



Induction of apically mistrafficked epiregulin disrupts epithelial polarity via aberrant EGFR signaling

Bhuminder Singh, Galina Bogatcheva, Evan Krystofiak, Eliot T. McKinley, Salisha Hill, Kristie Lindsey Rose, James N. Higginbotham and Robert J. Coffey
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First decision letter

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MS TITLE: Induction of apically mistrafficked EREG disrupts epithelial polarity via aberrant EGFR signaling

AUTHORS: Bhuminder Singh, Galina Bogatcheva, Evan Krystofiak, Eliot T McKinley, Salisha Hill, Kristie Lindsey Rose, James N Higginbotham, and Robert J Coffey
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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 have substantial enthusiasm for the study and its conclusions but raise a number of substantial criticisms that prevent me from accepting the paper at this stage, including concerns about the depth of mechanistic analysis and the integration of somewhat distinct subthemes into a coherent story. 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

The manuscript by Singh et al. is a follow-up to a previously published PNAS manuscript in which the Singh-Coffey duo first reported that the apical mistargeting of EREG Y156 or V159 mutants in MDCK cells results in apical (and basolateral) EGFR activation, and the formation of poorly differentiated/invasive tumors when Y156A-expressing MDCK cells are injected into nude mice. In the current study, a Y156A mutant of EREG is shown to exhibit delayed delivery to the surface, EREG Y156 is shown to be phosphorylated, and a Y156F mutant also assumes an apical steady-state distribution. Additional studies demonstrate that EREG Y156A expression (either constitutive or induced) leads to aberrant lumen formation in matrix-cultured MDCK cell cysts, phenomena that may depend on EGFR activation. Most intriguingly, the most abundant cancer-associated mutation in EREG, R147stop, also exhibits apical mistrafficking. The work is potentially significant as it may implicate aberrant EGFR signaling in response to EREG polarity defects as contributing to carcinomas.

Comments for the author

I have the following comments/suggestions:

Major comments:

While the manuscript is generally focused on EREG, it appears to be an amalgam of smaller stories that are not always taken to completion. Examples include the analyses of EREG Y156 basolateral targeting roles for metalloproteinase cleavage and EGFR activation, and studies of the R147stop mutant. Specific comments follow below.

1. One of the substories is that phosphorylation of EREG Y156 may promote basolateral targeting. We learn that Y156 is phosphorylated, and that a Y156F mutant is apically missorted (as is a Y156A mutant), but the authors do not perform relatively straight-forward experiments showing whether a phospho-mimetic Y156D/E mutant is basolaterally targeted. Could the authors also conceive of using a pulse-chase-biotinylation approach to show if the pool of newly synthesized, basolaterally trafficked, EREG is phosphorylated? Perhaps this could be shown by use of a phosphorylation-dependent shift in PAGE mobility or use of a phospho-specific antibody?

2. The studies of ectopic lumen formation in MDCK cells are interesting and indicate that mistargeting of EREG has significant consequences for cell polarity and growth. The authors note that the inducible expression of EREG Y156A leads to an increase in lumen diameter. While the authors describe this finding as unexpected, they have previously reported that a pool of EGFR is on the apical surface of MDCK cells.

Furthermore, a study from Enrique Rodriguez-Boulán's group (Caceres PNAS 116:11796, 2019) confirms not only apical expression of EGFR, but also of ERBB2 and ERBB3, all of which exhibit an A:B ratio of somewhere around 15:85. While the focus on apical proteins in the cyst studies is warranted, the authors should also consider looking at one or two basolateral proteins to assess whether basolateral polarity/trafficking is affected.

3. Another example of an incomplete substory is the potential role of metalloproteinase and EGFR signaling in aberrant lumen formation. Here, the investigators rely solely on single pharmacological tools (BB-94 or EKI-785). In these experiments, it seems that the authors should show some evidence EGFR activation, including phosphorylation or not of Y1045, and/or activation of downstream EGFR-regulated signaling pathways. While it may be difficult to see these changes in polarizing cell cultures, the authors could use siRNA approaches to confirm the EGFR dependence

(and possible roles for ERBB2 and ERBB3 signaling as well). Likewise, siRNA could be used to screen potential metalloproteinase candidates.

4. Perhaps the most interesting observation of this manuscript is that the most common cancer causing mutant of EREG, 147stop, is apically trafficked. However, this is another example of a substory that is given short shrift. Does expression of EREG147stop lead to ectopic lumen formation? Does expression of this mutant lead to EGFR activation? Will its expression lead to tumor formation when injected into nude mice?

Minor comments:

1. A small concern, but the rationale for making the Y156F mutant is not very satisfying given that Y156A is also not phosphorylatable.

