EMBO reports

Manuscript EMBOR-2014-39218

SOS1 and Ras regulate epithelial tight junction formation in the human airway through EMP1

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Review timeline:

Submission date:	25 June 2014
Editorial Decision:	23 July 2014
Revision received:	23 September 2014
Editorial Decision:	13 October 2014
Revision received:	23 October 2014
Accepted:	24 October 2014

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Barbara Pauly

1st Editorial Decision 23 July 2014

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. You will see that all reviewers appreciate the interest of your findings and are, in principle, supportive of publication of your study in our journal. However, they are also in agreement that several aspects of your data need to be improved before the paper can be published. For example, all three reviewers agree that the stage at which the SOS1/Ras/EMP1 axis controls tight junctions should be more clearly defined. Referees 1 and 3 state that evidence needs to be provided that the observed effects on tight junctions are not secondary, with the primary target being adherens junctions. Referee 1 further points out that the data on the causal, direct link between SOS1, Ras, and EMP1 in the regulation of tight junctions needs to be strengthened and provides suggestions on how to achieve this. Referee 3 feels that the data on the localization of SOS1 and EMP1 at tight junctions should be strengthened, that it should be excluded that SOS1 affects RhoA signaling and that it should formally be shown that the effects of MEK inhibition are mediated by SOS1/EMP1. With regard to this referee's suggestion to investigate whether this is a more general phenomenon in different epithelial cells, we think it would be sufficient to discuss this.

Overall, and given the reviewers' constructive comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 29,000 characters (including spaces and references). While you may consider displaying peripheral results as supplementary information, the materials and methods required for the understanding of the main experiments may not be displayed in the supplementary section only.

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

REFEREE REPORTS:

Referee #1:

This is a potentially interesting manuscript that shows that different players in the Ras/Erk kinase pathway can regulate tight junctions in airway epithelia. Specifically the authors show that interference with the GEF SOS1 interferes with tight junctions. As SOS1 has two separate GEF domains for either Ras or Rac the authors next used dominant negative Ras to show that this also interferes with tight junctions. Using MEK and ERK inhibitors they then implicate ERK activity in the regulation of ZO-1 containing tight junctions. They then identify EMP1, a member of the claudin superfamily, as being transcriptionally regulated by Ras/MEK/ERK and show that downregulation of EMP1 disturbs junctions. Finally, they correlate reduced EMP1 expression with lung cancer.

There are three main problems with the paper:
1. The data convincingly implicate SOS1, dominant negative RAS, the MEK/ERK inhibitors and EMP1 in the regulation of tight junctions. Although tempting to suggest a linear pathway, the relation between SOS1, RAS/ERK and EMP1 are not causally established in the experiments. Would a SOS1 domain that has the Rac GEF deleted indeed be able to rescue TJs upon depletion of SOS1? Vice versa would a SOS1 mutant that lacks the RAS GEF interfere with TJs? Is there indeed increased Ras/ERK activity in the SOS1-depleted cells? Is EMP1 downregulated upon depletion of SOS1 and is overexpression sufficient to rescue? The argument that transcriptional regulation is necessary based on inhibiting MEK/ERK either in a 4 hour calcium assay versus 4 days of inhibitor treatment is rather weak and indirect. Can exogenous EMP1 indeed rescue TJs upon ERK inhibition?

2. It is not clear at which stage the proposed pathway interferes with tight junctions. The authors state that it interferes with tight junction formation. However, the experimental set up does not allow this conclusion as they only assess after 4 days. In fact, when they do a short term interference using a Calcium switch assay junctions can be properly formed upon inhibition of ERK, suggesting that perhaps it is tight junctional maintenance that requires Erk signaling. Tight junction assembly and maintenance can easily be followed by TER measurements over time. No experiment addresses assembly and disassembly.

3. What is the mechanism? The data are not sufficient to conclude a direct effect of SOS1, RAS/ERK and EMP1 on tight junction function. How can the authors rule out that this is a specific effect on tight junctions? ZO-1 in many epithelial cell types is initially in adherens junctions and all the effects the authors are observing might be through regulating cadherin and/or nectin mediated adhesion and adherens junction formation. Are adherens junctions normally formed? Is EMP1 really

at tight junctions? Does it bind ZO-1? What is the importance of MEK/ERK at the junctions when its effect is apparently regulated through transcription?

