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Intersectin-s interaction with DENND2B facilitates recycling of epidermal growth factor receptor

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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 02 March 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings. However, referees 1 and 2 also point out several technical concerns and have a number of suggestions for how the study should be strengthened. Referee 3 is concerned that the role of Intersectin-DENND2B in recycling is not rigorously shown with the current assays. Upon further discussion with the referees we think that the following major experiments are essential:

- Perform EGFR recycling assays to clearly prove a role for DENN2B in EGFR recycling.
- Validate the knockdown efficiency and specificity and improve the description in the methods.

The rescue experiment with the S30A mutant (referee 1) as well as point 4 of referee 3 would certainly strengthen the paper but might be beyond the scope of a revision - as already indicated in the referee's reports. Therefore, it is not required to experimentally address these points for a successful revision. Textual changes and toning down the conclusions and the title will be sufficient in this respect.

Moreover, a domain cartoon as suggested by all three referees would certainly be helpful to guide the reader and to ease the understanding.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

The study by Ioannou supports a nice model in which DENND2B helps recycling of EGFR through interaction with Intersectin-s in a fashion that is inhibited through EGF-dependent phosphorylation of DENND2B via Protein-Kinase D. Thus, PKD phoshphorylation of DENND2B mediates its binding to 14-3-3 proteins and withdraws it from its functional role in EGFR-recycling.
The experiments are well done and the scope of the story is appropriate (in my view) for EMBO Reports.

My three main concerns are the knockdown procedure for DENND2B as used in Fig. 4 and connecting the dots on the role of S30 phosphorylation of DENND2B and its binding to ITSN-s. The first two deal with technical aspects, which hopefully are quite feasible for the authors to deal with, although may rely on DENND2B antibodies. The other is an increase in scope, which the authors may deem unfeasible, but I think would cement the importance of the DENND2B~ITSN-s interaction and the phospho-regulation of that interaction.

1. The procedure for knocking down DENND2B is not described in material and methods nor is it well described in the previous paper referred to. The actual RNA sequences used are not given nor where they hybridize to the target mRNA.

2. The method used to verify knockdown is not focused on endogenous proteins, but rather FLAG-tagged DENND2B expressed in an unspecified way (transient transfection, stably integrated?). There is no way to tell here how much DDND2B is left in the cell. In addition, the figure showing knockdown is in HEK-293T cells, whereas the functional assays are done in MCF-10A cells. No data are reported for efficiency of knockdown in the MCF-10A cells. One solution could blots of endogenous levels of DENND2B in MCF-10A cells.

3. The regulatory mechanism here revolves around EGF stimulating phosphorylation of DENND2B at primarily S30. Reconstituting MCF-10A knockdown cells with normal and S30A mutant DENND2B would strengthen this paper and tie it together more cohesively. This would also speak to the larger issue of what interaction with ITSN-s does and if it is important for EGFR-reycling. The authors conclude in the Discussion that 'ITSN-s couples EGF-independent EGFR internalization with its recycling by binding DENND2B'. This conclusion seems a bit of a reach. While the binding and effect of phosphorylation are clear, a functional assay to show this interaction per se (or its regulation via S30) would really enhance this work.

Minor:

Figure 2D legend mentions PKD-constitutively active was used. This is not clear from figure as there is no label for this.

The paper could include a diagram of the domain structure of DENND2B and what must be its multiple SH3-binding motifs together with a diagram of ITSN-s and some of where its key interactors bind. This would help readers understand the biochemistry and how these proteins come together.

Is DENND2B's effect on EGFR recycling specific for a subset of proteins or is TfR recycling affected?

The authors state that 'Using mass spectrometry we confirmed that Ser-30 is phosphorylated upon OA treatment (data not shown).' I think it is worthwhile to show these data - there is ample figure space to do so. Also, since there is residual binding with the anti-phospho PKD substrate antibody after IP of the S30A DENND2B mutant, it would be helpful to point out other putative PKD phospho-sites (possibly reviewing mass-spectrometry data for their detection).

The authors say 'Consistent with previous studies, the ability of DENND2B to enhance MAPK activity is independent of its GEF activity as expression of the DENN domain alone does not activate MAPK (Fig S1D-F).' This does not seem like a fair conclusion given the reason cited. There are probably lots of ways a naked DENN domain would not activate MAPK because its missing all of its other regulatory features that would hook the DENN domain up properly.

The authors motivate the survey for SH3-containing proteins that bind DENND2B with the observation that 'deletion of the Grb2-binding site on DENND2B does not affect the ability of DENND2B to enhance MAPK signaling. This suggests that DENND2B interacts with additional SH3 containing proteins important for EGFR signalling'. This particular reason is confusing as it is saying that when the known SH3-domain binding motif is removed from DENND2B, there is no effect on function, therefore there must be other SH3-domain proteins that bind DENDD2B ? ...and similarly have no effect either? A smoother rationale here would help the flow of the text.

Referee #2:

Intersectin-s interaction with DENND2B determines the recalling fate of EGFR.

This work form the McPherson lab reveals a novel mode of EGFR recycling control in which Intersectin-s binds the Rab13 exchange factor DENND2B to modulate return to the cell surface. Interestingly, EGF treatment results in increased PKD-dependent DENND2B phosphorylation that dissociates Intersectin-s and DENND2B, shifting the balance from EGFR recycling into degradation (as would be expected w EGF treatment).

Overall this work is of high quality and biologically interesting, no major criticisms.

This may be beyond the scope of the present work, but the idea that ubiquitin modification of EGFR overrides the recycling signal seems like an interesting topic for additional resolution that may establish a paradigm. (Steric hinderance vs opposing post-translational modifications vs ?)

