THE EMBO JOURNAL

Manuscript EMBO-2010-73942

USP9x-mediated deubiquitination of EFA6 regulates de novo tight junction assembly

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Review timeline:

Submission date:	4 June 2009
Editorial Decision:	25 June 2009
New Submission Received:	15 February 2010
Editorial Decision:	25 February 2010
Revision received:	02 March 2010

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 25 June 2009

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments to the authors are provided below. As you can see, there is clearly an interest in your study and the findings that USP9x plays a role in TJ assembly. However, the referees also raise many concerns with the paper and the conclusiveness of the findings reported. The referees raise concerns with the key experiments and it is clear that at present that the analysis is not well suited for publication here. Given that extensive work would have to be carried out to resolve the raised concerns as well as the uncertainty of the experimental outcome, I am afraid that I cannot offer to commit to a revised manuscript at this stage. I therefore see no other choice but to reject the manuscript. However, given the interest in the topic, I am not excluding to take a look at another submission should you be able to add data that would address the concerns raised in full and to strengthen the findings along the lines as suggested by the referees. However, I should point out that such a submission will be treated as a new submission, rather than a revision. For resubmissions we consider the novelty of data at the time of resubmission and may, if needed, bring in new referee(s).

For the present submission, I am sorry that we cannot be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

EFA6 activates Arf6 and plays a role in tight junction (TJ) formation. The authors observed that in MDCK cells EFA6B protein level temporally increased after calcium switch. The authors also found that EFA6B was poly-ubiquitinated in MDCK cells by using an anti-ubiquitin antibody. In USP9x knockdown cells, TJ formation was delayed; when EFA6 was overexpressed in these cells, TJ formation was restored. This manuscript is the first one to show that the deubiquinating enzyme USP9x protects EFA6 protein and functions during TJ assembly.

Major concern:

EFA6A and EFA6B are similar in Sec7 and PH domains, however, they have distinct expression pattern in tissues. The authors used EFA6A to rescue EFA6B knockdown cells. This is confusing since we don't know whether EFA6A and EFA6B functions the same in cells. Indeed, the authors couldn't get a complete TJ barrier function rescue by expressing EFA6A in USP9x knockdown cells. Furthermore, they couldn't co-precipitate USP9x and EFA6B from epithelial cells (MDCK), despite of PHCter domain from EFA6A interacting with USP9x.

Other Issues:

1)The authors mentioned Derrien's paper to address that there are four isoforms of EFA6[1] and a reference for the EFA6B antibody[2]. cDNA of EFA6B encodes 1056 amino acids and its estimated molecular weight (MW) is 116 kDa and Derrien showed the overexpressed protein migrates with a MW of 180 kDa in two different cell types. However, in the manuscript, the authors state that EFA6B expresses as a 66 kDa protein when not ubiquitinated. The reference for their EFA6B antibody does not address the antibody at all. The authors must address the discrepancy in the MW of EFA6B, since the interpretation of their data can become completely different on the specificity of the antibody. For example, in figure 1.d, the total level of EFA6B is increased between 30 and 60 min after calcium repletion. However, the level of total immunoprecipitated EFA6B is increased only at 60 min after calcium repletion in figure 2.d. Also, a band slightly bigger than 97 kDa was not detected with anti-ubiquitin antibody, FK-1 in figure 2.d. There is no clear mention about whether MG-132 or lactacystin was treated in the ubiquitination studies after figure 2.c. Ubiquitination was visible only in the presence of proteasome inhibitor in figure 2.c. However, in figure 2.d, band shifts with ubiquitination is clearly visible, though the intensity of shifted bands is dramatically decreased after 60 min time point. Regardless of whether the authors treated MG-132 to visualize ubiquitinated EFA6B in panel d, top band weakly visible above 200 kDa, not shown in panel c, seems to be less convincingly ubiquitinated. Immunoblotting of ubiquitinated ectopic EFA6A shows multiple bands. However, in figure 2. c and d that show ubiquitination of EFA6B, this reviewer can see only one band convincingly ubiquitinated and still, its MW looks less than reported MW of EFA6B.

The authors propose that USP9x is responsible for the protection of EFA6 from deubiquitination of EFA6. Though the authors show the level of ectopic EFA6A, USP9x in figure 3.c, total level of ectopically expressed ubiquitin is not shown. Dot blot analysis of the level of ubiquitin will be enough to validate the reduced ubiquitination of EFA6A in the presence of ectopic WT USP9x. In figure 3.e, the authors try to address that USP9x is a specific deubiquitination enzyme for EFA6 from specific gene knock down of USP7 or USP9x. This reviewer wonders if the experiment was done in the presence of proteasome inhibitor or not. Considering that shifted bands were seen only at 60 min time point in USP7 gene knock down, I assume that proteasome inhibitor was not added. However, again, figure 3.e is contradictory to figure 2.c in that in figure 2.c, ubiquitination was visible only in the presence of proteasome inhibitor.

3)if the total level of EFA6 is increased briefly after formation of adherence junctions, immunofluorescence observation should validate it by showing an increase and subsequent possible reduction of EFA6 signals in junction formation. The authors in previous MBC papers biochemically showed that EFA6 association to junction is increased after calcium repletion. It would be helpful to show whether the level of EFA6 on plasma membrane persists or decreases, in accordance with the change in total level, in the process of tight junction formation.