2. Could a role for transcytosis contribute to the delayed surface presentation in the EREG Y156A mutant?

3. Page 5, line 13, should refer to Fig. S4 and not S3.

4. The Materials and Methods section lacks important details:

Please describe how stocks of BB-94, EKI-785, galardin were prepared (what diluent?) and how they were stored. It prepared fresh, please state this.

Please give the catalog number for the anti-GFP and anti-ZO1 primary antibodies and information about what dilution these antibodies were used at.

When performing studies, were the MDCK cells maintained in complete DMEM medium throughout? How were pH fluctuations prevented?

For the stable, dox-inducible clones, which selection markers were used, how were cells cloned, how was the polarity of the non-induced clones verified, and how leaky or not was the expression of the EREG constructs?

For cell lysis/IP analysis - please state the rotor and model number of the centrifuge used in these studies or give the g-force employed

Reviewer 2

Advance summary and potential significance to field

The manuscript by Singh et al. describes consequences of inducing expression of epiregulin (EREG) on the apical surface of epithelial MDCK cells. This EGFR ligand is normally expressed basolaterally, and cleaved via metalloproteinases to signal via receptors localized to that surface. The authors show that mutation of the critical phosphorylated tyrosine residue present within a YxxO sorting determinant causes apical missorting and that when expression of this mutant form is induced in polarized epithelial cell cysts grown in 3D Matrigel culture it promotes the formation of ectopic lumens. This activity is dependent on cleavage of missorted EREG by an unidentified metalloproteinase on the apical surface and apically localized EGFR. Importantly, the authors show that a common cancer-associated mutation in EREG produces a form of the protein that is apically missorted. Although there are many unresolved questions concerning how EREG signaling from the apical membrane promotes ectopic lumenogenesis, the studies presented are carefully executed and interpreted. EREG trafficking and signaling are less studied than other EGFR ligands, and mutations that promote mistrafficking have been associated with cancer so this study is of broad potential significance.

Comments for the author

Major points.

1. The authors should be careful not to state that apical mistrafficking of mutant EREG leads to a “loss of polarity” (eg. p. 5, paragraph 2). The title also should be modified, because it isn’t accurate to state that polarity is disrupted. The cells appear to be polarized, because the mutant EREG is confined to the apical surface and junctional complexes form.

2. In the same sentence, the authors claim that mistrafficking of mutant EREG was associated with a “transformed phenotype”. Aside from promoting formation of ectopic lumens, how else was transformation assessed? Do cells expressing Y156A EREG exhibit altered growth or adhesive properties compared to cells expressing wild type EREG?

3. The authors should comment on why they think apical EREG signaling would impact cell behavior differently than basolaterally EREG signaling.

Minor points.

1. The authors should refrain from using acronyms in the manuscript title, where they have not yet been defined (ie. EREG).

2. On pg. 4, paragraph 3, it would be more appropriate to state “5 minutes of pervanadate treatment”, as opposed to “stimulation”, because pervanadate inhibits phosphatase activity.

3. On pg. 5, the citation for Fig. S3 should be for Fig. S4.

4. Pg. 5, paragraph 2: “Within 3 days of induction, we observed an ? of EREG on the epithelium”. There seems to be a word missing from this sentence.

5. Pg. 9, paragraph 2, citation reads “Fig. S3” but should read “Fig. 3”.

Reviewer 3

Advance summary and potential significance to field

In this manuscript, Singh and collaborators performed a characterization of (Y156) EREG mutant missorting in MDCK cells grown in 3D Matrigel culture and their putative implication in ectopic lumen formation. They demonstrate that EREG is phosphorylated at the tyrosine 156. The overexpression of the (Y156A) EREG mutant is missorted to the apical membrane domain and induce the formation of ectopical lumens. The characterization of Y156 phosphorylation is well documented. However, the observation of the apical missorting of Y156A mutant and their association with transformation and loss of polarity was published by the same group (Singh et al. 2013). The Y156 phosphorylation and the ectopic lumen formation induced by the phosphomutant (Y156A) is the novel part of the manuscript but the mechanism to explain the phenotype is not well supported. The authors attribute the induction of ectopic lumen formation to the metalloprotease activity and aberrant EGFR signaling. Both conclusion are made with just one pharmacological experiment with no proper quantification.

Comments for the author

Major and minor points:

Figure 1: By pulse-chase experiment the authors argue that (Y156A)EREG mutant has a delay in the secretory pathway compare to the wild type, but to confirm the identity of biosynthetic intermediates additional assay like Endo H sensibility should be performed. To compared adequately the different molecular weight of each biosynthetic intermediates the wild type and mutant samples must be load in the same gel.

Figure 3: This figure is unnecessary. The figure 4 show in more detail (including quantifications) the effect of the (Y156A)EREG mutant expression in the ectopic lumen formation. Beside, in this figure is shown that (Y156A)EREG mutant expression also induce the formation of inward grow lumens (arrow), this could be interesting to study in depth.