It would be helpful to provide the reader with domain cartoons for DENND2B and ITSN.

In figure 1E the lowest arrow in the lower left panel (Flag-ITSN-s) is not pointing to the same spot as it is in the mCH-Rab13 or merged image.

Efficiency of DENND2B knock-down efficiency in Figure 4? (Are the observed differences simply due to efficiency of KD?)

A summary model would benefit the non-aficiando and would serve as a great bullet point for advertising!

Referee #3:

In the present manuscript authors described Intersectin as a novel partner of DENND2B, a GEF for the recycling Rab protein, Rab 13. Their interaction is negatively regulated by serine phosphorylation of DENND2B by protein kinase D (PKD) at a conserved serine residue (Ser30, within a PKD consensus site), which is increased upon EGF stimulation. Indeed, Intersectin and DENND2B interact in basal condition, and EGF stimulation reduces their interaction. This is due to recruitment of 14-3-3 proteins to the phosphorylated Ser30 of DENN2B, which compete for Intersectin binding, displacing it.

The molecular mechanism at the basis of DENND2B-Intersectin interaction and its regulation are well dissected. The biochemical data are carefully performed and convincing. However, there is no demonstration for a role of Intersectin-DENND2B interaction in EGFR recycling. More experiments are needed to prove the model proposed and to justify the title of the manuscript.

Major issues:

1) No EGFR recycling assay is provided. Measurement of surface EGFR is just an indirect evidence for a putative role of DENND2B in recycling. Indeed, the reduced EGFR levels upon DENND2B KD might be the result of different mechanisms, including reduced recycling, increased endocytosis or reduced synthesis. I understand the difficulty of following constitutive EGFR recycling. But this can be done by they use of different approaches, either based on PM biotinylation/stripping

procedure (for a detailed protocol see, for instance, McGill MA, JBC, 2009) or labeling the EGFRs at the cell surface with an EGFR antibody recognizing the extracellular domain in vivo at 4C (e.g. Mab 108 from ATCC, or 13A9 from Genentech), followed by internalization at 16C for 1-2 h depending on the cell type (at this temperature recycling is blocked, while internalization proceeds). This will allow to load cells with antibody-bound EGFRs. Cells can be then shifted at 37C and recycling can be then followed by IF or FACS. If DENND2B affect EGFR recycling, pronged retention in intracellular compartments should be visualized, and/or delayed reappearance to the PM.

2) Authors should also exclude that, in their experimental setting, shRNA of DENND2B/Intersectin is not altering EGFR constitutive endocytosis. They could follow internalization by labeling again the cell in vivo with the anti-EGFR antibody, followed by internalization at different time points. Acid wash stripping prior fixation can be performed to follow only internalized EGFR.

3) While it is clear that DENND2B and Intersectin constitutively interact and their interaction is reduced upon EGF stimulation, it is not investigated whether this interaction plays a role in EGFR recycling. It is not tested a possible effect of Intersectin KD on EGFR surface level nor in the constitutive recycling pathway of the EGFR. This must be shown in the experimental setting under scrutiny. Also DENN2B/Intersectin double KD should be included, in order to understand whether they are indeed acting in the same pathway.

4) Does DENN2B interact with Intersectin through its proline-rich region? This is inferred from the fact that the binding occurs with the SH3 domain of Intersectin, but it is not formally demonstrated. Rescue experiments with DENN2B mutant in the intersectin-binding region would be instrumental to definitively demonstrate that the interaction between DENND2B and Intersectin is critical for EGFR recycling. I realize, however, that these experiments could be highly demanding, if not unpractical. Alternatively, authors should tone down a bit their conclusions. I think that - with the additional experiments at previous points - data provided are highly relevant and with a level of novelty that would be of interest to the community of cell biologists at large.

Other issues:

1) In Fig. 1A and 2B, please provide Ponceau for GST protein levels.

2) Please show that Itersectin-SH3A has a reduced binding for Ser30A mutant and that Ser30A mutant is not detected by P-PKD substrate.

3) Is Ser30 close to the proline-rich region to explain the competition between 14-3-3 and Intersectin? Or there are other possible explanations? This issue should be better discussed. Maybe adding a scheme depicting the different domains of the proteins studied could help the reader.

4) In the results section (pag. 8), it is incorrectly stated that Grb2 is an adaptor protein that binds the EGFR in the endosomes. Grb2 is recruited to the activated EGFR at the PM, at very early time point after EGF addiction (~2-3 min, see, for instance, Fortian and Sorkin, 2014).

5) Please note that, upon EGF, not all EGFRs are targeted to degradation: EGF-occupied EGFR are still either recycled or degraded, depending if they are ubiquitinated. Please revise text to render this clearer.

1st Revision - authors' response 04 August 2017

Thank you very much for your review of our manuscript (EMBOR-2017-44034V1). We were delighted that reviewer 1 found that “The experiments are well done and the scope of the story is appropriate (in my view) for EMBO Reports” and that reviewer 2 indicated that “Overall this work is of high quality and biologically interesting, no major criticisms” and that reviewer 3 found that “The molecular mechanism at the basis of DENND2B-Intersectin interaction and its regulation are well dissected. The biochemical data are carefully performed and convincing.” We recognize that the reviewers raised several important critiques of the study, some of which overlap. We have worked diligently to address their comments. We are now submitting a revised version of the manuscript in which we address all of the comments raised by the reviewers, in many cases through the addition of new data. Most notably we:

1) Performed EGFR recycling and endocytosis assays to clearly prove a role for DENND2B in EGFR recycling.

