited moslocalization of ABC11C-b in ezrin-depleted cells is due to the possible interaction of ABC11C-b with other polarized proteins is problematic. If this was the case, would a partial mislocalization not be expected in all cells instead of in only 13% of the cells?
9. The title is misleading as the polarized localization of ABC11C-b is lost in only 13% of Ezrin-depleted MDA-MB-231 cells, and the effect of ezrin depletion on ABC11C-b localization in BaF3 cells is not shown.
10. The sentence in the abstract "..., suggesting that the ERM proteins particularly ezrin, are crucial for the polarized localization of ATP11C-b." is misleading because the knockdown of ezrin affect the polarized localization of ABC11C-b in only 13% of MDA-MB-231 cells. As the polarized distribution of ABC11C-b was not affected in ~87% (!) of the ezrin-depleted cells, the term

"crucial" seems much to0 strong. Likewise, the statement "... in BaF3 cells, in which C-terminally phosphorylated ERM proteins (pERMs) were enriched at the uropod," is misleading as in ~70% (!) of the BaF3 cells, pERM was not enriched in the uropod (figure 6).
12. Because of the limited mutual effect on each other's polarized localization and the lack of a function relevance of this interaction, the authors may wish to determine how strong the observed interaction between ezrin and ABC11C-b (figure 2 and 4) really is, and/or whether interaction between the two proteins can be demonstrated in a cell lysate (e.g., by co-IP) or, preferentially, in living cells.

Reviewer 3

Advance summary and potential significance to field

The dynamic control of the transbilayer phospholipid asymmetry by scramblases and flippases is involved in many important physiological processes. Therefore, mechanisms regulating membrane-bound P4-ATPases localization and activity are clearly of broad interest to the cell biology community. In the present article, Hiroki Inoue and colleagues nicely demonstrated the mechanism allowing the P4-ATPase, ATP11C-b, to localize only in restricted cell areas in 2 different types of motile cells, the breast cancer cells MDA-MB-231 and the murine interleukin-3 dependent pro-B cells BaF3. In particular the authors demonstrated that ATP11C-b interacts with ERM proteins, especially Ezrin. This interaction is particularly well characterized as, through various complementary means (2-hybrid assays, GST-pull down, truncated mutants, recombinant proteins…) they identified the Nt-FERM domain of ezrin and the LLxY motif of ATP11C-b. Next, the authors convincingly showed that perturbing the expression of Ezrin (especially the phosphorylated open conformation) or ATP11C-b itself impact the polarized distribution of ATP11C-b and Ezrin, respectively.
Overall, this is a very interesting study for which the work is conducted with numerous appropriate methodologies. This topic is clearly of interest to the readers of Journal of Cell Science. I suggest few minor points that need to be addressed to improve the manuscript and warrant definite publication.

Comments for the author

-It seems that FERM domains of Moesin and Radixin interact as efficiently with ATP11C-b. This might be explored in cellulo. Are these 2 ERM proteins also polarized with ATP11C-b? This might also be correlated with the fact that only 13% of ATP11C-b are mislocalized in absence of Ezrin whereas 3 time more mislocalization are observed in absence of the 3 ERM.

-Upon Ezrin silencing, the localization of ATP11C-b appears more as punctate structure in the cytoplasm (Figure 5B). Is it specific and if yes, would it be possible that ATP11C-b mislocalized to vesicular compartments?

-The number of BaF3 cells exhibiting ERM enrichment in uropod (around 30 %, figure 6) appears quite low to me. This means that 70% of cells do not have a polarized distribution of ERM and Ezrin. Could the authors comment on that?
On the same line, in figure 3D, I find that the polarized localization of Ezrin-T567A does not appear so different than the one of Ezrin-T567D.

-It could have been interesting to better discuss why aminophospholipids flipping need to be restricted in particular cellular hot spot in the context of cell motility.

-Some of the images appear blured and/or out of focus:
Figure 5C, left panel, ATP11C-b and phalloidin staining, Figure 3F, ATP11C-b staining. Please show better images in these two cases.

-Figure 3 legend: why using different combination of phalloidin and secondary antibodies. This is confusing.