Figure 4: In the Y axes of cyst diameter quantification what is the meaning of “density”. To attribute the ectopic cyst formation to the unphosphorylated form of EREG the author should overexpress also the (Y156F) EREG mutant and check the cyst formation phenotype.

Figure 6: This figure is unnecessary the video of ectopic cysts formation could be added to the supplemental data.

The image of ERGE-GFP cyst is not completed, the image should be modified. The arrow is mentioned in the text but not in the figure legend.

Figure 7: The authors support their idea of matalloproteases and EGFR involve in the ectopic lumen formation using just a pharmacological approach without any quantification and statistical analysis. The authors use the BB-94 metalloprotease inhibitor to block the ectopic lumen formation, but in the figure S1 they use galardin inhibitor to show the shedding of the (Y156A)ERGE-GFP, is there any reason for this modification, do they have effect? Besides, the authors could make the cleavage

assay in filter grow MDCK to know if the mistargeting apical (Y156A)ERGE-GFP is subject to cleavage.

First revision

Author response to reviewers' comments

Dear Dr. Ewald:

Thank you for allowing us to resubmit our manuscript “**Transformation of polarized epithelial cells by apical mistrafficking of epiregulin**”. We thank the reviewers for their encouraging comments and insightful suggestions. We also thank the editor for allowing additional time to complete the revision. We have addressed each of their comments and added key experiments in this revision. We have added an additional analysis in panel C of Fig. 7 and several new experiments that are included in three new supplementary figures (Figs. S5-7). By incorporating the reviewers' recommendations, we feel that the manuscript is much improved and we are hopeful that it is now suitable for publication in the *Journal of Cell Science*. We have edited the manuscript and figures to adhere to the journal's formatting guidelines.

Below, we address each of the reviewers' concerns in detail. The major changes in the revised manuscript are highlighted in red.

Editor's comments:

As you will see, the reviewers have substantial enthusiasm for the study and its conclusions but raise a number of substantial criticisms that prevent me from accepting the paper at this stage, including concerns about the depth of mechanistic analysis and the integration of somewhat distinct subthemes into a coherent story. They suggest, however, that a revised version might prove acceptable, if you can address their concerns...

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...

Response: We thank the editor and reviewers for their helpful suggestions to improve the quality of the manuscript and strengthen the conclusions drawn. Part of the reason for the delay in this revision was the tragic death of a valued Research Specialist due to COVID-19 in January 2021.

Reviewer 1 comments:

- I. *Advance Summary and Potential Significance to Field: The manuscript by Singh et al. is a follow-up to a previously published PNAS manuscript ... In the current study, a Y156A mutant of EREG is shown to exhibit delayed delivery to the surface, EREG Y156 is shown to be phosphorylated, and a Y156F mutant also assumes an apical steady-state distribution. Additional studies demonstrate that EREG Y156A expression (either constitutive or induced) leads to aberrant lumen formation in matrix-cultured MDCK cell cysts, phenomena that may depend on EGFR activation. Most intriguingly, the most abundant cancer-associated mutation in EREG, R147stop, also exhibits apical mistrafficking. The work is potentially significant as it may implicate aberrant EGFR signaling in response to EREG polarity defects as contributing to carcinomas.*

Response: Thank you for highlighting the importance of the work in a clinical context.

- II. *One of the substories is that phosphorylation of EREG Y156 may promote basolateral targeting. We learn that Y156 is phosphorylated, and that a Y156F mutant is apically missorted (as is a Y156A mutant), but the*
 - a. *authors do not perform relatively straight-forward experiments showing whether a phospho-mimetic Y156D/E mutant is basolaterally targeted.*

- b. *Could the authors also conceive of using a pulse-chase- biotinylation approach to show if the pool of newly synthesized, basolaterally trafficked, EREG is phosphorylated?*
- c. *Perhaps this could be shown by use of a phosphorylation-dependent shift in PAGE mobility or use of a phospho-specific antibody?*

Response: Below, we address each of these comments.

- a. Although we considered a similar approach using Y>D/E mutation as a phospho-mimetic, we reasoned these mutants would localize basolaterally and the results would be indistinguishable from the localization of wild-type EREG. Instead, we performed an *in vitro* kinase assay to identify potential candidates that can phosphorylate the EREG Y156 residue; going forward, we plan to examine the contribution of these potential candidates to EREG phosphorylation.
- b. We are in the process of generating tools and optimizing experimental conditions to perform the experiment suggested. To determine the phosphorylation status of newly synthesized EREG to a specific cell surface, we need to perform an initial pulldown with a pY156-EREG specific antibody followed by with Streptavidin pulldown, similar to a tandem GFP IP:Streptavidin pull down we performed previously (1). We are in the process of generating this antibody after which we plan to conduct these experiments.
- c. No commercial phospho-specific antibodies to EREG exist. As mentioned above, the pY156-EREG specific antibody may prove valuable for these experiments.