2) Validated the knockdown efficiency and specificity and improved the description in the methods.

3) At your suggestion, we modified the text and the title to tone down the conclusions regarding the role of DENND2B/ITSN interaction in EGFR recycling. We carefully considered rescue experiments with the S30A mutant but as explained in detail in response to the reviewers, these experiments would be fraught with technical difficulties.

4) We added a domain cartoon as suggested by all three referees, which indeed will be helpful to guide readers and ease understanding.

We feel that these and several other changes outlined in detail in the response to reviewers strengthen the manuscript and we thank the reviewers for their comments.

POINT-BY-POINT RESPONSE

Referee #1:

The study by Ioannou supports a nice model in which DENND2B helps recycling of EGFR through interaction with Intersectin-s in a fashion that is inhibited through EGF-dependent phosphorylation of DENND2B via Protein-Kinase D. Thus, PKD phoshphorylation of DENND2B mediates its binding to 14-3-3 proteins and withdraws it from its functional role in EGFR-recycling. The experiments are well done and the scope of the story is appropriate (in my view) for EMBO Reports.

We thank the reviewer for the positive comments regarding our study.

My three main concerns are the knockdown procedure for DENND2B as used in Fig. 4 and connecting the dots on the role of S30 phosphorylation of DENND2B and its binding to ITSN-s. The first two deal with technical aspects, which hopefully are quite feasible for the authors to deal with, although may rely on DENND2B antibodies. The other is an increase in scope, which the authors may deem unfeasible, but I think would cement the importance of the DENND2B~ITSN-s interaction and the phospho-regulation of that interaction.

1. The procedure for knocking down DENND2B is not described in material and methods nor is it well described in the previous paper referred to. The actual RNA sequences used are not given nor where they hybridize to the target mRNA.

We apologize for this oversight. We have expanded our materials and methods section in the revised manuscript, which now includes a more detailed description of virus production and knockdown of DENND2B. We have included the RNA sequences used to target DENND2B for knockdown in the materials and methods section of the revised manuscript.

2. The method used to verify knockdown is not focused on endogenous proteins, but rather FLAG-tagged DENND2B expressed in an unspecified way (transient transfection, stably integrated?). There is no way to tell here how much DDND2B is left in the cell. In addition, the figure showing knockdown is in HEK-293T cells, whereas the functional assays are done in MCF-10A cells. No data are reported for efficiency of knockdown in the MCF-10A cells. One solution could blots of endogenous levels of DENND2B in MCF-10A cells.

We agree with the reviewer that the optimal way to demonstrate efficient knockdown would be to blot endogenous DENND2B in MCF10A cells. Unfortunately, despite extensive efforts and resources, we have been unable to produce or purchase an antibody that recognizes endogenous DENND2B specifically. In addition to testing two commercially available antibodies from GenTex and Abcam, we tested 4 homemade polyclonal antibodies raised against 2 different DENND2B peptides, with limited success. We thus originally chose to validate our knockdown by showing that cells transduced with shRNA targeting DENND2B were unable to express the Flag-DENND2B construct. We reasoned that transient transfection with plasmid DNA would yield higher levels of mRNA than what is found endogenously, and thus prevention of expression would be a meaningful readout of knockdown. We have added additional information in the materials and methods section to specify how this experiment was performed. However, we understand that this method of validation does not reveal alterations in the level of endogenous DENND2B. We thus used real-time PCR on MCF10A cells following viral knockdown of DENND2B, revealing that DENND2B mRNA

levels are reduced to 13% and 23% of the control cells with the two distinct knockdown sequences. This new data, found in Figure 4C of the revised manuscript confirms the effectiveness of the knockdown. Furthermore, these new experiments address the reviewers concern regarding cell type used for the functional studies.

3. The regulatory mechanism here revolves around EGF stimulating phosphorylation of DENND2B at primarily S30. Reconstituting MCF-10A knockdown cells with normal and S30A mutant DENND2B would strengthen this paper and tie it together more cohesively. This would also speak to the larger issue of what interaction with ITSN-s does and if it is important for EGFR-reycling. The authors conclude in the Discussion that 'ITSN-s couples EGF-independent EGFR internalization with its recycling by binding DENND2B'. This conclusion seems a bit of a reach. While the binding and effect of phosphorylation are clear, a functional assay to show this interaction per se (or its regulation via S30) would really enhance this work.

We agree with the reviewer that a functional assay showing the importance of S30 regulation in EGFR recycling would strengthen the paper. However, these experiments are not straight-forward. First, we did not expect S30 to be directly involved in ITSN binding to DENND2B since S30 is not part of a PXXP motif that is required for ITSN SH3A interactions (Tong et al., 2000, EMBO J). To test this directly, we have now performed pulldown assays with GST-SH3A in cells expressing Flag-DENND2B WT or the S30A mutation and as expected, the S30A mutation did not influence ITSN interaction. This new data can be found in Figure 3F. Ideally to perform the requested functional assay we would use a S30E phospho-mimetic mutation to recruit 14-3-3 and disrupt ITSN binding. Unfortunately, these experiments are not possible because 14-3-3 proteins do not bind phospho-mimetic mutations as they require phosphorylated S/T and mutation to D/E changes the 14-3-3 recognition site (Muslin et al. 1996, Cell; Obsil & Obsilova, 2011, Semin Cell Dev Biol; Riou et al. 2013, Cell).

We have however strengthened our conclusions about the role of DENND2B in regulated control of EGFR recycling through two new sets of experiments. First, we have now determined that PKD enhances DENND2B phosphorylation when the two proteins are co-transfected in cells (revised Fig 2H/I). Second, we have used endocytosis and recycling assays to reveal that DENND2B expression enhances recycling of ligand-free EGFR without influencing its endocytosis (revised Fig 4H-K).