4) Gene knock down of afadin is shown to hamper the recruitment of adherens and tight junctional proteins together[3]. The author's data that gene knock down of USP9x caused a decrease in the level of afadin raises important questions in addition to the validation of their EFA6B antibody.
a) would there be also a decrease in the level of afadin in EFA6 gene knock down?
b) the authors state that overexpression of afadin did not rescue the effects of USP9x deficiency (Data not shown). More analysis is required

[1] Derrien V, Couillault C, Franco M, Martineau S, Montcourrier P, Houlgatte R, Chavrier P. A conserved C-terminal domain of EFA6-family ARF6-guanine nucleotide exchange factors induces lengthening of microvilli-like membrane protrusions. J Cell Sci. 2002;115:2867-79.
[2] Marshansky V, Bourgoin S, Londono I, Bendayan M, Vinay P. Identification of ADP-ribosylation factor-6 in brush-border membrane and early endosomes of human kidney proximal tubules. Electrophoresis. 1997;18:538-47.
[3] Sato T, Fujita N, Yamada A, Ooshio T, Okamoto R, Irie K, Takai Y. Regulation of the assembly and adhesion activity of E-cadherin by nectin and afadin for the formation of adherens junctions in Madin-Darby canine kidney cells. J Biol Chem. 2006;281:5288-99.

Referee #2 (Remarks to the Author):

The manuscript by Thèard et al. describes a role for the ARF6 guanine nucleotide exchange factor EFA6 for tight junction formation in polarizing epithelial cells. The authors have shown previously that ARF6 is transiently upregulated during Ca2+-switch (CS) -induced cell-cell contact formation and that ectopic expression of EFA6 accelerates TJ formation. In this manuscript, the authors find that EFA6 is constitutively degraded by the ubiquitin-proteasome pathway and that it is transiently stabilized during CS-induced cell-cell contact formation through its association with the deubiquitinating enzyme USP9x. The model derived from the data implicates USP9x as an EFA6-deubiquitinating enzyme responsible for the transient increase in EFA6 levels which regulate the activity of ARF6 during cell-cell contact and TJ formation. This is a very interesting paper providing a molecular mechanism by which the levels of a small GTPase are specifically upregulated during cell-cell contact formation by the activity of a deubiquitinating enzyme.

Specific Points:
1. One drawback of the paper is the lack of biochemical evidence for the endogenous interaction of EFA6 and USP9x in MDCK epithelial cells. The authors show co-immunoprecipitations from synaptosomal fractions which are obviously only suggestive. Given the functional data, it is to be expected that the two proteins exist in a complex, perhaps only transiently. The authors should try to co-immunoprecipitate the EFA6 and USP9x at different stages during CS-induced cell-cell contact formation, preferably 30 and 60 min after readditon of Ca2+. In addition, a chemical crosslinker could be used to stabilize the predicted interaction. Furthermore, a proteasome inhibitor could be used to increase the levels of EFA6. These experiments could be further complemented by in vitro direct binding assays using recombinant proteins. If a direct interaction can be demonstrated it would at least further support a physical association of EFA6 and USP9x.
2. The second drawback of the paper is the lack of convincing evidence for a co-localization of EFA6 and USP9x during cell-cell contact formation. In Fig. 4, the authors analyze the subcellular localization of EFA6 and USP9x. A clear co-localization of EFA6A and USP9x can be observed at the tips of filopodia which is not in contact with another cell (Fig. 4f). However, the evidence for co-localization at cell-cell contacts is only very weak if at all. The authors state in the text that during early stages of cell-cell contact formation, the two proteins co-localize at cell-cell contacts as well. However, the signal for USP9x is highly overexposed and as a consequence USP9x seems to localize throughout the entire cell (Fig. 4 h, h'). Therefore, any protein expressed by the adjacent cell and present at the contact area would result in a merge signal. In addition, these figures show the tip of a single filopodium. The predicted function of EAF6 to regulate ARF6 activity which in turn regulates the formation of the actin cytoskeleton at sites of cell-cell contact formation would clearly require the presence of not only EAF6 but also of USP9x during longer and/or later periods of lateral cell-cell contact formation (such as shown for EAF6 in Fig. 4b, c), not only at the very tip of the initial contact. Thus, the evidence of a co-localization of EFA6 and USP9x during cell-cell contact formation is too weak to support the notion that they co-distribute and that USP9x is present at cell-cell contacts during critical stages of cell-cell contact formation.

3. The authors do not comment on the regulation of USP9x during cell-cell contact formation. What are the levels of USP9x in MDCK cells during CS-induced cell-cell contact formation? Do they change?