- III. *While the focus on apical proteins in the cyst studies is warranted, the authors should also consider looking at one or two basolateral proteins to assess whether basolateral polarity/trafficking is affected.*

Response: We thank the reviewer for this excellent suggestion. We have now performed immunofluorescence of two basolateral proteins Na⁺/K⁺-transporting ATPase α 1 subunit and p120 catenin. These results are presented in Fig. S5. Compared to apical proteins (ezrin and gp135), these basolateral proteins are excluded from the central lumen as well as from ectopic lumens.

- IV. *Another example of an incomplete substory is the potential role of metalloproteinase and EGFR signaling in aberrant lumen formation. Here, the investigators rely solely on single pharmacological tools (BB-94 or EKI-785). In these experiments, it seems that the authors should show some evidence EGFR activation, including phosphorylation or not of Y1045, and/or activation of downstream EGFR-regulated signaling pathways. While it may be difficult to see these changes in polarizing cell cultures, the authors could use siRNA approaches to confirm the EGFR dependence (and possible roles for ERBB2 and ERBB3 signaling as well). Likewise, siRNA could be used to screen potential metalloproteinase candidates.:*

Response: We agree with the reviewer that a number of substories are incomplete. In the revised manuscript, we now show that EGFR is phosphorylated after induction of apically mistrafficked mutant EREG in polarized MDCK cells grown on Transwell filters (see new Fig. S6A). EGFR phosphorylation after apical EREG expression in polarized MDCK cells indicates that apical EREG mistrafficking is not a loss-of-function phenotype as it is able to activate EGFR signaling (Fig. S6A). In the future, we do intend to identify the specific metalloproteases and receptors that mediate the apical EREG phenotype. In fact, EREG also binds to ERBB4 and activates it. In an earlier comment, the reviewer pointed out that, like EGFR, a small but significant pool of ERBB2 and ERBB3 also localize to apical surface. Additionally, ERBB2 and ERBB3 may contribute to the aberrant polarity phenotype by heterodimerizing to EGFR or ERBB4 activated by apically mistrafficked EREG. EREG has been shown to be cleaved by ADAM17 metalloprotease. At this time, we cannot exclude that other family members like ADAM- 9, 10, 12, or 15 might also mediate apical EREG cleavage, although ADAM9, -10, and -17 have previously been shown to localize to the basolateral membranes (2-4). We respectfully submit that a detailed analysis of the receptors and metalloproteases involved is beyond the scope of the present study.

- V. *Perhaps the most interesting observation of this manuscript is that the most common cancer causing mutant of EREG, 147stop, is apically trafficked. However, this is another example of a substory that is given short shrift. Does expression of EREG147stop lead to ectopic lumen formation? Does expression of this mutant lead to EGFR activation? Will its expression lead to tumor formation when injected into nude mice?*

Response: Based on the reviewer's suggestion, we obtained a cancer cell line (HDQ-P1), which harbors a EREG R147stop mutation to perform the experiments suggested by the reviewer. HDQ-P1 cells are growth inhibited by cetuximab, supporting a role for EGFR signaling in this line. EGFR is

wild-type in this line, thus it appears that endogenous ligands are needed for its activation. When cultured in 3D in Matrigel, this line also forms cysts with ectopic lumens. These results have been added in the supplementary information (Fig. S7). We next planned to treat HDQ-P1 subcutaneous xenografts in athymic nude mice with cetuximab but, unfortunately, tumors failed to form within three months of injecting 5 million cells per animal. We have been unable to perform additional experiments within the time frame allotted for this revision.

Minor comments Reviewer 1

1. *A small concern, but the rationale for making the Y156F mutant is not very satisfying given that Y156A is also not phosphorylatable.*

Response: We substituted Y156 with phenylalanine, which is the most conservative mutation that is unable to be phosphorylated; phenylalanine lacks the phosphate-accepting hydroxyl group. With an alanine substitution, the tyrosine's aromatic ring is removed, in addition to removal of the hydroxyl group.

2. *Could a role for transcytosis contribute to the delayed surface presentation in the EREG Y156A mutant?*

Response: Although formally possible, it is unlikely to be a major contributor since within 20 minutes wild-type and mutant were localized to the basolateral and apical cell surfaces, respectively, as shown in our earlier publication (1). Appearance at apical and basolateral surfaces within 20 minutes of chase indicates direct delivery for the newly synthesized EREG isoforms to their respective cell surfaces (1). We cannot exclude that recycling and/or transcytosis may contribute at later time points to modulate steady state levels.