Finally, we have toned down the argument that our study proves the interaction between DENND2B and ITSN regulates EGFR trafficking (including changing the title). We have revised the manuscript to point out that much remains to be determined regarding how ITSN and DENND2B regulate this process. We have proposed a potential mechanism for future studies involving how the interaction could influence EGFR ubiquitination.

Minor:

Figure 2D legend mentions PKD-constitutively active was used. This is not clear from figure as there is no label for this.

Thank you for pointing this out. We did not use PKD-constitutively active in this figure panel, only PKD-dominant negative. We have corrected our mistake in the figure legend of the revised manuscript.

The paper could include a diagram of the domain structure of DENND2B and what must be its multiple SH3-binding motifs together with a diagram of ITSN-s and some of where its key interactors bind. This would help readers understand the biochemistry and how these proteins come together.

In figure 3H of the revised manuscript we have added a domain diagram of DENND2B with ITSN illustrating how phosphorylation of Ser30 in the first proline-rich domain recruits 14-3-3, thereby disrupting ITSN binding. This figure makes it easier for the reader to follow the manuscript, and we thank the reviewer for the suggestion.

Is DENND2B's effect on EGFR recycling specific for a subset of proteins or is TfR recycling affected?

Indeed, the substrate for DENND2B, Rab13, is reported to facilitate recycling of numerous cargo types such as transferrin receptor, integrin, GLUT4, vascular endothelial growth factor receptor, occluden and claudin. Therefore, we would predict that DENND2B would regulate recycling of these proteins as well. While performing recycling experiments on the various cargo proteins is

beyond the scope of this study, we have modified the discussion section of the revised manuscript to include the potential of DENND2B to regulate recycling of various cargo proteins.

The authors state that 'Using mass spectrometry we confirmed that Ser-30 is phosphorylated upon OA treatment (data not shown).' I think it is worthwhile to show these data - there is ample figure space to do so. Also, since there is residual binding with the anti-phospho PKD substrate antibody after IP of the S30A DENND2B mutant, it would be helpful to point out other putative PKD phospho-sites (possibly reviewing mass-spectrometry data for their detection).

As suggested, we now include the phospho-mass spectrometry data, found in Figure 2C of the revised manuscript. We compare % phosphorylated DENND2B under DMSO and okadaic acid treatment of the most abundant peptides detected. We have also added Supplemental Table 1 where we provide a complete list and indicate the total numbers of detected peptides. There are 4 additional putative PKD phosphorylation sites on DENND2B, S107, T157, S531 and S681. Since none of these sites were detected in the mass spectrometry screen, we decided that it does not add value to mention these sites specifically. However, we agree that it is important to mention the possibility of additional PKD phosphorylation sites. Therefore, we have revised the manuscript to indicate this possibility.

The authors say 'Consistent with previous studies, the ability of DENND2B to enhance MAPK activity is independent of its GEF activity as expression of the DENN domain alone does not activate MAPK (Fig S1D-F).' This does not seem like a fair conclusion given the reason cited. There are probably lots of ways a naked DENN domain would not activate MAPK because its missing all of its other regulatory features that would hook the DENN domain up properly.

We agree with the reviewer's criticism regarding the conclusions we had originally drawn from overexpressing the DENN domain and examining MAPK activity. Indeed, we have previously shown that the DENN domain alone fails to properly localize to actin filaments (Ioannou et al. JCB, 2015). Thus, it is possible that the GEF activity contributes to MAPK signalling but requires proper localization to do so. To avoid confusion, we have removed this data from the revised manuscript.

The authors motivate the survey for SH3-containing proteins that bind DENND2B with the observation that 'deletion of the Grb2-binding site on DENND2B does not affect the ability of DENND2B to enhance MAPK signaling. This suggests that DENND2B interacts with additional SH3 containing proteins important for EGFR signalling'. This particular reason is confusing as it is saying that when the known SH3-domain binding motif is removed from DENND2B, there is no effect on function, therefore there must be other SH3-domain proteins that bind DENDD2B? ...and similarly have no effect either? A smoother rationale here would help the flow of the text.

We apologize for not being clear in our rationale. Our original rationale was that there are two defined proline-rich domains (PRD) in DENND2B, both of which contribute to MAPK signaling downstream of EGFR. Grb2 binds specifically to PRD2, however it you delete PRD2, expression of DENND2B continues to enhance MAPK signalling (Majiji et al., JBC, 1998). Therefore, we reasoned that through its PRD1 domain DENND2B likely interacts with additional SH3 containing proteins important for EGFR signaling. However, we did not focus our study on investigating the effects of PRD1 versus PRD2 and while we were able to map the 14-3-3 (non-SH3 protein) binding to PRD1 we did not map the ITSN (SH3 protein) binding to this region. Therefore, we have modified the rationale in the manuscript and removed the statement related to deletion of the Grb2-binding site. We now focus on the fact that DENND2B contains multiple proline-rich motifs and likely interacts with SH3 domain-containing proteins in addition to Grb2.

Referee #2:

Intersectin-s interaction with DENND2B determines the recalling fate of EGFR.
This work form the McPherson lab reveals a novel mode of EGFR recycling control in which Intersectin-s binds the Rab13 exchange factor DENND2B to modulate return to the cell surface. Interestingly, EGF treatment results in increased PKD-dependent DENND2B phosphorylation that dissociates Intersectin-s and DENND2B, shifting the balance from EGFR recycling into degradation (as would be expected w EGF treatment). Overall this work is of high quality and biologically interesting, no major criticisms.