Small points:
Figure1: Western blot 1B should be labeled.

Figure 1: as the small GTPases all affect each other, and often constitutive active forms and dominant negative forms have similar effects, especially on tight junctions, how does dominant negative RAS affect Rho and CDC42 signaling?

Figure 2G: the pERK staining at junctions is rather unconvincing.

It would be good to show at least for one experiment the absolute TER values and not the relative values compared to control treated cells.

For me the relationship between the data implicating this pathway and EMP1 in TJs and then showing altered expression of EMP1 in lung cancer is unclear and actually do not really make sense. Although cancer indeed may downregulate junctions, this may be completely different from the suggested pathway described here. Especially as to my knowledge several types of lung cancer are associated with a strong increase in EGFR/Map kinase signaling. More relevant models might be lung diseases that are more strongly connected to disturbed barrier function.

Data in a recent paper actually suggest the opposite from what is presented here: cytokine-induced activation of Erk induces disassembly of junctions in airway epithelia. Although the effect was not great, they did use Erk inhibitors and found that these did not further enhance disassembly but instead seemed to rescue. These data should at least be acknowledged in some way.

Referee #2:

Durgan et al. used an RNAi screen to identify activators of GTPase signalling that regulate junction formation. They identify SOS1, a GEF for Rac and Ras, and subsequently use different strategies to test whether Ras or Rac signalling are responsible for the defect in junction formation upon Sos1 depletion. The paper then further demonstrates that Ras signalling regulates expression of EMP1, which is also required for junction formation and may contribute to loss of differentiation in lung cancer.

This is an interesting paper that describes new and intriguing observations that link Ras signalling to the maintenance of epithelial junctions. The paper should be of interest not only to scientists working on epithelial junctions but to a wider readership including scientists with interests in tissue development and engineering, and cancer. The data are very solid and mostly convincing. However, there are a few important gaps that should be addressed prior to publication.

1) The two SOS1 shRNAs that are able to deplete the protein to some extent are very different in their phenotype. One of them only yields a minor phenotype. The specificity of the more efficient SOS1 shRNA should be tested by complementation. The authors seem very firm in excluding that defects in Rac signalling contribute to the phenotype without showing any evidence. The complementation approach using GEF mutants could also be used to test whether the Rac activation domain of SOS1 can really be excluded from contributing to the phenotype.

2) Cell proliferation and cell numbers: The authors mention at one point that MEK inhibitors did not cause reduced cell proliferation and state in the discussion again that Ras/Erk signalling inhibition does not affect proliferation in their model. These are crucial data and should be shown, as effects on cell density may affect the differentiation potential of the cells and junction formation. Most images shown seem to suggest that cells with reduced SOS/Ras signalling are at lower densities. One also wonders whether there is an increase in cell death at the time when junctions start to disintegrate.

3) The implication of the data is that EMP1 is not required for junction formation but junction

maintenance; hence, the question arises when it is recruited. In the calcium switch experiments, when is EMP1 expressed and recruited to cell-cell contacts? One would also wish for higher resolution localisation experiments that provide information about which type of junction EMP1 associates with.

Referee #3:

The manuscript by Durgan and colleagues identifies the Rho family guanine nucleotide exchange factor SOS1 as regulator of tight junctions and epithelial barrier formation in cultured lung-derived epithelial cells. This activity of SOS1 seems to be mediated by its activity on Ras, since ectopic expression of dominant-negative Ras results in a similar defect in tight junction formation as SOS1 depletion and as SOS1 depletion inhibits ERK1/2 phosphorylation. The authors find that inhibiting the Ras-Raf-MAPK pathway prevents the formation of a linear ZO-1 localization along intercellular contacts as well as the formation of functional tight junctions, implicating this pathway in cell contact and tight junction formation. Finally, the authors identify the integral membrane protein EMP1 as transcriptional target of the SOS1-Raf-MAPK pathway and find a role for EMP1 in epithelial barrier formation. This study is interesting as it provides evidence for the Ras-Raf-MEK pathway as regulator of epithelial barrier formation and identifies one upstream and one downstream component of this pathway. If this pathway operates specifically in lung epithelial cells and if it targets specifically the tight junctions remains unclear.