3. *Page 5, line 13, should refer to Fig. S4 and not S3.*

Response: This has now been corrected.

4. *The Materials and Methods section lacks important details:*

- *Please describe how stocks of BB-94, EKI-785, galardin were prepared (what diluent?) and how they were stored. If prepared fresh, please state this.*

Response: BB-94, EKI-785, and galardin were prepared at 1-10 mM stocks in anhydrous DMSO and stored as small aliquots (50-100 μ l) and stored in -20 °C. Each aliquot was thawed immediately before use and was promptly refrozen after use; each aliquot was typically reused 2-5 times. This information has now been added to the manuscript.

- *Please give the catalog number for the anti-GFP and anti-ZO1 primary antibodies and information about what dilution these antibodies were used at.*

Response: Rabbit polyclonal GFP (Cat #A-11120) and ZO-1 (Cat #61-7300) antibodies were purchased from Invitrogen. GFP antibodies were used at 1:2000 (1 μ g/ml) for western blots and 2 μ g (1 μ l) for immunoprecipitation. ZO-1 antibodies were used at 1:600 dilution for immunofluorescence (0.25 mg/ml stock). This information has now been added to the manuscript.

- *When performing studies, were the MDCK cells maintained in complete DMEM medium throughout? How were pH fluctuations prevented?*

Response: MDCK cells were maintained in complete DMEM medium throughout, except for the EGFR phosphorylation experiment added in Fig. S6 where cells were serum-starved for 24 hours. Media was changed every other day to avoid buildup of acidic metabolites and cells were constantly monitored with the pH indicator dye Phenol Red included in the medium.

- *For the stable, dox-inducible clones, which selection markers were used, how were cells cloned, how was the polarity of the non-induced clones verified, and how leaky or not was the expression of the EREG constructs?*

Response: For dox-inducible EREG-EGFP expression, pINDUCER20 plasmid with a G418 selection marker was obtained from Stephen J Elledge (5). After 10-14 days of selection (G418), individual GFP-positive cells were cloned by FACS in 96-well dishes. Individual clones were allowed to grow and tested for their leakiness by western blotting for EREG-EGFP expression with or without addition of doxycycline. Clones with tight expression were used for subsequent studies. To maintain tight control, low-doxycycline/tetracycline-containing serum was used to culture inducible clones. These additional details have now been added to the manuscript.

- For cell lysis/IP analysis - please state the rotor and model number of the centrifuge used in these studies, or give the g-force employed

Response: As requested, these details have been included in the revised manuscript.

Reviewer 2 comments:

- I. *The manuscript by Singh et al. describes consequences of inducing expression of epiregulin (EREG) on the apical surface of epithelial MDCK cells. ... Although there are many unresolved questions concerning how EREG signaling from the apical membrane promotes ectopic lumenogenesis, the studies presented are carefully executed and interpreted. EREG trafficking and signaling are less studied than other EGFR ligands, and mutations that promote mistrafficking have been associated with cancer so this study is of broad potential significance.*

Response: Thank you for commenting on the impact this manuscript has to the study of cancer.

- II. *The authors should be careful not to state that apical mistrafficking of mutant EREG leads to a “loss of polarity” (eg. p. 5, paragraph 2). The title also should be modified, because it isn’t accurate to state that polarity is disrupted. The cells appear to be polarized, because the mutant EREG is confined to the apical surface and junctional complexes form.*

Response: We agree that “loss of polarity” may cover multiple aspects of epithelial polarity. It is a broad term that we should have defined more precisely. We have now replaced that phrase with “disruption of selective aspects of epithelial polarity.” We consider formation of unilamellar epithelial (MDCK) cysts with single central lumen in 3D Matrigel cultures to be a normal polarized structure. From that perspective, deviations from that appearance constitute disruption of polarity. The text in introduction, results, and discussion has been revised to clarify that inducible expression of mutant EREG results in a temporal disruption of selected aspects of epithelial polarity as manifest by ectopic lumen formation.

- III. *In the same sentence, the authors claim that mistrafficking of mutant EREG was associated with a “transformed phenotype”. Aside from promoting formation of ectopic lumens, how else was transformation assessed? Do cells expressing Y156A EREG exhibit altered growth or adhesive properties compared to cells expressing wild type EREG?*

Response: Transformation is classically defined as the ability of cells to form tumors in athymic nude mice. In our earlier report, we showed that MDCK cells expressing apical Y156A EREG formed tumors in nude mice when injected subcutaneously (1).

- IV. *The authors should comment on why they think apical EREG signaling would impact cell behavior differently than basolaterally EREG signaling.*

Response: We have updated discussion to include this aspect: “We observed that basolateral addition of EREG to polarized MDCK cells led to the expected robust yet transient EGFR phosphorylation. In contrast, apical EREG addition led to weaker but sustained EGFR phosphorylation (1).”