Minor points:
- in the graphs displaying TER, the Y-axis label must be ohms.cm2 but not ohms.cm-2 due to the inverse relationship between area and ohmic resistance
- in the Introduction, the authors ignore the described role of the Par - aPKC complex during the temporal and spatial regulation of TJs in response to de novo E-cadherin-mediated cell-cell adhesion

Referee #3 (Remarks to the Author):

This manuscript from Thèard and colleagues is focused on the mechanisms underlying the temporal regulation of EFA6 during TJ biogenesis. Through a series of complementary biochemical and cell biological approaches, the authors make a series of key findings. They show that enzymatically active EFA6 is required for de novo TJ assembly using an siRNA approach. They further make the critical observation that EFA6 stability is regulated by the ubiquitin-proteasome system and that the deubiquitinating enzyme, USP9x, counters EFA6 turnover early in TJ biogenesis. Corroborating evidence for the interplay between EFA6 and USP9x is provided in the form of pulldown assays, co-localization studies, and siRNA/rescue experiments. Further, the authors show that USP9x over-expression can reduce the levels of ubiquitin-modified EFA6. Overall, the data are of high quality, suitable specificity controls are included in most experiments, and this works represents a significant advance in our understanding of how TJ biogenesis can be modulated by specific enzymes.

Prior to publication, the below list of shortcomings should be appropriately addressed.
1. In the legend for Supplemental Fig. 1C, reference is made to upper and lower panels but the authors intend to indicate left and right panels.
2. In Supplemental Fig. 1C, the half-life of EFA6B looks bi-phasic with only the first phase having a steeper slope than the two comparison proteins. An explanation or comment should be provided by the authors regarding this bi-phasic pattern.
3. In Fig. 2B, the authors show that GST-S5a can precipitate myc-ubiquitin conjugates from a transfected cell lysate but as described, this experiment does not prove that these conjugates are attached to EFA6B (which appears to be the interpretation the authors make from the data). The authors should precipitate ubiquitin-modified EFA6B from the cells, elute the precipitated proteins, and then demonstrate that GST-S5a can selectively recover the ubiquitin-conjugated, eluted EFA6B.
4. In Fig. 2D, although the 105 kDa band is visible on the anti-EFA6B blot (right panel), it is not visible on the anti-ubiquitin blot (left panel).
5. Regarding the data presented in Fig. 3B, is the PH domain sufficient for the interaction between EFA6B and USP9x? This should be tested and shown in the manuscript. Also, the authors should test if over-expression of the minimal USP9x-binding domain of EFA6A can phenocopy the data obtained with siRNA-targeting USP9x.
6. In Fig. 3C, the arrow next to vsvg-EFA6A is pointing to a blank area of the blot.
7. In Fig. 3C, an explanation should be provided for why the levels of vsvg-EFA6A in lane 1 are higher than the levels in lane 3 where USP9x expression should be increasing the levels of unmodified vsvg-EFA6A by reducing the levels of ubiquitin-modified vsvg-EFA6A.
8. The authors should show that over-expression of a different deubiquitination enzyme (e.g., USP7) does not reduce the ubiquitination of vsvg-EFA6A.
9. The authors should refer the reader to the movies in the results section when appropriate rather than only mentioning the movies in the figure legends.

New Submission Received 15 February 2010

Referee #1 (Remarks to the Author):

Major concern:

EFA6A and EFA6B are similar in Sec7 and PH domains, however, they have distinct

expression pattern in tissues. The authors used EFA6A to rescue EFA6B knockdown cells. This is confusing since we don't know whether EFA6A and EFA6B functions the same in cells. Indeed, the authors couldn't get a complete TJ barrier function rescue by expressing EFA6A in USP9x knockdown cells.

We cannot completely exclude significant differences between EFA6A and EFA6B function as suggested by the Reviewer. The Chavrier's lab has extensively characterized the two isoforms. They showed that EFA6A and EFA6B function similarly regarding their ability to catalyze Arf6 nucleotide exchange activity, their subcellular distribution, and their effects on the actin cytoskeleton organization. Further, the PH-Cter constructs of EFA6A and EFA6B both localize and affect the actin cytoskeleton organization similarly[1]. We have extended these observations to polarized cells[2,3]. We have shown that the levels of expression of the transfected EFA6A in polarizing MDCK cells follow the same pattern of variation in a calcium switch, though to a lower extent and a with temporal delay, when compare to the endogenous EFA6B[2] (and this manuscript). Also, we showed that over-expression of EFA6A accelerates the assembly of the TJ by contributing to the reorganization of the apical actin cytoskeleton associated to the TJ[2]. For all these reasons, we have used our cell line expressing stably EFA6A under the Tet-off repressible system to rescue the depletion of EFA6B by siRNA.

We agree that the rescue is not complete, which is typical of many RNAi rescue experiments. Indeed, the kinetics of EFA6A increase in response to calcium switch may not perfectly emulate the kinetics of endogenous EFA6B changes. Nonetheless, the fact that EFA6A can rescue EFA6B knockdown at all in this complex biological system argues strongly that their functions are significantly overlapping. Thus, we use EFA6A to rescue EFA6B knockdown as a robust experimental system to establish a proof-of-principle about the role of EFA6 in TJ biogenesis. We do not claim that the physiological functions of EFA6A and EFA6B are equivalent in all respects. We agree with the reviewer that these points may not have been clear enough in the original manuscript and have now significantly emphasized these issues in the Introduction of the revised manuscript.

Furthermore, they couldn't co-precipitate USP9x and EFA6B from epithelial cells (MDCK), despite of PHCter domain from EFA6A interacting with USP9x.