Specific points

1. The authors describe the SOS1-Ras signaling pathway as a regulator of epithelial TJ and barrier function formation (eg in the title). However, SOS1 depletion disrupts the localization of the adherens junction component E-cadherin as well (Suppl. Fig.1a), suggesting that SOS1 could regulate primarily adherens junction formation, which is considered as prerequisite for tight junction formation. It remains unclear if the effects on tight junctions are secondary to the disruption of adherens junctions.
2. The authors claim that the cell lines used (16HBE, BCi-NS1.1) form an apical junctional complex including tight junctions. However, confocal Z-section and co-stainings with typical tight junction components such as Occludin or ZO-1 are missing throughout the manuscript. This would be particularly important for the localization of SOS1 (no stainings shown) and EMP1 (claimed to be localized at the apical junction, Fig.4e). From the immunofluorescence studies shown in the manuscript, it is not possible to judge the specific localization of the two proteins along intercellular junctions.
3. To address the question how SOS1 and EMP1 regulate junction formation it would be important to know when they appear at junctions in the course of junction formation. Scratch wounding assays and staining of SOS1 and EMP1 with markers of puncta (e.g. E-cadherin or ZO-1) and markers for more maturated junctions (e.g. Occludin or Claudins) could provide some first ideas.
4. The authors suggest that SOS1 mediates its effect on junction formation through the Ras-Raf-ERK1/2 pathway. However, SOS1 might act through its activity on RhoA as well. Rescue experiments using dominant-active or fast-cycling mutants of Ras and Rac1 in SOS1-depleted cells could tell.
5. Why do the authors focus in their study on lung epithelial cells? Is the role of SOS1 and EMP1 specific for lung epithelial cells? Epithelial cell lines derived from other organs (e.g. Caco-2, MDCK) should be used to address the question if the role of the SOS1-Raf-MAPK-EMP1 pathway is specific for lung epithelium. These polarized epithelial cells could also provide a clearer picture on the subcellular localization of SOS1 and EMP1 (optical z-sections on filter-grown cells, see point 2). Finally, these cells could be used to study the role of the Ras-Raf-MAPK pathway and the identified upstream and downstream components in a 3-dimensional environment, which in the author's laboratory has proven to be a useful tool to distinguish gate and fence functions of tight junctions.
6. The studies applying acute and chronic MEK inhibition suggest a role for the Ras-Raf-MAPK pathway after chronic but not acute treatment with MEK inhibitors (Fig.3a). However, it is not clear if these effects are regulated by SOS1 and mediated through EMP1. A stable cell line with inducible SOS1 knockdown would allow to address this issue by depleting SOS1 in confluent cells.

7. It is interesting that inhibition of the Ras-Raf-MAPK pathway blocks both the gate and the fence function of tight junctions. It would be important to know if EMP1 as downstream effector of the pathway regulates both functions as well. The authors, therefore, should analyze both gate and fence function in EMP1 knockdown cells (Fig.4 shows only the gate function).

Minor points

1. The presence/absence of a linear ZO-1 staining cannot be generally considered as indication for functional/non-functional tight junctions since only the simultaneous absence of all three ZO proteins (ZO-1, -2, -3) prevents TJ strand formation (Umeda et al 2005, Cell)
2. Several references appear to be incomplete.

1st Revision - authors' response 23 September 2014

Referee #1:

1. The data convincingly implicate SOS1, dominant negative RAS, the MEK/ERK inhibitors and EMP1 in the regulation of tight junctions. Although tempting to suggest a linear pathway, the relation between SOS1, RAS/ERK and EMP1 are not causally established in the experiments. Would a SOS1 domain that has the Rac GEF deleted indeed be able to rescue TJs upon depletion of SOS1? Vice versa would a SOS1 mutant that lacks the RAS GEF interfere with TJs? Is there indeed increased Ras/ERK activity in the SOS1-depleted cells? Is EMP1 downregulated upon depletion of SOS1 and is overexpression sufficient to rescue? The argument that transcriptional regulation is necessary based on inhibiting MEK/ERK either in a 4 hour calcium assay versus 4 days of inhibitor treatment is rather weak and indirect. Can exogenous EMP1 indeed rescue TJs upon ERK inhibition?