We thank the reviewer for the positive comments regarding our study.

-This may be beyond the scope of the present work, but the idea that ubiquitin modification of

EGFR overrides the recycling signal seems like an interesting topic for additional resolution that may establish a paradigm. (Steric hinderance vs opposing post-translational modifications vs ?)

This is an interesting idea since ubiquitination is also critical for determining the trafficking fate of EGFR. ITSN activates Cbl (ubiquitin ligase) by binding to Spry2 and disrupting the inhibitory Spry2/Cbl interaction. Interestingly, Spry2 binds specifically to the ITSNís SH3A domain (Okur et al., Mol Cell Biol, 2012). So itís possible that DENND2B binding to ITSN outcompetes Spry2 causing Cbl to remain inactive. This would occur in the absence of EGF stimulation when DENND2B and ITSN bind tightly. Consistent with this idea, Baumdick et al (eLife, 2015) found that spontaneously activated EGFR is not ubiquitinated and continuously recycles back to the plasma membrane, whereas EGF stimulated EGFR is ubiquitinated and trafficked to the lysosome for degradation. We have added a brief discussion of this possibility to the revised manuscript.

-It would be helpful to provide the reader with domain cartoons for DENND2B and ITSN.

We agree with this comment, which was also raised by reviewer 1. Thus, we have added a domain diagram of DENND2B with ITSN illustrating how phosphorylation of Ser-30 in the first proline-rich domain recruits 14-3-3 binding thereby disrupting ITSN binding. This schematic can be found in figure 3H of the revised manuscript. We believe that the new figure makes it easier for the reader to follow and thank the reviewer for the suggestion.

-In figure 1E the lowest arrow in the lower left panel (Flag-ITSN-s) is not pointing to the same spot as it is in the mCH-Rab13 or merged image.

We have corrected the misplaced arrows in this figure.

-Efficiency of DENND2B knock-down efficiency in Figure 4? (Are the observed differences simply due to efficiency of KD?)

Indeed we consistently see that shRNA2 shows greater efficiency than shRNA1 when we examine knockdown of overexpressed protein. This is consistent with seeing a more robust phenotype with shRNA2 in our functional assays. However, in response to the concern of reviewer 1 regarding our use of overexpressed protein to validate the efficiency of DENND2B knockdown, we quantified mRNA levels of DENND2B following knockdown using real-time PCR. This new data can be found in figure 4C of the revised manuscript. Compared to control cells, we observed that DENND2B mRNA levels are reduced to 13% and 23% using shRNA1 and shRNA2 respectively. This data does not support that the differences in phenotype strength are due to differences in knockdown efficiency. We believe the only way to confidently make this claim is if the levels of endogenous protein following knockdown mirror the phenotype strength. Unfortunately, we have spent a great amount of time and resources trying to obtain an antibody that recognizes endogenous DENND2B with no success. In addition to testing two commercially available antibodies from GenTex and Abcam, we tested 4 homemade polyclonal antibodies raised against 2 different DENND2B peptides, with limited success. Therefore, given the new mRNA data, we feel it would be misleading to make any claims relating knockdown efficiency to phenotype strength.

-A summary model would benefit the non-aficiando and would serve as a great bullet point for advertising!

As the reviewer suggested we have added a summary model of EGFR trafficking. This new model can be found in figure 5G of the revised manuscript. We agree that the new figure makes it easier for the reader to follow and thank the reviewer for the suggestion.

Referee #3:

In the present manuscript, authors described Intersectin as a novel partner of DENND2B, a GEF for the recycling Rab protein, Rab 13. Their interaction is negatively regulated by serine phosphorylation of DENND2B by protein kinase D (PKD) at a conserved serine residue (Ser30, within a PKD consensus site), which is increased upon EGF stimulation. Indeed, Intersectin and DENND2B interact in basal condition, and EGF stimulation reduces their interaction. This is due to recruitment of 14-3-3 proteins to the phosphorylated Ser30 of DENN2B, which compete for

Intersectin binding, displacing it. The molecular mechanism at the basis of DENND2B-Intersectin interaction and its regulation are well dissected. The biochemical data are carefully performed and convincing.

We thank the reviewer for the positive comments regarding our study.

However, there is no demonstration for a role of Intersectin-DENND2B interaction in EGFR recycling. More experiments are needed to prove the model proposed and to justify the title of the manuscript.

Major issues:

1) No EGFR recycling assay is provided. Measurement of surface EGFR is just an indirect evidence for a putative role of DENND2B in recycling. Indeed, the reduced EGFR levels upon DENND2B KD might be the result of different mechanisms, including reduced recycling, increased endocytosis or reduced synthesis. I understand the difficulty of following constitutive EGFR recycling. But this can be done by they use of different approaches, either based on PM biotinylation/stripping procedure (for a detailed protocol see, for instance, McGill MA, JBC, 2009) or labeling the EGFRs at the cell surface with an EGFR antibody recognizing the extracellular domain in vivo at 4C (e.g. Mab 108 from ATCC, or 13A9 from Genentech), followed by internalization at 16C for 1-2 h depending on the cell type (at this temperature recycling is blocked, while internalization proceeds). This will allow to load cells with antibody-bound EGFRs. Cells can be then shifted at 37C and recycling can be then followed by IF or FACS. If DENND2B affect EGFR recycling, pronged retention in intracellular compartments should be visualized, and/or delayed reappearance to the PM.