First revision

Author response to reviewers' comments

Response to reviewers

Major changes in Figures

Newly added data

Figure 6C and 6D
Supplementary Figure S2F and S2G
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S7

Modification of existing Figures
Figure 3F and Figure 5C panels

Modification of text
Highlighted with blue letters

Reviewer 1 Advance Summary and Potential Significance to Field:
Please see attachedThis manuscript aims to understand the mechanism mediating the polarized localization to contractile cell regions of ATP11C-b (P4-ATPase Flippase) that translocates phosphatidylserine (PS) and Phosphatidylethanolamine (PE) from the outer leaflet to the inner leaflet of the plasma membrane (PM). ATP11C encodes for three splice variants, and only ATP11C-b shows polarized localization in contractile regions of migrating breast cancer cells (MDA-MB-231) and pro B-cells (BaF3), while the other spliceoforms are evenly distributed. The authors show that replacing three amino acids (LLY) of the ATP11C-b C-terminal with alanines causes uniform localization of ATP11C-b but does not influence the enzymatic flippase activity, indicating that the LLxY motif is required for polarized localization. Yeast two-hybrid and pull down assays show that the ATP11C-b LLxY motif specifically interacts with the FERM domain of the cortical actin linker protein ezrin, and weakly with other ERM proteins moesin and radixin. Studies with phospho-ezrin mutants show that ATP11C-b preferentially binds to the active ezrin confirmation (T567D), with no effect on its flippase activity. Ezrin (and ERM) knockdowns show they are required for polarized distribution of ATP11C-b, while ATP11c knockouts show a minor effect on activated ERM protein distribution.

Reviewer 1 Comments for the Author:
This paper is a straightforward and well-executed study of the mechanism of localization of a lipid flippase isoform, and reveals an interesting interaction with the cortical actin membrane-linking proteins of the ERM family. The experiments are well-performed and thorough. The paper will make a nice contribution to JCS once the following issues are dealt with.
We deeply appreciate a reviewer’s positive review and constructive comments.

Major comments-
1)Authors show that Atp11c-knockout can lead to the redistribution of p-ERM in the whole-cell. However, ERM proteins are well-known to bind to PI (3,4) P2, to mediate their localization. Does Atp11c knockout effect PI (3,4) P2 localization? Or is the effect on ERM proteins of Atp11c loss solely through the their interaction with each other. Pharmacological disruption of PI (3,4) P2 levels and/or biosensor localization in the knockout cells would answer this question.
It has been shown that PI(4,5)P2 play a role for ezrin recruitment to the plasma membrane and ezrin activation (Senju et al., 2017 PNAS; Fievet et al., 2004 JCB). We could not find any literature showing that PI(3,4)P2 is required for the function of ezrin. Therefore, we do not think that it is useful to perform the experiments for the current manuscript. On the other hand, we have tested PI(4,5)P2 localization in MDA-MB-231 cells by transient expression but it does not show polarized localization and concentrated in membrane ruffles (Figure below). Therefore we do not further perform experiments to quantify the PI(4,5)P2 amounts.

***NOTE**: We have removed unpublished data that had been provided for the referees in confidence.*

Left, EGFP-tagged PLCd-PH domain was transiently expressed in MDA-MB-231 cells

2)ERM protein activity is important to maintain the uropod and back identity of the cells. Do authors see loss of cytoskeletal polarity in the Atp11c-knockout cells? This should be examined and quantified.
We performed new experiment to quantify the fluorescence of MPAct and phalloidin in Atp11c-knockout cells and parental BaF3 cells (new supplementary figure S7). The MPAct and phalloidin polarity was largely unaffected in the knockout cells. We added sentences in page 9.
We think that cell polarity may not be determined by a single factor, such as ATP11C-b or ezrin, but by combinatorial protein-protein and/or protein-lipid interactions. Indeed, CD44 (an ezrin-binding membrane protein) retains the polarized localization to the uropod in neutrophils lacking ezrin and moesin (Panicker et al., 2020 Blood Adv). We have discussed this point in the original manuscript (p.11, the last paragraph).

3)Discussion section para 6 where authors discuss Atp11c depletion did not cause a dramatic impact on steady-state polarization and migration might be due to only 50% reduction in PE-flipping activity. This hypothesis could be tested by pharmacological inhibition of PE activity in Atp11c depleted cells (as shown in Das et al., 2012), but at the least should be discussed.
In yeast studies including Das et al. 2012, toxins such as papuamide B and cinnamycin, are often used for measuring PS and PE exposure at the cell surface in steady states. As far as I know that the toxins, especially papuamide B, are not commercially available and should be produced by ourselves. And thus it is difficult to perform the experiments for the current manuscript. Moreover, we think that the PS or PE exposure in the lamellipodia and/or pseudopods in transiently polarized mammalian cells are very dynamic and transient, and the exposure level should be very low. It may not be easy to detect the transient PS and PE exposure.