In our original submission, we proposed a linear pathway operating from SOS1 to Ras, to MEK and then ERK, which regulates the expression of EMP1. We are not sure why the reviewer is unconvinced by this series of relationships. We showed that: (a) Depletion of SOS1 (Fig 2d), expression of DN Ras (Fig 1d) and inhibition of MEK (Fig 2a) all lead to the inhibition of ERK activation (as shown by western blotting for p-ERK). (b) Expression of DN Ras, inhibition of MEK, or inhibition of ERK itself, all reduce EMP1 expression (as shown by microarray and QPCR, Fig 3c-d). However, to further reinforce the conclusion that this pathway is linear, we have now provided an additional experiment showing that depletion of SOS1 reduces EMP1 expression (new Fig 4a), as requested.

Our existing data demonstrate that the Ras pathway controls airway epithelial morphogenesis through EMP1, but as the referee implies, it remains possible that SOS1 also functions as a Rac GEF in the context of junction formation. The potential role of Rac in junction assembly is outside the scope of this current work, which is focused on Ras signaling and EMP1. However we have now included a comment to clarify that our results do not exclude a possible role for Sos1 in regulating Rac (p4).

We also agree that that our initial argument for transcriptional regulation was somewhat indirect, but feel that the follow up experiments of the microarray analysis and the EMP1 depletion experiments have validated this hypothesis.

The question of whether exogenous EMP1 expression is sufficient to rescue Ras/MEK/ERK inhibition is interesting but not straightforward to address, since it is only one of many genes whose transcription is regulated by the ERK MAP kinase pathway (see Fig. 3c). We are currently examining the other candidates to see if any of these also contribute to the junctional defects observed after blocking this signaling pathway.

2. It is not clear at which stage the proposed pathway interferes with tight junctions. The authors state that it interferes with tight junction formation. However, the experimental set up does not allow this conclusion as they only assess after 4 days. In fact, when they do a short term interference using a Calcium switch assay junctions can be properly formed upon inhibition of ERK, suggesting that perhaps it is tight junctional maintenance that requires Erk signaling. Tight junction assembly and maintenance can easily be followed by TER measurements over time. No experiment addresses assembly and disassembly.

The reviewer raises an important question regarding the precise role of the ERK MAP kinase pathway: does it control the formation or maintenance of tight junctions? In our original submission, we did not address this issue directly. We previously compared acute versus chronic ERK MAP kinase pathway inhibition (as a preliminary means of investigating whether ERK functions through short-term direct phosphorylation or longer-term transcriptional changes). We clearly demonstrated that chronic, but not acute, ERK inhibition was required to disrupt junctions. We concluded that chronic ERK inhibition caused a defect in junction formation, because only primordial puncta assembled. However, as inferred by the reviewer, during the time-course of our chronic pathway inhibition assay (4 days), control junctions have in fact been both formed and maintained. As such, we agree that we had not adequately determined whether junction formation or maintenance was impaired by long-term pathway suppression.

To clarify this important issue, we have added new data as follows:

(a) Fig. 3a. Our original figure compared cells acutely inhibited for MEK (calcium switch) to cells chronically inhibited (seeded sparsely in the inhibitor, then assayed at day 4). We acknowledge that this is not an ideal comparison and have now improved the experimental design and replaced the figure to clarify the confusion. As explained in the new legend, cells in panels 3 and 4 (chronic) were seeded sparsely and incubated for 4 days in either DMSO (panel 3) or with the MEK inhibitor (panel 4). After reaching confluence, a calcium switch recovery assay was performed with DMSO (panel 3), or with MEK inhibitor (panel 4), to assay only the formation of *de novo* junctions. We find that in cells that were chronically MEK inhibited, the formation of new junctions after the calcium switch is severely inhibited.

(b) To specifically address the issue of junctional maintenance, we have also added data to Supp. Fig 2. Control cells were cultured to confluence, to form mature monolayers with established apical junctions. The cells were then incubated for a further 4 days -/+ MEK inhibitor, to analyse the effect of chronic MEK inhibition on already formed junctions (Supp. Fig.2a). We confirmed that MEK is efficiently inhibited under these conditions, through blotting of p-ERK (Supp. Fig. 2b). We observed no detectable effect on junctions, suggesting that the ERK MAP kinase pathway is not required for junctional maintenance.

Together, our data now clearly demonstrate that the ERK MAP kinase pathway is essential for bronchial tight junction formation, but dispensable for junctional maintenance.