The reviewer raises an important concern in that changes in EGFR on the surface could be due to alterations in endocytosis, recycling or synthesis/degradation. By western blot, we observed no change in the total levels of EGFR with DENND2B knockdown. This can be found in Figure 4E and quantified in Figure 4G of the revised manuscript. Thus the differences observed in surface levels of EGFR cannot be accounted for by alterations in EGFR synthesis or degradation. Moreover, we have now performed new experiments to demonstrate that DENND2B does not affect endocytosis. These experiments are described in detail in the response to reviewer 3, comment 2.

In order to address the influence of DENND2B on recycling of EGFR we performed recycling assays as suggested by the reviewer. Using an antibody that labels the extracellular domain of EGFR, we labelled surface EGFR at 4∞C, shifted the cells to 16∞C to allow internalization without recycling, acid washed the cells to remove surface labeled EGFR, and then shifted the cells to 37∞C to allow for recycling followed by acid wash and quantification of the remaining internal pool EGFR by immunofluorescence. A schematic of the experimental design can be found in Fig 4H of the revised manuscript. Indeed, we observed that DENND2B expression decreased the internal pool of EGFR following the shift to 37∞C, indicating enhanced EGFR recycling. This new data can be found in Fig 4K and quantified in Fig 4J of the revised manuscript. These experiments provide further support that DENN2B promotes EGFR recycling.

2) Authors should also exclude that, in their experimental setting, shRNA of DENND2B/Intersectin is not altering EGFR constitutive endocytosis. They could follow internalization by labeling again the cell in vivo with the anti-EGFR antibody, followed by internalization at different time points. Acid wash stripping prior fixation can be performed to follow only internalized EGFR.

As mentioned in comment 1 of the reviewer, alterations in the surface levels of EGFR could be caused by differences in endocytosis. To address this, we performed endocytosis assays as suggested by the reviewer. Using an antibody that labels the extracellular domain of EGFR, we labelled surface EGFR, shifted to 16∞C, acid washed the cells and quantified the amount of internalized EGFR by immunofluorescence. A schematic of the experimental design can be found in Fig 4H of the revised manuscript. We found that expression of DENND2B had no effect on the amount of internalized EGFR. This new data can be found in Fig 4K and quantified in Fig 4I of the revised manuscript. This data shows that DENND2B does not affect EGFR endocytosis, further supporting that DENND2B regulates EGFR recycling.

3) While it is clear that DENND2B and Intersectin constitutively interact and their interaction is reduced upon EGF stimulation, it is not investigated whether this interaction plays a role in EGFR recycling. It is not tested a possible effect of Intersectin KD on EGFR surface level nor in the constitutive recycling pathway of the EGFR. This must be shown in the experimental setting under scrutiny. Also DENN2B/Intersectin double KD should be included, in order to understand whether they are indeed acting in the same pathway.

We certainly understand the reviewer's concerns. As the reviewer noted, surface levels of EGFR can be altered by changes in endocytosis, recycling and degradation, and ITSN has been shown to regulate all three of these processes! For example, the effects of ITSN knockdown on EGFR endocytosis have been previously examined. Martin et al. (Mol. Pharm., 2006) show that ITSN knockdown decreases internalization of EGFR. ITSN knockdown has also been demonstrated to decrease internalization of other proteins such as transferrin receptor (Thomas et al., J Biol. Chem, 2009). This is consistent with ITSN localizing to endocytic sites and interacting with several components of the endocytic machinery. Therefore, we feel it is not necessary to repeat these studies. However, we have revised the manuscript to better highlight that these studies have been performed previously. In terms of recycling, because ITSN is a large multi-domain scaffold protein that interacts with numerous proteins, knockdown experiments will not be easy to interpret. For example, ITSN can determine the trafficking fate of EGFR by regulating its ubiquitination. ITSN activates Cbl (ubiquitin ligase) by binding to Spry2 and disrupting the inhibitory Spry2-Cbl interaction (Okur et al., Mol Cell Biol, 2012). In the absence of ubiquitination, EGFR would be recycled back to the surface. Therefore we donít expect ITSN loss-of-function or gain-of-function experiments to mirror those of DENND2B. Instead, we expect ITSN knockdown to enhance EGFR recycling. However, these findings are still consistent with our model where the interaction between ITSN and DENND2B promote EGFR recycling. ITSN disrupts the inhibitory Spry2-Cbl interaction by binding Spry2 via its SH3A domain (Okur et al., Mol Cell Biol, 2012). It is possible that DENND2B binding to ITSN outcompetes Spry2 causing Cbl to remain inactive. Thus, in the absence of EGF, DENND2B binding to ITSN could enhance recycling by preventing ubiquitination. Conversely, EGF dissociates DENND2B from ITSN, leaving ITSN free to activate Cbl, and EGFR is ubiquitinated and degraded. For these reasons, we do not believe ITSN single or double knockdown experiments would confirm or disprove that ITSN and DENND2B act in the same pathway. Although we feel that testing our proposed mechanism of regulating ubiquitination is beyond the scope of the current study, we have added a brief discussion of this possibility to the revised manuscript.

4) Does DENN2B interact with Intersectin through its proline-rich region? This is inferred from the fact that the binding occurs with the SH3 domain of Intersectin, but it is not formally demonstrated. Rescue experiments with DENN2B mutant in the intersectin-binding region would be instrumental to definitively demonstrate that the interaction between DENND2B and Intersectin is critical for EGFR recycling. I realize, however, that these experiments could be highly demanding, if not unpractical. Alternatively, authors should tone down a bit their conclusions. I think that - with the additional experiments at previous points - data provided are highly relevant and with a level of novelty that would be of interest to the community of cell biologists at large.