Minor comments-
1)Results section para 1, line 11, ATP11C-a distribution in BaF3 cells is shown in figure 1Da, not 1Ca.
We have corrected it (page 4).

2)Results section para 5, mutations of L1108…. in yeast two-hybrid assay is shown in Figure 2E, not 2D.
We have corrected it (page 5).

3)Discussion section para 1, authors cite the ERM proteins contribute to neutrophil polarization by inhibiting Rac signaling (Liu et al., 2015), this is an over the interpretation of the paper cited. ERM protein knockdown leads to a decrease in overall active Rac, Rho, and Cdc42 levels.
We have corrected it (page 10).

4)Discussion section para 3, statement ‘Therefore, ezrin and MPA linked to plasma membrane….protrusions’ is not very unclear. Also, the authors conclude ‘MPAct is concentrated in the ATP11C-b localized plasma membrane region, Ezrin links ATP11C-b to the cell region where MPA is enriched’, this seems to be an over-interpretation of data because, in 3ala mutants, MPAct localization is not impacted when ATP11c-b is redistributed to the whole cell. How does p-ERM look in this condition?
We have revised the sentence in page 11.

We did not perform an experiment to investigate the dominant negative effect of ATP11c-b(3ala) on the localization of p-ERM. Because we have already shown that the mislocalization of p-ERM in *Atp11c*-KO cells cannot be rescued by expression of ATP11C-b(3ala) (Figure 6). Therefore, the interaction of ezrin with ATP11C-b is required for the polarized localization of pERM.

Reviewer 2 Advance Summary and Potential Significance to Field:
The authors addressed the regulatory mechanism underlying the ATP11C-b localization to the restricted plasma membrane region in polarized motile cells.
Complementary methodological approaches were used. They identify the ezrin (and other ERM

family proteins) as an interaction partner of A~~BC~~TP11C-b. They find that A~~BC~~TP11C-b expression is to a limited extent required for the polarized distribution of phosphorylated ezrin and that the expression of ezrin and/or other ERM family members is required for the polarized distribution of A~~BC~~TP11C-b in 13% / 35% of the cells. The enzymatic activity of A~~BC~~TP11C-b does not appear to be involved and the interaction between A~~BC~~TP11C-b and ezrin/ERM does not appear to affect steady state cell polarity. No functional assays have been performed. The data indicate that the interaction between A~~BC~~TP11C-b and ezrin affects each other's polarized distribution to some extent with unclear functional relevance.

Reviewer 2 Comments for the Author:
The conclusions drawn from the presented data are too strong and need adjustment (see comments below). Also, some experiments are performed in one cell type and other experiments are performed in another cell type. If the purpose of using two cell types is to demonstrate that any effect is not cell type-specific but applies to multiple polarized cell types, then all experiments should be performed in both cell types. There is a lack of functional relevance of the A~~BC~~TP11C-b-ezrin interaction.
We thank to reviewer for the critical comments. As we described in the discussion, although it remains to be solved the physiological roles of polarized localization of ATP11C-b, we were able to demonstrate the mechanism of polarized localization partly among ezrin, MPA and ATP11C-b in the manuscript.

Specific comments:
1. In figures 1-3 only on cell per conditionis depicted. However, for example Figure 6 shows that the polarized distribution of pERM is seen in only ~30% of all BaF3 cells (why is this?). Is this also the case for the exogenouslyexpressed phospho-mimetic ezrin(TD)? If yes, then the images shown in figure 3 are not representative for the cell population. If no, then the discrepancy must be explained. For clarity, figures 1-3 require quantification and it should be shown to what extent do A~~BC~~TP11C-b and (endogenous) pERM colocalize in those cells where pERM is not confined to the cell body or uropod.
Ezrin(TD) mutant and ATP11C-b are exclusively localized to the uropod. The quantitative analysis of ATP11C-b was shown in the previous our paper and it was exclusively localized to the uropod (almost 100%) (Takayama et a., 2019 JCS). Here we included additional quantification data of ezrin(TD) and ezrin(WT) localization in supplementary figure S3.
Moreover, we added new quantification data of pERM localization in the revised manuscript (Figure 6C and D). pERM is localized to the entire plasma membrane but polarized in significant population of cells. We think ezrin can be phosphorylated not only in the uropod but also in pseudopods and the dynamic phosphorylation/dephosphorylation cycle of ezrin may play an important role in pseudopods formation. Indeed, the dephosphorylation (inactivation) of moesin at the leading edge is required for the neutrophil polarization (Liu et al., 2015 JEM). The phosphorylated ezrin can be accumulated and stabilized at the uropod and the cell body via interaction with MPA(membrane-proximal F-actin) and membrane proteins by crosslinking each other. Therefore, we think that the phosphomimetic mutant which cannot cycle between phosphorylation and dephosphorylation was predominantly accumulated in the uropod and the cell body region.