3. What is the mechanism? The data are not sufficient to conclude a direct effect of SOS1, RAS/ERK and EMP1 on tight junction function. How can the authors rule out that this is a specific effect on tight junctions? ZO-1 in many epithelial cell types is initially in adherens

junctions and all the effects the authors are observing might be through regulating cadherin and/or nectin mediated adhesion and adherens junction formation. a) Are adherens junctions normally formed? b) Is EMP1 really at tight junctions? c) Does it bind ZO-1? d) What is the importance of MEK/ERK at the junctions when its effect is apparently regulated through transcription?

The reviewer recommends that we address whether the SOS/Ras/MEK/ERK axis may control adherens junction formation in 16HBE. In our original submission, we did show disruption of E-cadherin staining upon SOS1 depletion, DN Ras expression or MEK inhibition (see Supp. Fig 1). We have now added data to show that depletion of EMP1 also disrupts E-cadherin, as well as ZO-1, localisation (Fig. 4b). Our findings indicate that inhibition of the SOS1/Ras/MEK/ERK pathway disrupts both adherens and tight junction formation, as only punctate primordial junctions assemble.

To look more closely at the association of EMP1 with tight junctions, we have included confocal z-stacks of mature 16HBE monolayers, co-stained for EMP1 and ZO-1 (Fig. 4g). These images show that EMP1 colocalises with ZO-1 at apical tight junctions. Published protein-protein interaction work is also consistent with the idea that EMP1 associates with tight junctions, as EMP1 can interact with both ZO-1 and occludin in mouse brain endothelial cells (Ref. 40: Bangsow *et al*, 2008), as noted on p6.

Finally, the reviewer raises an interesting point: what is the relevance of MEK/ERK staining at junctions when their effects are apparently transcriptional? Perhaps MEK/ERK are regulated at nascent junctions to provide feedback control on the transcriptional regulation of EMP1? At present we are not able to comment more specifically on this point. Although we cannot fully explain the finding, we have retained these images in our figure as the information may be useful for others in the field.

Small points:
Figure1: Western blot 1B should be labeled.

Western blot 1b is positioned directly above Graph 1c, with lanes and bars aligned, such that the labels relate to both data sets. We have amended the figure legend to make this clearer.

Figure 1: as the small GTPases all affect each other, and often constitutive active forms and dominant negative forms have similar effects, especially on tight junctions, how does dominant negative RAS affect Rho and CDC42 signaling?

Addressing the interplay between Ras signaling and Rho/Cdc42 is an interesting avenue, but we feel this is beyond the scope of this short report, which is focused on the MEK/ERK pathway and its transcriptional effects.

Figure 2G: the pERK staining at junctions is rather unconvincing.

We agree that the pERK staining at junctions is less intense than for other proteins we have visualised in this report. The signal is relatively weak and is not dramatically enriched in comparison to other regions of the cell, presumably because pERK is localised in other areas too. Nevertheless, the signal we detect is highly reproducible and we have no reason to doubt that a population of the protein does reside in this region, co-localised with its upstream activator MEK. As such, we have retained this image in our figure.

It would be good to show at least for one experiment the absolute TER values and not the relative values compared to control treated cells.

We have added example TER values, expressed in ohms/cm^2, into the text for both 16HBE and BCi-NS1.1 cells.

For me the relationship between the data implicating this pathway and EMP1 in TJs and then showing altered expression of EMP1 in lung cancer is unclear and actually do not really make sense. Although cancer indeed may downregulate junctions, this may be completely different from the suggested pathway described here. Especially as to my knowledge several types of lung cancer are associated with a strong increase in EGFR/Map kinase signaling. More relevant models might be lung diseases that are more strongly connected to disturbed barrier function.

We agree that the relationship between EMP1, epithelial junctions and cancer is likely to be complex, and that our findings may not be relevant to all lung cancers, particularly those with amplified EGFR or activated Ras. Nevertheless the pattern of EMP1 loss was significant across multiple lung cancers in the study indicated (as well as several others in the Oncomine database, data not shown). As such, we feel it reasonable to present this interesting correlation.

Data in a recent paper actually suggest the opposite from what is presented here: cytokine-induced activation of Erk induces disassembly of junctions in airway epithelia. Although the effect was not great, they did use Erk inhibitors and found that these did not further enhance disassembly but instead seemed to rescue. These data should at least be acknowledged in some way.