We agree with the reviewer that a rescue experiment with a DENND2B mutant deficient in ITSN binding would be definitive in demonstrating that the interaction between DENND2B and ITSN is critical for EGFR recycling. As the reviewer points out though, these experiments are indeed highly demanding. The minimal binding site for SH3 interaction is PXXP and DENND2B contains 15 PXXP motifs both within and outside of its defined proline-rich regions. Thus, we did not map the ITSN binding specifically to a proline-rich region. Instead we have toned down our conclusions as the reviewer has suggested. In the revised manuscript we have eliminated statements claiming that our data proves that the interaction of DENND2B with ITSN regulates EGFR trafficking, replacing them with statements indicating that our data suggests such a conclusion. We have revised the manuscript to point out that much remains to be determined regarding how ITSN and DENND2B regulate EGFR trafficking. We have proposed a potential mechanism for future studies involving how the interaction could influence EGFR ubiquitination (described in the comment above). We feel these modifications improve the paper as they better describe the data that we have while opening up questions for future studies to come.

Other issues:

1. In Fig. 1A and 2B, please provide Ponceau for GST protein levels.

We agree with the reviewer's recommendation to include ponceaus as they illustrate that the differences observed in protein binding are not caused by differences in the amount of GST-protein used in the assay. Therefore we have added the ponceaus to figures 1A and 2B of the revised manuscript.

2) Please show that Itersectin-SH3A has a reduced binding for Ser30A mutant and that Ser30A mutant is not detected by P-PKD substrate.

We did not expect S30 to be directly involved in ITSN binding to DENND2B since S30 is not part of a PXXP motif that is required for ITSN SH3A interactions (Tong et al., 2000, EMBO J). To test this directly, we have now performed pulldown assays with GST-SH3A in cells expressing Flag-DENND2B WT or the S30A mutation and as expected, the S30A mutation did not influence ITSN interaction. This new data can be found in Figure 3F of the revised manuscript. Ideally, instead of using the S30A phospho-deficient mutation, we would use a S30E phospho-mimetic mutation to recruit 14-3-3 to disrupt ITSN binding. Unfortunately, these experiments are not possible because 14-3-3 proteins do not bind phospho-mimetic mutations as they require phosphate groups and not simply negatively charged side chains (Muslin et al. 1996, Cell; Obsil & Obsilova, 2011, Semin Cell Dev Biol; Riou et al. 2013, Cell). We find that DENND2B Ser30A can still be detected by the phospho-PKD substrate antibody. We suspect this is because additional PKD substrate motifs exist on DENND2B that can be detected. Therefore, we have now revised the manuscript to reflect the likelihood of additional PKD phosphorylation sites on DENND2B.

2) Is Ser30 close to the proline-rich region to explain the competition between 14-3-3 and Intersectin? Or there are other possible explanations? This issue should be better discussed. Maybe adding a scheme depicting the different domains of the proteins studied could help the reader.

Indeed S30 is within a proline-rich domain (PRD1). However, this does not necessarily mean that ITSN binding also occurs within PRD1. 14-3-3 proteins bind to their substrates as dimers and depending on how the protein is folded and where the second 14-3-3 binding site is located, 14-3-3 could outcompete ITSN by inducing a conformational change in DENND2B or by steric hindrance, even if the ITSN binding site is far from PRD1. This is a very interesting point raised by the reviewer and we have added these possibilities to the revised manuscript. Furthermore, as the reviewer suggested, we have added a domain diagram of DENND2B with ITSN illustrating how phosphorylation of S30 in the first proline-rich domain recruits 14-3-3 binding thereby disrupting ITSN binding. This schematic can be found in figure 3H of the revised manuscript. We believe that the new figure makes it easier for the reader to follow and thank the reviewer for the suggestion.

4) In the results section (pag. 8), it is incorrectly stated that Grb2 is an adaptor protein that binds the EGFR in the endosomes. Grb2 is recruited to the activated EGFR at the PM, at very early time point after EGF addiction (~2-3 min, see, for instance, Fortian and Sorkin, 2014).

We agree with the reviewer that our explanation of Grb2 recruitment to EGFR was incomplete. Indeed, Grb2 is recruited to activated EGFR at the plasma membrane and remains associated with EGFR on endosomes following internalization (see Di Guglielmo GM et al. 1994 EMBO J). The Fortian and Sorkin study mentioned by the reviewer concludes that endosomes are the main intracellular location of EGFRñGrb2 complexes and these EGFR-Grb2 positive endosomes can be observed for up to 60 minutes following EGF treatment. Therefore we have revised the manuscript to provide a clear description that Grb2 is recruited to EGFR at the plasma membrane and remains associated with EGFR on endosomes following internalization.

5) Please note that, upon EGF, not all EGFRs are targeted to degradation: EGF-occupied EGFR are still either recycled or degraded, depending if they are ubiquitinated. Please revise text to render this clearer.

We agree that ubiquitination is a critical modification in determining the trafficking fate of EGFR and should be clearly explained in our paper. Therefore we have revised the manuscript as the reviewer suggested to say that the majority of EGF-activated receptor is ubiquitinated and targeted for degradation in the lysosome, and that in the absence of ubiquitination, EGF-activated EGFR is recycled back to the surface.

2nd Editorial Decision 05 September 2017

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below. As you will see, all referees are now positive about the study and support publication in EMBO reports. Browsing through the manuscript myself, I noticed several things that we need before we can proceed with the acceptance of your study.

- shorten the manuscript. Your paper will be published as Scientific Report and the main text (w/o materials and methods) plus the figure legends should not exceed 25,000 {plus minus} 2,000 characters. Please shorten your manuscript text where possible to get at least somewhat closer to this limit. Please also review the separate Discussion section you have currently at the end of the manuscript and integrate this text into the combined "Results and Discussion" paragraph.

