EMBO reports

Prominin-1-Radixin Axis controls hepatic gluconeogenesis by regulating PKA activity

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1st Editorial Decision 7th Nov 2019

Dear Prof. Ko,

Thank you for the submission of your research manuscript to our journal, which was now seen by two referees, whose reports are copied below.

As you can see, the referees express interest in the proposed role of Prom1 in regulation of hepatic gluconeogenesis. However, they also raise a number of concerns that need to be addressed to consider publication here.

Regarding the major point raised by referee #1, it is essential to discuss possible hypotheses on how a membrane restricted complex can lead to phosphorylation of CREB in the cytoplasm if this point cannot be addressed experimentally.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as 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.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1. A data availability section providing access to data deposited in public databases is missing (where applicable).
2. Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter blots in these cases. No statistics can be calculated if n=2.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<http://embor.embopress.org/authorguide>).

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <http://embor.embopress.org/authorguide#expandedview>.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <http://embor.embopress.org/authorguide#sourcedata>.

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct

from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <http://embor.embopress.org/authorguide#datacitation>.

9) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

In the present manuscript the authors have explored the role of the membrane protein prominin-1 that resides in lipid rafts in hepatocytes and liver. In an elegant series of experiments, they first demonstrate that prominin-1 is necessary for hepatic control of gluconeogenesis by glucagon and adrenergic stimuli. They go on to show that the molecular mechanism for this dependency involves prominin-1 binding of the ERM protein radixin and its function as an A-kinase anchoring protein to localize PKA.

Major comment:

The authors present strong evidence for the requirement of a prominin-1/radixin-anchored pool of PKA to mediate the regulatory effects of glucagon and adrenalin to provide the necessary spatiotemporal control of glucagon signaling. They also discuss a recent series of papers from the Scott laboratory demonstrating that the PKA holoenzyme does not even dissociate when bound to an AKAP yielding a short dynamic range. On this background, it appears somewhat illogical that the substrate the authors present for the prominin-1/radixin-anchored pool of PKA and that would regulate gluconeogenesis is CREB that is phosphorylated in the cytoplasm and subsequently needs to shuttle to the nucleus to mediate its effects as the authors hypothesize. Alternatively, it may be that there is a chain of events leading to CREB phosphorylation and an intermediate substrate between the anchored PKA and CREB. PKA is not the only kinase that may phosphorylate CREB and it could be a kinase cascade effect. It would strengthen the work if the authors were to identify a spatially restricted substrate for the anchored complex and/or a mechanism for signaling to the nucleus. In the absence of such findings, the authors should at least discuss different hypotheses for how the signal may be propagated from the membrane proximal spatially restricted complex to regulate gluconeogenesis more widely than the present discussion.

Minor comments:

1) Page 5, line 10: what does Fig EV1B mean?
2) Page 9, line 6 from bottom: not clear that Fig S4D is the right figure reference here?
3) Fig 1B vs 1E, why is the regulation seen in hepatocytes in the resting situation, but only upon fasting in liver?
4) Fig 1F, pCREB panel, fasting WT vs KO - what is the % down regulation here? In 3 experiments? And why does the effect appear much more pronounced in Fig 2B?
5) Fig 2D: are the differences untreated and across treatments in KO animals significant/not significant? I.e. when comparing in KO untreated vs glucagon -/+ IBMX.
6) Fig S1A: what is the difference between the left and right columns of IF pictures? WT vs KO?
7) Fig S2A and S2C: as in Fig 2D - would be good also to know whether KO differences as significant or not across treatments.