Minor points.

1. *The authors should refrain from using acronyms in the manuscript title, where they have not yet been defined (ie. EREG).*

Response: This has now been corrected; EREG in the title has been changed to epiregulin.

2. *On pg. 4, paragraph 3, it would be more appropriate to state “5 minutes of pervanadate treatment”, as opposed to “stimulation”, because pervanadate inhibits phosphatase activity.*

Response: This has now been corrected.

3. *On pg. 5, the citation for Fig. S3 should be for Fig. S4.*

Response: This has now been corrected.

4. *Pg. 5, paragraph 2: “, we observed an ? of EREG on the epithelium”. There seems to be a word missing from this sentence.*

Response: This has now been corrected.

5. *Pg. 9, paragraph 2, citation reads “Fig. S3” but should read “Fig. 3”.*

Response: This has now been corrected.

Reviewer 3 comments:

- I. *In this manuscript, Singh and collaborators performed a characterization of (Y156) EREG mutant missorting in MDCK cells grown in 3D Matrigel culture and their putative implication in ectopic lumen formation. ... The authors attribute the induction of ectopic lumen formation to the metalloprotease activity and aberrant EGFR signaling. Both conclusion are made with just one pharmacological experiment with no proper quantification.*

Response: Quantification is now performed and shared as a new panel in Fig. 7C.

Major and minor points:

- II. *Figure 1: By pulse-chase experiment the authors argue that (Y156A)EREG mutant has a delay in the secretory pathway compare to the wild type, but to confirm the identity of biosynthetic intermediates additional assay like Endo H sensibility should be performed. To compared adequately the different molecular weight of each biosynthetic intermediates the wild type and mutant samples must be load in the same gel.*

Response: We agree that for absolute quantification samples must be loaded on the same gel; however, no quantitative conclusions were drawn comparing wild-type and mutant EREG in the results section. It was only in the discussion section that we speculated on the transit of the two isoforms through different intermediates. To prevent any appearance of a direct comparison, we have kept wild-type and Y156A mutant EREG in separate panels in Fig. 1A and 1B, respectively.

- III. *Figure 3: This figure is unnecessary. The figure 4 show in more detail (including quantifications) the effect of the (Y156A)EREG mutant expression in the ectopic lumen formation. Beside, in this figure is shown that (Y156A)EREG mutant expression also induce the formation of inward grow lumens (arrow), this could be interesting to study in depth.*

Response: Based on the reviewer's suggestion, we had submitted the revision where we had moved Fig. 3 to the supplementary section. However, the manuscript was returned with the note that the number of supplementary figures must be equal to or less than the number of main figures. To adhere to this JCS policy, we have now moved this figure back as a main figure. We did not remove this figure altogether since the reviewer pointed out that it has important additional information, that is, formation of inward growth in lumens.

- IV. *Figure 4: In the Y axes of cyst diameter quantification what is the meaning of "density". To attribute the ectopic cyst formation to the unphosphorylated form of EREG the author should overexpress also the (Y156F) EREG mutant and check the cyst formation phenotype.*

Response: Density (y-axis) corresponds to the fraction of cysts with that diameter (x-axis). (Y156F)EREG -EGFP is expressed constitutively; we did not have time to generate an inducible version of that mutation within the time frame allotted for this revision. Consequently, we have refrained from stating that unphosphorylated EREG leads to formation of ectopic lumens, but that only induction of apical EREG leads to formation of ectopic lumens.

- V. *Figure 6: This figure is unnecessary the video of ectopic cysts formation could be added to the supplemental data.*

Response: We would like to keep this figure, since it will be included in the print version, which may prompt readers to access the time-lapse video in supplementary information.

- VI. *The image of ERGE-GFP cyst is not completed, the image should be modified. The arrow is mentioned in the text but not in the figure legend.*

Response: An arrow description is now included in the figure legend in Fig. 6. Additionally, Video 2 includes the complete duration of cyst imaging.

- VII. *Figure 7: The authors support their idea of matalloproteases and EGFR involve in the ectopic lumen formation using just a pharmacological approach without any quantification and statistical analysis. The authors use the BB-94 metallopretease inhibitor to block the ectopic lumen formation, but in the figure S1 they use galardin inhibitor to show the shedding of the (Y156A)EREG-GFP, is there any reason for this modification, do they have effect? Besides, the authors could make the cleavage assay in filter grow MDCK to know if the mistargeting apical (Y156A)ERGE-GFP is subject to cleavage.*

Response: Both Galardin and BB-94 are broad-spectrum metalloprotease inhibitors that inhibit EGFR ligand cleavage, and we sometimes use them interchangeably as shown in Fig. S1 for inhibition of cleavage of EREG-EGFP. In the new supplementary data (Fig. S6B), we show that both Galardin and BB-94 can inhibit cleavage of (Y156A)EREG-EGFP.