We have now expanded our discussion to include an extra reference to this paper.

Referee #2:

1) The two SOS1 shRNAs that are able to deplete the protein to some extent are very different in their phenotype. One of them only yields a minor phenotype.
a) The specificity of the more efficient SOS1 shRNA should be tested by complementation. b) The authors seem very firm in excluding that defects in Rac signalling contribute to the phenotype without showing any evidence. The complementation approach using GEF mutants could also be used to test whether the Rac activation domain of SOS1 can really be excluded from contributing to the phenotype.

We would contend that the two SOS1 shRNAs do not yield different phenotypes, but rather induce the same junctional defect to different levels. Quantification shows that the penetrance of the phenotype correlates closely with the level of SOS1 knockdown induced (i.e. strong knockdown gives a strong phenotype, partial knockdown gives a partial phenotype), which is convincing evidence for a titratable, SOS1 specific role. Furthermore, we are able to phenocopy loss of SOS1 through inhibition of numerous downstream pathway members (Ras, MEK, ERK), indicating that disruption of this axis at multiple levels inhibits junction formation. As such, we feel that we feel our existing data are sufficiently strong to support a specific role for SOS1 as upstream GEF.

In our original submission, we did not comment on a possible role for Rac but certainly did not intend to formally exclude this (we simply chose to focus on the more novel Ras branch of this pathway in the current study). We have amended the text to clarify this (p4).

2) Cell proliferation and cell numbers: The authors mention at one point that MEK inhibitors did not cause reduced cell proliferation and state in the discussion again that Ras/Erk signalling

inhibition does not affect proliferation in their model. These are crucial data and should be shown, as effects on cell density may affect the differentiation potential of the cells and junction formation. Most images shown seem to suggest that cells with reduced SOS/Ras signalling are at lower densities. One also wonders whether there is an increase in cell death at the time when junctions start to disintegrate.

We agree that this is an important point to clarify. Chronic inhibition of the SOS/Ras/MEK/ERK pathway induces only a modest reduction in 16HBE cell number, which we have now noted in the text for completion (GSK inhibitor reduces cell number by 26±7% at day 3, as compared to DMSO). As cell number is not dramatically reduced, a monolayer is able to form, in which junctions can be analysed effectively. These MEK/ERK inhibitors have been employed across numerous experiments and we have not observed any obvious morphological signs of cell death in cells lacking intact junctions, under the conditions tested.

3) The implication of the data is that EMP1 is not required for junction formation but junction maintenance; hence, the question arises when it is recruited.
a) In the calcium switch experiments, when is EMP1 expressed and recruited to cell-cell contacts?
b) One would also wish for higher resolution localisation experiments that provide information about which type of junction EMP1 associates with.

The reviewer raises an important question regarding the stage at which EMP1 controls epithelial junctions. We have now provided data demonstrating that EMP1 is essential during *de novo* junction formation, but dispensable for junctional maintenance. Please see the response to Reviewer 1, point 2.

As suggested, we have also explored when EMP1 is recruited to junctions. Images are included in the new Supplementary Fig 4. Briefly, our new data indicate that EMP1 is not obviously recruited to E-cadherin/ZO-1 positive primordial puncta, but rather gradually accumulates with a more linear pattern, similar to the related protein, claudin-1.

To localise EMP1 more precisely, we have included confocal z-stacks of mature 16HBE monolayers, co-stained for EMP1 and ZO-1. These images are presented in Fig 4g and indicate that EMP1 is co-localized with ZO-1 at tight junctions.

Referee #3:

1. The authors describe the SOS1-Ras signaling pathway as a regulator of epithelial TJ and barrier function formation (eg in the title). However, SOS1 depletion disrupts the localization of the adherens junction component E-cadherin as well (Suppl. Fig.1a), suggesting that SOS1 could regulate primarily adherens junction formation, which is considered as prerequisite for tight junction formation. It remains unclear if the effects on tight junctions are secondary to the disruption of adherens junctions.