In line with the observations in Baf3 cells, what percentage of MDA-MB-231 cells show a polarized distribution of pERM?
Although ezrin(TD) mutant was exclusively concentrated in the cell body region, pERM was not concentrated in the cell body region and also localized to the lamellipodia and membrane ruffles in MDA-MB-231 cells. Therefore, we think that ERM phosphorylation/dephosphorylation occurs in the lamellipodia and pseudopods where membrane shape changes actively occur.

2. Figure 3C and 3F should also show the (lack of) co-localization of A~~BC~~TP11C-b and ezrin(TA).
The data was added in the Supplementary Figure S2F and G in the revised manuscript.

3. Figure 5. Why only show MDA-MB-231 cells? The effect of ezrin/ERM knockdown on A~~BCB~~TP11C-b distribution should also be shown in BaF3 cells.
It is impossible to transfect siRNA into BaF3 cells transiently, therefore we have performed the knockdown experiment in MDA-MB-231 cells.

4. Figure 5. It would be on interest to see whether expression of the (dominant-negative) ezrin(TA) mutant affects the polarized distribution of A~~BC~~TP11C-b.
We have added Supplementary Figure S2F and G. The ATP11C-b localization was not affected upon expression of ezrin(TA) mutant.

5. Figure 6. Why only show BaF3 cells? The effect of A~~BC~~TP11C-b knockout on pERM distribution should also be shown in MDA-MB-231 cells.
We have not established ATP11C-b-KO using MDA-MB-231 cells. Sometimes it is problematic to establish KO cells using cancer cells due to uncertain karyotype. So we firstly established KO cells using BaF3 cells.

6. Figure 6. In the majority of cells, pERM is not enriched in the uropod. What is the reason for this? What is the distribution of A~~BC~~TP11C-b in the cells where pERM is not enriched in the uropod?
Please see the response of number 1. We showed that ATP11C-b was exclusively localized to the uropod (almost 100%) (Takayama et al., 2019 JCS).

7. Figures 6 and 7. How is enrichment of pERM and MPAct in the uropod operationally defined? By quantitative analyses of microscopy images? This needs to be explained in the methods section.
Please see the response of number 1. In addition, we also compared the fluorescence intensities of MPAct and phalloidin from the back to the front of BaF3 cells by line-scan and added new data in new Supplementary Figure S7.

8. The suggestion that the limited moslocalization of A~~BC~~TP11C-b in ezrin-depleted cells is due to the possible interaction of A~~BC~~TP11C-b with other polarized proteins is problematic. If this was the case, would a partial mislocalization not be expected in all cells instead of in only 13% of the cells?

Ezrin depletion inhibited cell adhesion and cell spreading as we described in the original manuscript (page 8, in the first paragraph). We also added a sentence in the discussion (page 10). The more ezrin is depleted, the more cells are detached from the cover slips and thus, we may underestimate the effect of ezrin knockdown.

9. The title is misleading as the polarized localization of A~~BC~~TP11C-b is lost in only 13% of Ezrin-depleted MDA-MB-231 cells, and the effect of ezrin depletion on A~~BC~~TP11C-b localization in BaF3 cells is not shown.
We have revised the title.