Importantly, based on this reviewer's suggestion, we have performed the experiments in Fig. S6A,B on Transwell filters, which further confirms that apical (Y156A)EREG-EGFP is subject to cleavage, which was blocked by metalloprotease inhibitors.

Sincerely

Robert J. Coffey, MD

Bhuminder Singh, PhD

Epithelial Biology Center

10415F, MRB IV

Vanderbilt University Medical Center

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Second decision letter

MS ID#: JOCES/2020/255927

MS TITLE: Induction of apically mistrafficked epiregulin disrupts epithelial polarity via aberrant EGFR signaling

AUTHORS: Bhuminder Singh, Galina Bogatcheva, Evan Krystofiak, Eliot T McKinley, Salisha Hill, Kristie Lindsey Rose, James N Higginbotham, and Robert J Coffey

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 are generally satisfied with the scope of revisions. Reviewers 1 and 2 raise some minor points that are worth addressing. You do not need to respond to Reviewer 3. I

hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from Reviewers 1 and 2.

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

Establish that a common mutation found in cancers (R147STOP) is apically targeted, disrupting epithelial polarity upon EGFR activation.

Comments for the author

The revised manuscript by Singh et al. has addressed many of the reviewer's initial concerns. I have a few minor concerns that the authors may consider remedying:

1. Fig. 1 - it is stated that the Y156A mutant is slower to process. However, in the single blot provided, the processed form of the construct (species 3) appears much faster in Y156A mutant than the wild-type protein.

It looks like the mutant may get processed more quickly than the wild-type protein.

2. Fig.2B - could the authors include the same data for the wild-type protein (ERE=EGFP)?

3. Fig. S4. - could the authors include an image of MDCK cell xenografts expressing the control construct (ERE-EGFP)

Reviewer 2

Advance summary and potential significance to field

The manuscript by Singh et al. describes consequences of inducing expression of epiregulin (ERE) on the apical surface of epithelial MDCK cells. Normally, this EGFR ligand is expressed basolaterally and cleaved via metalloproteinases to bind and signal via receptors localized there. Authors show that mutation of a critical phosphorylated tyrosine residue present within a known basolateral sorting motif causes apical missorting of EREG. When expression of this mutant form is induced in polarized epithelial cell cysts grown in 3D Matrigel culture, it promotes formation of ectopic lumens. This activity is dependent on cleavage of missorted EREG by an unidentified metalloproteinase on the apical surface and apically localized EGFR. Importantly, the authors show that a common cancer-associated mutation in EREG produces a form of the protein that is apically missorted. EREG trafficking and signaling are less studied than other EGFR ligands, and mutations that promote mistrafficking have been associated with cancer so this study is of broad potential significance.

Comments for the author

In this revised manuscript, the authors have addressed most of the previously raised concerns with inclusion of new data, rewritten passages or clarifications in the rebuttal letter. Effort has been made to round out several sub-stories that were considered somewhat thin in the original submission, and the paper is much improved as a result. Although there are still some unanswered questions concerning the role of phospho-Y156 in promoting basolateral trafficking of EREG in this reviewer's opinion those results are not critical to this paper.

Reviewer 3*Advance summary and potential significance to field*

In this manuscript Singh and colleagues show that the mutated form of epiregulin (Y156A) results in direct and complete delivery of mutant epiregulin to the apical cell surface in MDCK cysts. Further, epiregulin-expressing cysts form ectopic lumens in mutant, but not wild-type, cells within three days of induction. Interestingly, these ectopic lumens form de novo rather than budding from the central lumen and depend on metalloprotease cleavage of epiregulin and EGFR activity. Moreover, the most frequent EREG mutation in human cancer (R147stop) results in its apical mistrafficking in MDCK cells. Thus, induction of epiregulin apical trafficking is sufficient to disrupt normal lumenogenesis of a preformed polarized epithelium.

Comments for the author

In this new version of the manuscript sent for publication by Singh and coauthors, new details are provided on the role of epiregulin in EGFR signalling in the process of epithelial morphogenesis and tumour transformation. I understand that the authors have made a significant effort to answer these questions given the limitations that we have in these times and the limited time is given to them to answer the reviewers' questions. Indeed, the authors have carried out a series of experiments to answer the questions asked, and many of them have been satisfactorily answered. I believe the initial observation is quite interesting: the expression of the mutant of epiregulin that localizes in the apical region is associated with the formation of ectopic lumens that depend on the activity of metalloproteases and EGFR. Also, they found that the most frequent EREG mutation in human cancer (R147stop) results in its apical mistrafficking in MDCK cells, and compromised the formation of lumens in epithelial tissues. Still, this new version of the manuscript provides little new mechanistic insights on how these effects of epiregulin in EGFR signalling compromise the integrity of the epithelium. The authors in the discussion suggest two possibilities that could explain the mechanism/s that are undoubtedly possible: defects in the orientation of the mitotic spindle and dysregulation of apoptosis. However, they do not make any approach to investigate if they have any relevant role in this process. It is somewhat surprising since they are relatively simple experiments to perform in this cell model, as shown by the extensive previous literature on this matter.