As suggested by the reviewer, it is certainly possible that adherens junction defects may precede tight junction disruption in our model. As noted, we previously showed that depletion of SOS1, expression of DN Ras, or inhibition of MEK/ERK disrupts both E-cadherin and ZO-1 localisation, suggesting defects in both adherens and tight junction formation. We have now included additional data to show that depletion of EMP1 has the same dual phenotype (Fig.4b). Whether the SOS1/Ras/MEK/ERK/EMP1 axis regulates tight junctions directly, or secondary to adherens junctions, the functional consequences remain, ie both fence and gate functions are perturbed upon pathway inhibition. As such, we feel that our title is accurate and have left it unchanged.

2. The authors claim that the cell lines used (16HBE, BCi-NS1.1) form an apical junctional complex including tight junctions. However, confocal Z-section and co-stainings with typical tight junction components such as Occludin or ZO-1 are missing throughout the manuscript.
a) This would be particularly important for the localization of SOS1 (no stainings shown) and
b) EMP1 (claimed to be localized at the apical junction, Fig.4e).
From the immunofluorescence studies shown in the manuscript, it is not possible to judge the specific localization of the two proteins along intercellular junctions.

The reviewer raises an important point and we agree that we had not provided sufficient data to localise our proteins of interest precisely. As recommended, we have now added confocal z-stacks of mature 16HBE monolayers that more clearly demonstrate co-localization of EMP1 with ZO-1 at tight junctions (Fig 4g). We have also tried staining cells for SOS1, as requested. Although we have observed a positive staining at the junctional region, the signal is quite weak and not detectable in all cells. For this reason, we are not confident to comment definitively on SOS1 localisation and have not included this data in our report.

3. To address the question how SOS1 and EMP1 regulate junction formation it would be important to know when they appear at junctions in the course of junction formation. Scratch wounding assays and staining of SOS1 and EMP1 with markers of puncta (e.g. E-cadherin or ZO-1) and markers for more maturated junctions (e.g. Occludin or Claudins) could provide some first ideas.

Please see the response to Reviewer 2, Point 3. Briefly, we have analysed EMP1 recruitment during junction formation in the context of a calcium switch assay. These data are presented in Supplementary Fig 4. We have not included data on SOS1, for the reasons described above.

4. The authors suggest that SOS1 mediates its effect on junction formation through the Ras-Raf-ERK1/2 pathway. However, SOS1 might act through its activity on RhoA as well. Rescue experiments using dominant-active or fast-cycling mutants of Ras and Rac1 in SOS1-depleted cells could tell.

We are not sure why this reviewer feels that SOS1 might be acting through RhoA. We agree that Rac may also be involved and have now made this possibility clear in the text. We feel that a more comprehensive analysis of Rac – whether it is involved and if so whether it is controlled by SOS1 signalling - is beyond the scope of this report.

5. Why do the authors focus in their study on lung epithelial cells?
a) Is the role of SOS1 and EMP1 specific for lung epithelial cells? Epithelial cell lines derived from other organs (e.g. Caco-2, MDCK) should be used to address the question if the role of the SOS1-Raf-MAPK-EMP1 pathway is specific for lung epithelium.
b) These polarized epithelial cells could also provide a clearer picture on the subcellular localization of SOS1 and EMP1 (optical z-sections on filter-grown cells, see point 2).
c) Finally, these cells could be used to study the role of the Ras-Raf-MAPK pathway and the identified upstream and downstream components in a 3-dimensional environment, which in the author's laboratory has proven to be a useful tool to distinguish gate and fence functions of tight junctions.

Our current study was initiated to investigate morphogenesis in airway epithelia, in which tight junctions are essential to provide a barrier against inhaled pathogens, allergens and other xenobiotics. To date we have not explored a wider role for SOS1 or EMP1 in cells derived from other organs. This is certainly an interesting avenue for future work, but goes well beyond the topic

of our current short report. In line with the editor's recommendation, we have added text to discuss this issue (p7). Similarly, analysis of this pathway in a 3D environment is a goal of ongoing work.

To address the issue of subcellular localisation, we have included confocal z-stacks of mature 16HBE monolayers, co-stained for EMP1 and ZO-1 (Fig 4g).

6. The studies applying acute and chronic MEK inhibition suggest a role for the Ras-Raf-MAPK pathway after chronic but not acute treatment with MEK inhibitors (Fig.3a). However, it is not clear if these effects are regulated by SOS1 and mediated through EMP1. A stable cell line with inducible SOS1 knockdown would allow to address this issue by depleting SOS1 in confluent cells.