- shorten the title to 100 characters incl. spaces

- provide up to five keywords

- provide an Author contribution section after the Acknowledgements section, and provide the Conflict of interest statement as separate paragraph.

- move the legend for Figure EV1 to the end of the figure legends in a separate section called "Expanded View Figure legends"

- change the name of the Supplemental table to Table EV1 and also change the corresponding callouts in the text.

- regarding statistics: Fig. 4 J, K show the values from two independent experiments. If n <3 please show the individual data points as scatter plot instead of means.

- You have submitted the raw quantification data and the original Western blots as source data. I very much appreciate this and I think it will be of value to publish these data alongside the paper. The only thing that we would need in this case: could you please provide a separate pdf and excel file per figure? I.e. split the Excel file into five, one for each figure and do the same for the pdf? Thank you!

- All corresponding authors are required to supply an ORCID for their name to ensure unambiguous name assignment. Please also link the ORCID of Maria Ioannou to her profile in the online submission system. Please find the instructions on how to link your ORCID to your profile in our online Authors Guidelines.

We look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors have responded appropriately to all of the concerns and suggestions voiced. The conclusions fit the data and some needed clarifications have been made. It still would be great to see transferrin recycling since this is a well used and explored assay that would connect the the magnitude of DENND2B's effect much better to the literature for comparison. But the scope of this paper is sufficient in my view.

Referee #2:

The authors did a nice job of responding to reviewer critiques. Manuscript has been strengthened as a result.

Referee #3:

The authors have convincingly addressed all my concerns. The data are sound and technically well performed. The manuscript is suitable for publication without further revision.

2nd Revision - authors' response 11 September 2017

The authors made the requested changes and submitted a further revised version of their manuscript.

3rd Editorial Decision 17 September 2017

Thank you for the submission of your revised manuscript to EMBO reports and for the incorporation of all requested changes. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- Figure 4 J, K: Thank you for changing the graph to show the individual data points. I however note that the number of independent biological experiments is only two in this case and the calculation of a p-value is therefore not very meaningful and statistically not appropriate.

- Please upload table EV1 as .doc file.

- Please split the source data file for Figure 4 into two, one showing the data for Fig. EV1 and one showing the data for Fig. 4.

Moreover, I noticed some inconsistence between the source data and the figure panels, as follows:

- Source data Fig 5A: the data shown in Figure 5A for P-PKD does not correspond to the boxed bands in the source data file. The figure sows the leftmost lanes, while the source data file indicates that the rightmost bands from the P-PKD blot have been used. Please review this figure panel and ensure that also the correct and corresponding Hsc70 control bands are shown for this experiment in the figure.

- In panel 5C you appear to have rotated the slice for the P-PKD substrate a bit. Now the band shown in the 10% SM lane does not correspond well with the boxed area in the source data file. It might help to enlarge the red box a bit.

- Fig. EV1: the bands for MAPK shown in the figure panel and those in the source data file appear not to be the same. Could you please double-check this too?

Once you have made these minor revisions, please submit your corrected manuscript.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

3rd Revision - authors' response 19 September 2017

Thank you for all of your help working through this manuscript with us.

We have made all of the minor revisions as you have requested.

We removed the p-values from Figure 4J and 4K given that the number of independent biological experiments is two cases. In this case, we believe that it is more transparent to plot all of the individual data points and let the readers interpret the data for themselves as suggested by Vaux. DL (2012) Nature.

Our apologies for the discrepancies between the source data files and the figures themselves. They have now been corrected. In Figure 5A, we replaced the P-PKD panel in the actual figure so that it corresponds to the Hsc70 blots from the same experiment. Similarly we replaced the MAPK panel in Figure EV1 so that it corresponds to the P-MAPK and Hsc70 panels of the same experiment. The source data blots should now match the figures used.

4th Editorial Decision 20 September 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ioannou MS & McPherson PS
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44034V1

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	n= or >3, in all figure legends, following the convention in our field
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, all statistical tests used are described the figure legends corresponding to the quantified experiments. All statistical analysis were included along with the raw data values in the source data file.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The data is designed such that a normal distribution is expected. Normality tests (Shapiro-Wilk) were performed for experiments where n = or > 5 however such tests are unreliable for smaller sample sizes in which case normality was assumed.
Is there an estimate of variation within each group of data?	Each group of data is displayed as the mean +/- standard deviation. Therefore, the variance of the data can be infered from this information.
Is the variance similar between the groups that are being statistically compared?	When comparisons were made between two samples with unequal variance a Welch's t-test was used in place of a Student's t-test.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The following antibodies from commercial sources were used in the study and have been previously validated and extensively used: Mouse Flag (M2) Sigma-Aldrich. Mouse EGFR (recognizes EC domain) (ab30) Abcam Rabbit EGFR (D38B1) (#4267) CST Rat Hsc70 (1B5) Enzo LifeSciences Rabbit GFP (A6455) Invitrogen Mouse phospho-p44/42 MAPK (Thr202/Tyr204) (E10) (#9106) CST Mouse p44/42 MAPK (3A7) (#9107) CST Rabbit PKD/PKCμ (#2052) CST Rabbit phospho-(Ser/Thr) PKD substrate (#4381) CST Rabbit phospho-PKD/PKCμ (Ser916) (#2051) CST
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK-293T, HeLa and MCF10A cells are all originally from ATCC. These cell lines were routinely tested for mycoplasma contamination.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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http://www.selectagents.gov/	List of Select Agents

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

F- Data Accessibility

18. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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