In summary, an attractive, solid, and well-done work lacks a deeper mechanistic foundation to explain the observed phenotypes. I think that it does not make much sense to request a new round of review and, in my opinion, it could be accepted under the current conditions.

Second revisionAuthor response to reviewers' comments

Dear Dr. Ewald:

Thank you for allowing us to resubmit our manuscript "**Transformation of polarized epithelial cells by apical mistrafficking of epiregulin**". We thank the reviewers for their uniformly positive comments. Below, we address each of their remaining minor concerns and we are hopeful that it is now suitable for publication in the *Journal of Cell Science*. We have edited the manuscript and

figures to conform to the journal's formatting guidelines. The major changes in the revised manuscript are highlighted in red.

Reviewer 1 comments:

- I. *Fig. 1 - it is stated that the Y156A mutant is slower to process. However, in the single blot provided, the processed form of the construct (species 3) appears much faster in Y156A mutant than the wild-type protein. It looks like the mutant may get processed more quickly than the wild-type protein.*

Response: Thank you for this astute observation. The discussion now has been modified to bring out this point. *"Compared to WT, initial glycosylation of Y156A EREG appears to be slower (compare appearance of isoforms 2 at 0 and 20 min timepoints in Fig. 1A,B). However, subsequent accumulation of the processed isoform 3 appears to be faster for Y156A EREG."*

- II. *Fig.2B - could the authors include the same data for the wild-type protein (EREG=EGFP)?*

Response: We refer the reviewer to our previously published manuscript (PMID: 23671122, Fig. 1A) that includes the data requested. Moreover, based on the suggestion of Reviewer 2, this figure has now been removed (see below).

- III. *Fig. S4. - could the authors include an image of MDCK cell xenografts expressing the control construct (EREG-EGFP)*

Response: We thank the reviewer for this excellent suggestion. We have now included the data for MDCK xenografts expressing WT EREG-EGFP in updated Fig. S2.

Reviewer 2 comments:

- I. *In this revised manuscript, the authors have addressed most of the previously raised concerns with inclusion of new data, rewritten passages or clarifications in the rebuttal letter. Effort has been made to round out several sub-stories that were considered somewhat thin in the original submission, and the paper is much improved as a result. Although there are still some unanswered questions concerning the role of phospho-Y156 in promoting basolateral trafficking of EREG, in this reviewer's opinion those results are not critical to this paper.*

Response: Thank you for noting that the manuscript is much improved; in no small measure, this is due to the constructive comments provided by Reviewer 2. Based on the reviewer's recommendation, the sub-story related to EREG tyrosine phosphorylation has been removed.

Finally, we wish to thank Dr. Ewald for his guidance throughout the review process and encouraging us to take this manuscript across the finish line.

Sincerely

Robert J. Coffey, MD

Bhuminder Singh, PhD

Epithelial Biology Center

10415F, MRB IV

Vanderbilt University Medical Center

Third decision letter

MS ID#: JOCES/2020/255927

MS TITLE: Induction of apically mistrafficked epiregulin disrupts epithelial polarity via aberrant EGFR signaling

AUTHORS: Bhuminder Singh, Galina Bogatcheva, Evan Krystofiak, Eliot T McKinley, Salisha Hill, Kristie Lindsey Rose, James N Higginbotham, and Robert J Coffey

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.

Reviewer 1

Advance summary and potential significance to field

Authors were very responsive, and the manuscript is a welcome addition to our understanding of EGFR ligand biology.

Comments for the author

Authors were very responsive, and the manuscript is a welcome addition to our understanding of EGFR ligand biology.

Reviewer 2

Advance summary and potential significance to field

Singh et al demonstrate that a mutated form of epiregulin (Y156A) is missorted to the apical surface of MDCK cells and that Matrigel-cultured cysts expressing mutated epiregulin form ectopic lumens. They also show that ectopic lumen formation requires metalloprotease cleavage of epiregulin and EGFR activity. This is potentially relevant to cancer biology, because the most frequent EREG mutation in human (R147stop) causes the mutated protein to be incorrectly trafficked to the apical surface of MDCK cells. Although mechanistic questions remain to be answered, the fundamental finding of the study is very interesting.

Comments for the author

The authors have addressed all of the concerns of this reviewer.