10. The sentence in the abstract "..., suggesting that the ERM proteins, particularly ezrin, are crucial for the polarized localization of ATP11C-b." is misleading because the knockdown of ezrin affect the polarized localization of A~~BC~~TP11C-b in only 13% of MDA-MB-231 cells. As the polarized distribution of A~~BC~~TP11C-b was not affected in ~87% (!) of the ezrin-depleted cells, the term "crucial" seems much to0 strong. Likewise, the statement "... in BaF3 cells, in which C-terminally phosphorylated ERM proteins (pERMs) were enriched at the uropod," is misleading as in ~70% (!) of the BaF3 cells, pERM was not enriched in the uropod (figure 6).
We have revised the sentence (page 1).

12. Because of the limited mutual effect on each other's polarized localization and the lack of a function relevance of this interaction, the authors may wish to determine how strong the observed interaction between ezrin and A~~BC~~TP11C-b (figure 2 and 4) really is, and/or whether interaction between the two proteins can be demonstrated in a cell lysate (e.g., by co-IP) or, preferentially, in living cells.
We performed the IP experiment but it was not successful. We think that the interaction may not be stable enough to detect by IP.

Reviewer 3 Advance Summary and Potential Significance to Field:
The dynamic control of the transbilayer phospholipid asymmetry by scramblases and flippases is involved in many important physiological processes. Therefore, mechanisms regulating membrane-bound P4-ATPases localization and activity are clearly of broad interest to the cell biology community. In the present article, Hiroki Inoue and colleagues nicely demonstrated the mechanism allowing the P4-ATPase, ATP11C-b, to localize only in restricted cell areas in 2 different types of motile cells, the breast cancer cells MDA-MB-231 and the murine interleukin-3 dependent pro-B

cells BaF3. In particular the authors demonstrated that ATP11C-b interacts with ERM proteins, especially Ezrin. This interaction is particularly well characterized as, through various complementary means (2-hybrid assays, GST-pull down, truncated mutants, recombinant proteins…) they identified the Nt-FERM domain of ezrin and the LLxY motif of ATP11C-b. Next, the authors convincingly showed that perturbing the expression of Ezrin (especially the phosphorylated open conformation) or ATP11C-b itself impact the polarized distribution of ATP11C-b and Ezrin, respectively.
Overall, this is a very interesting study for which the work is conducted with numerous appropriate methodologies. This topic is clearly of interest to the readers of Journal of Cell Science. I suggest few minor points that need to be addressed to improve the manuscript and warrant definite publication.
We deeply appreciate the reviewer's constructive comments.

Reviewer 3 Comments for the Author:
-It seems that FERM domains of Moesin and Radixin interact as efficiently with ATP11C-b. This might be explored in cellulo. Are these 2 ERM proteins also polarized with ATP11C-b? This might also be correlated with the fact that only 13% of ATP11C-b are mislocalized in absence of Ezrin whereas 3 time more mislocalization are observed in absence of the 3 ERM.
We have added supplementary figure S4 showing localization of moesin(TD) and radixin(TD) and added a sentence in page 8. As reviewer mentioned, ERM proteins somewhat redundantly play a role in the polarized localization of ATP11C-b although the interaction was not as strong as ezrin (Figure 2D).

-Upon Ezrin silencing, the localization of ATP11C-b appears more as punctate structure in the cytoplasm (Figure 5B). Is it specific and if yes, would it be possible that ATP11C-b mislocalized to vesicular compartments?
No, we do not think so. Some punctate signals are shown along the plasma membrane and ruffles.

-The number of BaF3 cells exhibiting ERM enrichment in uropod (around 30 %, figure 6) appears quite low to me. This means that 70% of cells do not have a polarized distribution of ERM and Ezrin. Could the authors comment on that?
It is true. We think ezrin can be phosphorylated not only in the uropod but also in pseudopods although it is not clear whether the dynamic phosphorylation/dephosphorylation cycle of ezrin play a role in pseudopods. Indeed, the dephosphorylation (inactivation) of moesin at the leading edge is required for the neutrophil polarization (Liu et al., 2015 JEM). On the other hand, the phosphorylated ezrin can be accumulated and stabilized at the uropod and cell body by interaction with MPA and membrane proteins by crosslinking each other. Therefore, the phosphomimetic mutant which cannot cycle between phosphorylation and dephosphorylation was predominantly accumulated in the uropod and the cell body.
In the revised manuscript, we added new quantification data of pERM localization (new Figure 6C and D).