We are happy to report that we have now successfully co-immunoprecipitated endogenous EFA6B and USP9x from MDCK cells (Fig. 3a). We followed the recommendations of Referee #2 and performed the co-immunoprecipitation at the expected time of maximal interaction using cross-linking. We did not need to treat the cells with proteasome inhibitors. Under these conditions, we could co-immunoprecipitate USP9x with EFA6B only at times after calcium repletion ranging from 30 to 60 min but not at later time points. We have also performed pull-down experiments with purified proteins showing a direct interaction between EFA6A and USP9x (Fig. 3b).

Other Issues:

1)The authors mentioned Derrien's paper to address that there are four isoforms of EFA6[1] and a reference for the EFA6B antibody[2]. cDNA of EFA6B encodes 1056 amino acids and its estimated molecular weight (MW) is 116 kDa and Derrien showed the overexpressed protein migrates with a MW of 180 kDa in two different cell types. However, in the manuscript, the authors state that EFA6B expresses as a 66 kDa protein when not ubiquitinated. The reference for their EFA6B antibody does not address the antibody at all. The authors must address the discrepancy in the MW of EFA6B, since the interpretation of their data can become completely different on the specificity of the antibody.

We understand the Reviewer's confusion as the field of EFA6 is still expanding and thorough characterization of all the EFA6 isoforms is needed. Without reviewing the field at length, I would like to point out several critical points that I hope will help clarify this issue. The EFA6 belongs to a subfamily comprised of four members first cloned by Chavrierís lab: EFA6A, B, C and D[1,4.] EFA6A was the first member identified and cloned from a human brain library[4]. Later, by sequence homology the three other homologs of

EFA6A have been identified and described[1,5]. Derrien et al. have cloned a cDNA encoding for an EFA6B protein of a predicted MW of 116kD[1]. With an antibody raised against a C-terminal peptide (1000-1013) they identified by immuno-precipitation and immunoblot a protein with an apparent MW of 180kD. However, the immunoblot presented in the article does not exclude the existence of a 66kDa band. In fact, as the immunoblot was over-exposed to detect the 180kDa band, another large band is seen at around 66kDa above the heavy chain. Our antibody raised against the Sec7 domain (591-736) of human EFA6B immunoprecipitates a protein of 66kD from MDCK cells as previously published[2]. Our collaborator, Dr. Sakagami, generated two different anti-sera (B1 and B2) raised against the N-ter (1-308) of EFA6B, both of which identify by immunoblot a 66kD band in all tissues tested, and a ≈ 175kD band in some tissues. Another band of about 80kD is also detected in the brain. These results are now presented in the Supplementary Information, S1a. In addition, when we performed an immunoprecipitation from a MDCK lysate with our antibody and probed the membrane with the antibody B1 from Dr. Sakagami, a band at 66kD is detected that comigrates with the 66kD protein found in a kidney lysate. As a control, on the same immunoblot, the ≈ 175kD band was found in the lung. This result demonstrates that the two antibodies from different origins and raised against two different parts of the same protein recognize at least 3 different variants of EFA6B (Supplementary Information, S1b). Further, we have performed an exchange activity assay on the immunoprecipitated protein of 66kD using our antibody and detected an active catalytic exchange activity specific for Arf6 (data not shown). Finally, and most importantly, our siRNAs directed against specific sequences of EFA6B repress selectively the expression of EFA6B as shown in Fig. 1. Thus, we have strong evidence that the 66kD protein is a functional short EFA6B isoform which we refer to as EFA6B in the manuscript for simplicity.
This is not the first example as a short version has been recently described for EFA6A[6]. Further, messengers of at least three different sizes have been observed and by immunoblot at least 5 different bands have been detected[1,6-8]. The same situation applies to EFA6C where at least two different bands were detected in a cerebellum lysate or in transfected Cos-7 cells by immunoblot[9]. In conclusion, it is anticipated that the 4 genes encoding for EFA6A, B, C and D generate variants by alternative splicing that have yet-to-be cloned and characterized.

For example, in figure 1.d, the total level of EFA6B is increased between 30 and 60 min after calcium repletion. However, the level of total immunoprecipitated EFA6B is increased only at 60 min after calcium repletion in figure 2.d.

If one looks carefully at the Fig. 2d one sees an increase of EFA6B (66kD) at 30 min that peaks at 60 min similarly to what is shown in Fig. 1d. In addition, in Fig. 2d we detected the poly-ubiquitinated forms only at 60 min. This is consistent with our model, whereby EFA6B first accumulates to stimulate the formation of the TJ, after which it is poly-ubiquitinated and degraded which reduces levels back to baseline. Note that although the accumulation of total EFA6B is seen over several time points, we mostly detected the poly-ubiquitinated forms at one time point presumably because EFA6B is rapidly degraded after its poly-ubiquitination.

Also, a band slightly bigger than 97 kDa was not detected with anti-ubiquitin antibody, FK-1 in figure 2.d. There is no clear mention about whether MG-132 or lactacystin was treated in the ubiquitination studies after figure 2.c. Ubiquitination was visible only in the presence of proteasome inhibitor in figure 2.c. However, in figure 2.d, band shifts with ubiquitination is clearly visible, though the intensity of shifted bands is dramatically decreased after 60 min time point. Regardless of whether the authors treated MG-132 to visualize ubiquitinated EFA6B in panel d, top band weakly visible above 200 kDa, not shown in panel c, seems to be less convincingly ubiquitinated. Immunoblotting of ubiquitinated ectopic EFA6A shows multiple bands. However, in figure 2. c and d that show ubiquitination of EFA6B, this reviewer can see only one band convincingly ubiquitinated and still, its MW looks less than reported MW of EFA6B.