Confluent cells already have apical junctions and we have now shown in the paper that the ERK MAP kinase pathway is not required for junction maintenance (Supp. Fig.2). Whether this is because mature junctions no longer require EMP1 or whether EMP1 transcription becomes independent of ERK MAP kinase after junctional maturation we are currently exploring.

7. It is interesting that inhibition of the Ras-Raf-MAPK pathway blocks both the gate and the fence function of tight junctions. It would be important to know if EMP1 as downstream effector of the pathway regulates both functions as well. The authors, therefore, should analyze both gate and fence function in EMP1 knockdown cells (Fig.4 shows only the gate function).

As requested, we have performed a fence function assay, using FM 4-64 dye, in EMP1 depleted cells. We find that loss of EMP1 phenocopies inhibition of MEK, disrupting fence function to permit diffusion between apical and lateral membrane domains. Together with our previous work, this data indicates that loss of EMP1 disrupts both the fence and gate functions of bronchial tight junctions.

Minor points

1. The presence/absence of a linear ZO-1 staining cannot be generally considered as indication for functional/non-functional tight junctions since only the simultaneous absence of all three ZO proteins (ZO-1, -2, -3) prevents TJ strand formation (Umeda et al 2005, Cell)

We agree with the reviewer, but feel that we have satisfactorily addressed this issue through: a) staining for multiple tight junction components (occludin images are presented in Supplementary Fig 1), but more importantly b) through our functional gate (TER) and fence (FM 4-64 dye) assays.

2. Several references appear to be incomplete.

This error has been corrected in the updated text.

2nd Editorial Decision 13 October 2014

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. I am happy to let you know that all referees now in principle support publication of your manuscript in EMBO reports.

Nevertheless, both referees 1 and 3 still raise some issues and after having considered them I would

suggest the following: With regard to the issue of the contribution of EMP1 to the ERK phenotype, can you discuss which of the other, novel genes that are differentially regulated under conditions of HRas/MEK/ERK inhibition likely contribute to tight junction formation?

With regard to the SOS1 stainings (referee 3): I would recommend showing the results as supplementary information, even if the staining is weak/not visible in every cell. I do not think that it is necessary to complement the Ca switch assay with a scratch wound assay, as suggested by this reviewer.

Please do let me know if you need any further assistance at this point and I look forward to receive the final version in due time.

REFEREE REPORTS:

Referee #1:

The authors have by and large addressed my concerns and have added very nice new data to support their claims. My only remaining point is the rescue. I do understand that there are other targets that are transcriptionally regulated but even in this short report it would be nice to see how much of the Erk phenotype is mediated through EMP1, as it sheds light on to what extent this pathway coordinates junctions. Alternatively, as a minimum, of those 33 genes, how many might potentially directly or indirectly be involved in tight junction formation? Whether others are involved is indeed beyond this manuscript, I fully agree.

Referee #3:

The authors provide a revised manuscript, which contains some additional information. Some of the aspects raised were not addressed even though the experiments seem simple. For example, the localization of SOS1 during cell-cell contact formation and in fully polarized bronchial epithelial cells should not have been so difficult. Other antibodies or expression of GFP-tagged SOS1 could have been tried. Also, the putatively weak signals for SOS1 at junctions of polarized cells could nevertheless be shown, for example in the supplements. In addition, scratch-wounding assays provide a simple assay system to analyze early events of junction formation, and Ca2+-switch assay cannot compensate scratch assays as the assays follow different mechanisms. The manuscript has been slightly improved, several aspects remain unclear, e.g. the specificity of the SOS1-Raf-MAPK pathway for lung epithelial cells or its relation to adherens vs tight junctions.

2nd Revision - authors' response 23 October 2014

We are very pleased to submit an updated version of our manuscript '*SOS1 and Ras regulate epithelial tight junction formation in the human airway through EMP1*'.

As requested, we have made the following revisions:

1) A table is now provided in Supplementary Figure 3b summarising possible links between additional Ras/MEK/ERK target genes and the process of bronchial morphogenesis.
2) Images of SOS1 staining in 16HBE cells have been added to Supplementary Figure 1d.

We hope that our work will be suitable for publication and thank you for your help.

3rd Editorial Decision 24 October 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

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