On the same line, in figure 3D, I find that the polarized localization of Ezrin-T567A does not appear so different than the one of Ezrin-T567D.
We appreciate criticizing this point. Although ezrin(TD) mutant exclusively localized to the uropod, ezrin(TA) and ezrin(WT) localized uropod as well as pseudopods although it seems to be polarized to some extent. We revised the sentence in the manuscript (page 7) and added new quantification data for the localization of ezrin(WT) and ezrin(TD) (new supplementary figure s3).

-It could have been interesting to better discuss why aminophospholipids flipping need to be restricted in particular cellular hot spot in the context of cell motility.
We appreciate raising the point. We have revised the discussion part (page 12).

-Some of the images appear blured and/or out of focus:
Figure 5C, left panel, ATP11C-b and phalloidin staining, Figure 3F, ATP11C-b staining. Please show better images in these two cases.
We have exchanged with new images.

-Figure 3 legend: why using different combination of phalloidin and secondary antibodies. This is

confusing.
We additionally indicated each panel (A), (C), (D), and (F) in the figure legend to avoid confusion.

Second decision letter

MS ID#: JOCES/2021/258523

MS TITLE: The interaction of ATP11C-b with ezrin contributes to its polarized localization

AUTHORS: Hiroki Inoue, Hiroyuki Takatsu, Asuka Hamamoto, Masahiro Takayama, Riki Nakabuchi, Yumeka Muranaka, Tsukasa Yagi, Kazuhisa Nakayama, and Hye-Won Shin
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. In particular, reviewer #3 posed a question and a discussion point for you to address. I hope that you will be able to address these concerns because I would like to be able to accept your paper, depending on further comments from reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 2

Advance summary and potential significance to field
Several adjustments have been made in response to my earlier comments, but some minor issues remain.

Comments for the author

The authirs state that ATP11C-b localization was not affected upon expression of ezrin(TA) mutant. Would mislocalization of ATP11C-b not be expected in ezrin(TA)-overexpressing cells as the authors [ropose that ezrin phosphorylation is required to stabilize the polarized distribution of ATP11C-b?
A quick Google search shows that transient siRNA in BaF3 cells has succesfully been done.
I still think the effect of ABCTP11C-b knockout on pERM distribution should also be investigated in MDA-MB-231 cells. The authors choose to study two cell lines in parallel and key experiments should then also be performed in both.

The limited mutual effect on each other's polarized localization and the lack of a function relevance of the interaction between ezrin and ATPC11-
b remains an issue which should be more explicitely discussed.

Reviewer 3

Advance summary and potential significance to field

The dynamic control of the transbilayer phospholipid asymmetry by scramblases and flippases is involved in many important physiological processes. Therefore, mechanisms regulating membrane-bound P4-ATPases localization and activity are clearly of broad interest to the cell biology community. In the present article, Hiroki Inoue and colleagues nicely demonstrated the mechanism allowing the P4-ATPase, ATP11C-b, to localize only in restricted cell areas in 2 different types of motile cells, the breast cancer cells MDA-MB-231 and the murine interleukin-3 dependent pro-B cells BaF3. In particular the authors demonstrated that ATP11C-b interacts with ERM proteins, especially Ezrin. This interaction is particularly well characterized as, through various complementary means (2-hybrid assays, GST-pull down, truncated mutants, recombinant proteins…) they identified the Nt-FERM domain of ezrin and the LLxY motif of ATP11C-b. Next, the authors convincingly showed that perturbing the expression of Ezrin (especially the phosphorylated open conformation) or ATP11C-b itself impact the polarized distribution of ATP11C-b and Ezrin, respectively.
Overall, this is a very interesting study for which the work is conducted with numerous appropriate methodologies. This topic is clearly of interest to the readers of Journal of Cell Science. I recommend to accept this revised version for publication.

Comments for the author

All my concerns has been successfully addressed. Moreover, I think that the article has been nicely improved and warrant publication.

Second revision

Author response to reviewers' comments

Reviewer 2 Advance Summary and Potential Significance to Field...
Several adjustments have been made in response to my earlier comments, but some minor issues remain.

Reviewer 2 Comments for the Author...
The authirs state that ATP11C-b localization was not affected upon expression of ezrin(TA) mutant. Would mislocalization of ATP11C-b not be expected in ezrin(TA)-overexpressing cells as the authors [ropose that ezrin phosphorylation is required to stabilize the polarized distribution of ATP11C-b?
Unfortunately, I could not understand the question. Does reviewer mean ‘Would mislocalization of ATP11C-b **BE** expected in ezrin(TA)-overexpressing cells as the authors PROPOSE that ….’? If my understanding is correct, the answer is that the C-terminal region of ATP11C-b did not interact with ezrin(TA) mutant (Figure 2C) and thus expression of the TA mutant did not affect the localization of ATP11C-b.