We thank the Reviewer for noticing that the presence or absence of the proteasome inhibitor was not clearly stated as it is an important point of our experimental design and interpretation of the results. In Fig. 2a, 2b and 2c the cells were exposed to MG-132 as indicated on the figures. In Fig. 2d we had mentioned that the cells were not treated with proteasome

inhibitors. We have tried to better explain these experimental conditions as it is quite striking to detect such a strong signal of poly-ubiquitination in the absence of over-expression or treatment with inhibitors. Indeed, we feel this result actually strengthens the physiological relevance and robustness of our findings. In Fig. 2d, we agree with the Reviewer that the band at 105 kDa is not visible in the anti-ubiquitin immunoblot. It is in fact barely visible on the original figure. We have now corrected the text in the Results section accordingly. Nevertheless, we always observed a strong and robust poly-ubiquitination of EFA6B in our calcium switch experiments.

The authors propose that USP9x is responsible for the protection of EFA6 from deubiquitination of EFA6. Though the authors show the level of ectopic EFA6A, USP9x in figure 3.c, total level of ectopically expressed ubiquitin is not shown. Dot blot analysis of the level of ubiquitin will be enough to validate the reduced ubiquitination of EFA6A in the presence of ectopic WT USP9x.

The immunoblot of the total lysates corresponding to the immuno-precipitation experiments are now shown in the new Fig. 3e.

In figure 3.e, the authors try to address that USP9x is a specific deubiquitination enzyme for EFA6 from specific gene knock down of USP7 or USP9x. This reviewer wonders if the experiment was done in the presence of proteasome inhibitor or not. Considering that shifted bands were seen only at 60 min time point in USP7 gene knock down, I assume that proteasome inhibitor was not added. However, again, figure 3.e is contradictory to figure 2.c in that in figure 2.c, ubiquitination was visible only in the presence of proteasome inhibitor.

We apologize that we had not stated clearly enough the absence or presence of the proteasome inhibitors in our various experiments. We have now explicitly stated in the Results section and the Figure Legends the presence or not of the proteasome inhibitors for every single experiment. Indeed, in the previous Fig. 3e (now Fig. 4b) the experiment shown was performed in the absence of proteasome inhibitors while the experiment shown in the Fig. 2c was done with proteasome inhibitors. However, the two results are not contradictory. In Fig 3e (now Fig. 4b) the ubiquitination is observed in response to the calcium repletion. In Fig. 2c the cells were grown on plastic and submitted to no stimulus other than being exposed to the proteasome inhibitor.

3)if the total level of EFA6 is increased briefly after formation of adherence junctions, immunofluorescence observation should validate it by showing an increase and subsequent possible reduction of EFA6 signals in junction formation. The authors in previous MBC papers biochemically showed that EFA6 association to junction is increased after calcium repletion. It would be helpful to show whether the level of EFA6 on plasma membrane persists or decreases, in accordance with the change in total level, in the process of tight junction formation.

We agree with the Reviewer's comment. Indeed, the increase of GFP-EFA6A at the plasma membrane is visible in the Fig. 5 (previously Fig. 4) and the corresponding videos Fig. S3 that show the formation and maturation of a cell-cell contact labeled with E-cadherin-RFP. The intensity of the GFP-EFA6A staining increases as the primordial contact is maturing which is indicative of the accumulation of GFP-EFA6A. This is also followed by a reduction of the signal although not a total exclusion of GFP-EFA6A as observed for USP9x. We have now emphasized this point in the text to address the Reviewer's comment.

4) Gene knock down of afadin is shown to hamper the recruitment of adherens and tight junctional proteins together[3]. The author's data that gene knock down of USP9x caused a decrease in the level of afadin raises important questions in addition to the validation of their EFA6B antibody.
a) would there be also a decrease in the level of afadin in EFA6 gene knock down?

There is no effect of EFA6 depletion on the levels of afadin and as previously mentioned no detectable rescue by afadin in USP9x knockdown cells (our unpublished data) which does not totally exclude a role for afadin on an other pathway. We explicitly do not claim that EFA6 is

solely responsible for all of the regulation of the TJ assembly and given the complexity and redundancy of signaling networks, this is unlikely to be the case, but we do show that EFA6 is one of the key players. We have included a paragraph in the Discussion section to address the Reviewer's comment.

b) the authors state that overexpression of afadin did not rescue the effects of USP9x deficiency (Data not shown). More analysis is required.

We agree with the referee that the situation is complex and we briefly discuss a model in the Discussion section. However, please keep in mind that the goal of this paper is to analyze the regulation of EFA6 by the deubiquitinase USP9x and not to provide an exhaustive study of all the potential substrates of USP9x. The Reviewer is referring to a manuscript from Pr. Takaiís lab. The same group has more recently published a study on the cooperative role of Par-3 and afadin for AJ and TJ assembly[10]. Although, they nicely demonstrate a role for Par-3 and afadin in TJ assembly by siRNA and show a role for Par-3 upstream of afadin to promote its association with nectin, surprisingly they could not rescue Par-3 knockdown cells by reexpressing afadin alone or together with another downstream effector of Par-3, the constitutively activated Rac mutant. They concluded that Par-3 might control other pathway(s) of the formation of cell-cell adhesion. Thus, we agree that the situation is complex and that further work is needed to resolve all possibilities, but we believe that our manuscript makes a significant, novel, and clear contribution to an important but relatively less well studied TJ regulatory pathway.

Referee #2 (Remarks to the Author):

The manuscript by Thèard et al. describes a role for the ARF6 guanine nucleotide exchange factor EFA6 for tight junction formation in polarizing epithelial cells. The authors have shown previously that ARF6 is transiently upregulated during Ca2+-switch (CS) -induced cell-cell contact formation and that ectopic expression of EFA6 accelerates TJ formation. In this manuscript, the authors find that EFA6 is constitutively degraded by the ubiquitinproteasome pathway and that it is transiently stabilized during CS-induced cell-cell contact formation through its association with the deubiquitinating enzyme USP9x. The model derived from the data implicates USP9x as an EFA6-deubiquitinating enzyme responsible for the transient increase in EFA6 levels which regulate the activity of ARF6 during cell-cell contact and TJ formation. This is a very interesting paper providing a molecular mechanism by which the levels of a small GTPase are specifically upregulated during cell-cell contact formation by the activity of a deubiquitinating enzyme.

Specific Points:
1. One drawback of the paper is the lack of biochemical evidence for the endogenous interaction of EFA6 and USP9x in MDCK epithelial cells. The authors show coimmunoprecipitations from synaptosomal fractions which are obviously only suggestive. Given the functional data, it is to be expected that the two proteins exist in a complex, perhaps only transiently. The authors should try to co-immunoprecipitate the EFA6 and USP9x at different stages during CS-induced cell-cell contact formation, preferably 30 and 60 min after readditon of Ca2+. In addition, a chemical crosslinker could be used to stabilize the predicted interaction. Furthermore, a proteasome inhibitor could be used to increase the levels of EFA6.

We have followed the recommendations of the Reviewer and performed the coimmunoprecipitation at the expected time of maximal interaction using a cross-linker. We did not need to treat the cells with proteasome inhibitors. Under these conditions, we could coimmunoprecipitate USP9x with EFA6B only at times after calcium repletion around 30 to 60 min depending on the experiment (Fig. 3a). Thank you for the suggestion.

These experiments could be further complemented by in vitro direct binding assays using recombinant proteins. If a direct interaction can be demonstrated it would at least further support a physical association of EFA6 and USP9x.

We have now performed a pull-down assay using 4 fragments of USP9x fused to GST

incubated with the recombinant His-EFA6A. We could demonstrate a direct interaction between the two proteins and determine the region of binding within USP9x (Fig. 3b).

2. The second drawback of the paper is the lack of convincing evidence for a co-localization of EFA6 and USP9x during cell-cell contact formation. In Fig. 4, the authors analyze the subcellular localization of EFA6 and USP9x. A clear co-localization of EFA6A and USP9x can be observed at the tips of filopodia which is not in contact with another cell (Fig. 4f). However, the evidence for co-localization at cell-cell contacts is only very weak if at all. The authors state in the text that during early stages of cell-cell contact formation, the two proteins co-localize at cell-cell contacts as well. However, the signal for USP9x is highly overexposed and as a consequence USP9x seems to localize throughout the entire cell (Fig. 4 h, h'). Therefore, any protein expressed by the adjacent cell and present at the contact area would result in a merge signal.

We have prepared a new stable cell line co-expressing E-cadherin-RFP and GFP-EFA6A to better appreciate the co-localization of these two proteins together with USP9x. We now show images that clearly demonstrate the co-localization of the three proteins at early times of cellcell adhesion mediated by E-cadherin (Fig. 5e-g).

In addition, these figures show the tip of a single filopodium. The predicted function of EAF6 to regulate ARF6 activity which in turn regulates the formation of the actin cytoskeleton at sites of cell-cell contact formation would clearly require the presence of not only EAF6 but also of USP9x during longer and/or later periods of lateral cell-cell contact formation (such as shown for EAF6 in Fig. 4b, c), not only at the very tip of the initial contact. Thus, the evidence of a co-localization of EFA6 and USP9x during cell-cell contact formation is too weak to support the notion that they co-distribute and that USP9x is present at cell-cell contacts during critical stages of cell-cell contact formation.

We agree with the Reviewer's assumption and our new confocal immunofluorescence micrographs now show co-localization of USP9x and EFA6 at later time points (Fig. 5g) which is more coherent with our biochemical results and EFA6 functions. These observations were possible thanks to the new cell line described above which allowed us to perform a calcium assay and thus better control the kinetic of cell-cell adhesion formation. Yet, at much longer time points, EFA6 appears to reside at the cell-cell contact for longer times than USP9x. In the Discussion section we are proposing that once at the cell-cell contacts the translocated EFA6 could be protected from degradation until a certain stage of maturation of the contact when EFA6 becomes available for degradation. The protection could be due to EFA6 association with specific partners, such as cytoskeletal proteins which are highly concentrated at the contact zone, that compete with the E3 ligase. We have included a small paragraph in the Discussion section to address the Reviewer's comment.

3. The authors do not comment on the regulation of USP9x during cell-cell contact formation. What are the levels of USP9x in MDCK cells during CS-induced cell-cell contact formation? Do they change?