A quick Google search shows that transient siRNA in BaF3 cells has succesfully been done.
We have no idea about it. It is widely accepted that BaF3 cell lines are almost impossible to introduce plasmids by usual lipofection. In our hands, we failed transfection by lipofection in BaF3 cells.

I still think the effect of A~~BC~~TP11C-b knockout on pERM distribution should also be investigated in MDA-MB-231 cells. The authors choose to study two cell lines in parallel and key experiments should then also be performed in both.

As we describe previous responses, although ezrin(TD) mutant was exclusively concentrated in the cell body region, pERM was also localized to some regions of membrane ruffles in addition to the cell body in MDA-MB-231 cells. Here I attach figures to convince the reviewer. Therefore, we think that phosphorylation of ezrin also occurs in the membrane ruffles and phosphorylation/dephosphorylation cycle of ezrin might be important for proper lamellipodia and pseudopods formation. We thought that MDA-MB-231 cells might not be suitable for quantitative analysis. Specifying the peak fluorescence intensities of pERM between lamellipodia and cell body in MDA-MB-231 cells seemed to be very difficult, because pERM localized to internal side from the plasma membrane at the lamellipodia unlike ezrin and heterogeneously localized at the cell body and lamellipodia (Figure in this letter). Moreover, KO experiments could not be a minor revision. I hope the reviewer generously considers it.
We did not insist that the ATP11C-b is the only factor for the polarized localization of pERM and we described that 'Because ERM proteins can interact with other uropod components, such as CD44 and PSGL-1, these plasma membrane proteins could also contribute to pERM stabilization at the uropod' in the last paragraph in page 11 (red colored).

***NOTE**: We have removed unpublished data that had been provided for the referees in confidence.*

The limited mutual effect on each other's polarized localization and the lack of a function relevance of the interaction between ezrin and ATPC11-b remains an issue which should be more explicitely discussed.
We have already described the lack of a functional relevance in the first paragraph of page 12 in the original manuscript (red colored). And we discussed a putative function of the interaction between ezrin and ATP11C-b in the following sentences of the manuscript and we also discussed it in the first paragraph of page 11. Those sentences are what we can discuss based on our current data.

Reviewer 3 Advance Summary and Potential Significance to Field...

The dynamic control of the transbilayer phospholipid asymmetry by scramblases and flippases is involved in many important physiological processes. Therefore, mechanisms regulating membrane-bound P4-ATPases localization and activity are clearly of broad interest to the cell biology community. In the present article, Hiroki Inoue and colleagues nicely demonstrated the mechanism allowing the P4-ATPase, ATP11C-b, to localize only in restricted cell areas in 2 different types of motile cells, the breast cancer cells MDA-MB-231 and the murine interleukin-3 dependent pro-B cells BaF3. In particular the authors demonstrated that ATP11C-b interacts with ERM proteins, especially Ezrin. This interaction is particularly well characterized as, through various complementary means (2-hybrid assays, GST-pull down, truncated mutants, recombinant proteins…) they identified the Nt-FERM domain of ezrin and the LLxY motif of ATP11C-b. Next, the authors convincingly showed that perturbing the expression of Ezrin (especially the phosphorylated open conformation) or ATP11C-b itself impact the polarized distribution of ATP11C-b and Ezrin, respectively.
Overall, this is a very interesting study for which the work is conducted with numerous appropriate methodologies. This topic is clearly of interest to the readers of Journal of Cell Science. I recommend to accept this revised version for publication.

Reviewer 3 Comments for the Author...

All my concerns has been successfully addressed. Moreover, I think that the article has been nicely improved and warrant publication.
We appreciate all constructive comments.

Third decision letter

MS ID#: JOCES/2021/258523

MS TITLE: The interaction of ATP11C-b with ezrin contributes to its polarized localization

AUTHORS: Hiroki Inoue, Hiroyuki Takatsu, Asuka Hamamoto, Masahiro Takayama, Riki Nakabuchi, Yumeka Muranaka, Tsukasa Yagi, Kazuhisa Nakayama, and Hye-Won Shin
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.