This was shown in the previous Fig. 3e and is now presented in the new Fig. 4b. We have modified the text in the Results section to more clearly explain this point.

Minor points:
- in the graphs displaying TER, the Y-axis label must be ohms.cm2 but not ohms.cm-2 due to the inverse relationship between area and ohmic resistance

We have changed the labeling according the Reviewer's recommendation

- in the Introduction, the authors ignore the described role of the Par - aPKC complex during the temporal and spatial regulation of TJs in response to de novo E-cadherin-mediated cell-cell adhesion

As suggested by the Reviewer, we have commented on the role of the polarity complexes, including the Par3/Par6/aPKC complex, in the Introduction and the Discussion.

Referee #3 (Remarks to the Author):

This manuscript from Thèard and colleagues is focused on the mechanisms underlying the temporal regulation of EFA6 during TJ biogenesis. Through a series of complementary biochemical and cell biological approaches, the authors make a series of key findings. They show that enzymatically active EFA6 is required for de novo TJ assembly using an siRNA approach. They further make the critical observation that EFA6 stability is regulated by the ubiquitin-proteasome system and that the deubiquitinating enzyme, USP9x, counters EFA6 turnover early in TJ biogenesis. Corroborating evidence for the interplay between EFA6 and USP9x is provided in the form of pulldown assays, co-localization studies, and siRNA/rescue experiments. Further, the authors show that USP9x over-expression can reduce the levels of ubiquitin-modified EFA6. Overall, the data are of high quality, suitable specificity controls are included in most experiments, and this works represents a significant advance in our understanding of how TJ biogenesis can be modulated by specific enzymes.

Prior to publication, the below list of shortcomings should be appropriately addressed.

1. In the legend for Supplemental Fig. 1C, reference is made to upper and lower panels but the authors intend to indicate left and right panels.

We have incorporated the correction in the new Supplemental Information, Fig. S1.

2. In Supplemental Fig. 1C, the half-life of EFA6B looks bi-phasic with only the first phase having a steeper slope than the two comparison proteins. An explanation or comment should be provided by the authors regarding this bi-phasic pattern.

Typically, half-life curves display 3 phases observed for all proteins. The first one within 10 min after the pulse corresponds to the fact that the pulsed proteins are not yet all matured and out of the secretory pathway and thus one only observes the degradation of the very first cohort of proteins that were pulse-labeled. The second phase is linear and represents the rate of degradation. The third phase corresponding to a flatter part of the curve reflects the slower degradation of a small fraction of the protein that has not been efficiently sorted towards degradation. Since EFA6B has a very short half-life the second phase is rapid and more marked than the ones of TfnR and occludin. It occurs between 15 and 30 min, while for the two other proteins this second phase lasts between 15 and 60 min reflecting their slower rate of degradation.

3. In Fig. 2B, the authors show that GST-S5a can precipitate myc-ubiquitin conjugates from a transfected cell lysate but as described, this experiment does not prove that these conjugates are attached to EFA6B (which appears to be the interpretation the authors make from the data).

We have corrected the error of labeling of the figure (old Fig. 2b). The immunoblot was performed with the anti-vsvg (vsvg-EFA6A) and not with the anti-myc (myc-ubiquitin). We have modified the text in the Results section to more clearly explain the experiment and its interpretation. This figure is now presented in the supplementary Fig.2c.

4. In Fig. 2D, although the 105 kDa band is visible on the anti-EFA6B blot (right panel), it is not visible on the anti-ubiquitin blot (left panel).

Indeed, the band at ≈ 105 kDa is not visible in the anti-ubiquitin immunoblot. It is in fact very faint on the original figure and does not show up on the digital figure. We have now corrected the text in the Results section accordingly.

5. Regarding the data presented in Fig. 3B, is the PH domain sufficient for the interaction between EFA6B and USP9x? This should be tested and shown in the manuscript.

We have now performed the experiment and show that the PH domain interacts with USP9x

in the pull-down assay (Fig. 3c).

Also, the authors should test if over-expression of the minimal USP9x-binding domain of EFA6A can phenocopy the data obtained with siRNA-targeting USP9x.

We thank the Reviewer for prompting us to perform this experiment. We have made a new cell line expressing the PH domain fused to GFP and show that indeed it acts as a dominant negative by blocking the assembly of the TJ (Fig. 3d and Supplementary Information).

6. In Fig. 3C, the arrow next to vsvg-EFA6A is pointing to a blank area of the blot.

The arrow is pointing to the position of the non-ubiquitinated vsv-G to help the reader to appreciate the shift of the molecular weight due to poly-ubiquitination. We have modified the legend to better explain the significance of this arrow.

7. In Fig. 3C, an explanation should be provided for why the levels of vsvg-EFA6A in lane 1 are higher than the levels in lane 3 where USP9x expression should be increasing the levels of unmodified vsvg-EFA6A by reducing the levels of ubiquitin-modified vsvg-EFA6A.

We understand the point of the reviewer but one has to be careful not to over-interpret this experiment (Fig. 3e) as the proteins are transfected and thus their levels are not only dependent on their ubiquitination and subsequent proteasomal degradation but also to their rate of transfection. However, one sees that co-expression of EFA6A with Ub (lane2) leads to a marked reduction of the levels of EFA6A. When USP9x is co-expressed (lane 3) this reduction is not as strong suggesting that USP9x partially protected the transfected EFA6A from degradation. This protection is not observed with the catalytically mutated USP9x (lane 4). We have modified the text in the Results section to address this concern.

8. The authors should show that over-expression of a different deubiquitination enzyme (e.g., USP7) does not reduce the ubiquitination of vsvg-EFA6A.

We had tested USP7 and USP19 but over-expression of these DUBs led to a general deubiquitination visible on the whole cell lysate questioning the specificity of the reaction. This seems to be the case for many DUBs, but not so much for USP9x. Indeed, as requested by Reviewer #1 we are now showing the corresponding lysates of USP9x over-expression that only show a slight reduction of the general ubiquitination signal. As a better control of specificity, we reasoned that depletion by siRNA that is shown in Fig. 4 would be more informative.

9. The authors should refer the reader to the movies in the results section when appropriate rather than only mentioning the movies in the figure legends.

We have made this correction in the new manuscript.

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1st Editorial Decision 25 February 2010

Thank you for submitting your manuscript to the EMBO Journal. This submission is an invited resubmission of MS 71532 that was rejected after review in 2009. I asked the original three referees to review the resubmission and both referees #2 and 3 were available to do so. I have now received the comments back and as you can see below the referees find that the added data has strengthen the paper and they support publication in the EMBO Journal. I am therefore very pleased to proceed with the acceptance of the paper for publication here. Before doing so there are a few minor points that have to be sorted out in a final revision. When you send us your revision, please include a cover letter with an itemised list of changes made in response to comments from review.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

Thèard et al. provide a new manuscript in which they carefully addressed the reviewers concerns. In this new manuscript, the authors now provide important new information of the physical association between EFA6 and USP9x. Most importantly, the authors demonstrate the existence of an endogenous complex of EFA6 and USP9x within a short time window during cell-cell contact formation. In addition, the authors find that the two proteins directly interact and that the PH domain of EFA6 is involved in the interaction and critically important for the function of EFA6. Finally, the authors provide evidence that both proteins transiently co-localize at cell-cell contacts during the process of cell-cell contact formation. In summary, with the new data, the manuscript makes a much stronger point for EFA6 and USP9x as important and novel regulators of cell-cell contact and tight junction formation. I strongly recommend publication of the manuscript in the EMBO Journal.

Minor points:

- the authors could show the transient nature of the interaction between EFA6B and USP9x (Fig. 3a) by showing several instead of only a single time point (45 min); based on the authors comments in the manuscript, the experiment has been done and thus could be incorporated in the figure

- in the text the authors refer to Fig. 2d, which, however, does not exist in the resubmitted manuscript; (must mean Fig.2c in the text)

Referee #3 (Remarks to the Author):

Figure 2D was missing from the figures.

Other than this omission, the authors successfully addressed the critiques of all the reviewers and in

the process of doing so, greatly bolstered the manuscript with new data and revised text. In particular, the inclusion of the GFP-PH dominant negative studies and the endogenous interactions between USP9x and EFA6 strengthened the authors' findings.

1st Revision - authors' response 02 March 2010

Referee #2 (Remarks to the Author):

- the authors could show the transient nature of the interaction between EFA6B and USP9x (Fig. 3a) by showing several instead of only a single time point (45 min); based on the authors comments in the manuscript, the experiment has been done and thus could be incorporated in the figure

The figure has been modified to add a time point at 15 min and another one at 90 min.
In consequence in we made the following changes:

In the Results section the sentences “To co-precipitate USP9x and EFA6B from MDCK cells, we performed a calcium switch and lysed the cells 45 min after calcium repletion. Under these conditions, USP9x could be co-precipitated together with EFA6B (Fig. 3a). The co-imunoprecipitation was not observed for periods of calcium repletion longer than 1 hr indicating that the interaction between endogenous EFA6B and USP9x is transient and occurs predominantly at early time points during cell-cell adhesion.î were replaced by ìTo co-precipitate USP9x and EFA6B from MDCK cells, we performed a calcium switch and lysed the cells at various times after calcium repletion. USP9x could be co-precipitated together with EFA6B only at 45 min after calcium repletion indicating that the interaction between endogenous EFA6B and USP9x is transient and occurs predominantly at early time points during cell-cell adhesion (Fig. 3a).”

In the corresponding Legend the sentence “MDCK cells grown on 24-mm filters and submitted to a calcium switch were solubilized in NP-40 lysis buffer 45 min after calcium repletion.îwas replaced by ìMDCK cells grown on 24-mm filters and submitted to a calcium switch were solubilized in NP-40 lysis buffer at the indicated times after calcium repletion.”

- in the text the authors refer to Fig. 2d, which, however, does not exist in the resubmitted manuscript; (must mean Fig.2c in the text)

Indeed, we made an error. After having tranferred the Fig. 2c into the supplemetal material we forgot to change Fig. 2d by Fig. 2c. We have now replaced Fig. 2d by Fig. 2c in the Results section.

Referee #3 (Remarks to the Author):

Figure 2D was missing from the figures.

See our answer above.

In the Discussion, first sentence of the 3rd paragraph we have replaced the abbreviation UPS to spell it out as ubiquitin-proteasome system.