Referee #2:

Dear Colleagues,

The manuscript entitled "Prominin-1-Radixin Axis controls hepatic gluconeogenesis by regulating PKA activity" (ID: EMBOR-2019-49416-T) by Lee and colleagues describes the potential implication of a Prom1-radixin axis in the glucagon/-adrenergic receptor-mediated gluconeogenesis. Based on their data, the authors conclude that Prom1 regulates hepatic PKA signaling via radixin functioning as an A kinase-anchored protein. This manuscript contains a large volume of data that make a potentially interesting study. Numerous new findings (e.g., Prom1-radixin interaction, implication of lipid rafts in these interactions, Prom1-radixin axis as a key signaling pathway that regulates cAMP-mediated PKA activation etc.) are described and inter-connected. Understanding the role of Prom1 is also highly important given that this molecule as a cell surface marker enables the identification of stem and cancer stem cells in various organs. Furthermore, various retinal diseases are associated with mutations in the PROM1 gene. Beyond cells with stem cell properties, Prom1 is expressed in fully differentiated cells in numerous tissues and organs. Consequently, the identification of new Prom1 interacting partner and new pathway associated with it deserve a great attention. Unfortunately, numerous claims throughout the manuscript in its current state lack solid demonstration, which raises various questions that need to be experimentally addressed. The literature cited in the present work should be improved, notably regarding the current knowledge about the expression of Prom1/CD133 in murine and human liver.

Major points

1. The authors stressed in the Introduction that Prom1 is localized in plasma membrane protrusions, and this in various cell types. Where is this protein found in mouse liver and primary hepatocytes? The subcellular localization of Prom1 should be investigated by electron microscopy using primary hepatocytes.

2. Do only hepatocytes express it in the liver? Indeed, did any publication report the expression of Prom1 in human or mouse hepatocytes? These publications should be cited. Yoshikawa S et al. have reported that mature hepatocytes were negative for CD133 (World J Gastroenterol 2009). What exactly are we looking at in Fig. S1? Can we exclude that Prom1 staining (in red) corresponds to vascular sinusoids or other liver cell types, e.g., oval cells or bile duct cells (see Yovchev MI et al., Hepatology 2006; Rountree CB et al., Stem Cells 2007; Shmelkov SV et al., J Clin Invest 2008)? What is the difference between the left and right panels (WT versus Prom1 KO mice) of Fig. S1? Proper cellular- and subcellular-localization are important to understand the role of Prom1 in the liver, and its potential impact on radixin localization. Prom1 has been reported to be associated with the endosomal compartment among other intracellular structures in addition to cellular protrusions.

3. In addition to the data provided by proximity ligation assay (Prom1-radixin), a co-localization of Prom1 and radixin or actin should be documented by double-immunofluorescence microscopy (e.g., as in Fig. 4K). Does the radixin level change upon Prom1 knockdown?

4. Did the authors also observe a redistribution of actin upon silencing of Prom1 or radixin by means of sh technology? This would strengthen the data obtained with Prom1-/- cells. Does radixin influence the localization of Prom1? Was any morphological alteration detected in Prom1-deficient cells? Does Prom1 interact directly with actin or is radixin required? These questions can be addressed using radixin-deficient cells and Prom1 immunofluorescence and immunoprecipitation. In Fig. 4J, actin immunoblot should be presented. The interaction of Prom1 and actin was proposed (Yang Z et al., J Clin Invest 2008).

5. Since most investigators are using non-ionic Triton X-100 for the isolation of lipid rafts, the rational for using Brij 35 as detergent should be explained. Brij 35 was described to poorly solubilize both endoplasmic reticulum and plasma membrane forms of Prom1 and only the latter were associated with lipid rafts. This suggests that Prom1-associated Brij 35 insoluble complexes are not necessarily related to lipid raft (Roper K et al., Nat Cell Biol. 2000). Experiments showing that cholesterol depletion affects the association of Prom1 and radixin with lipid rafts and their subcellular localization would demonstrate the role of lipid rafts in the phenomena described here and hence strengthen the manuscript. A membrane protein marker of non-lipid raft domains should be presented in Fig. 4L. The data regarding flotillin as a lipid raft marker should be mentioned in the main text.

Other points
1. The rationale for studying Prom1 in gluconeogenesis in the liver is ill exposed in the Introduction. The authors need to justify with adequate referencing their choice that appears abrupt at the moment.

2. The authors mentioned "... indicating that Prom1 might be necessary for the formation of membrane extrusions (Zacchigna et al., 2009)" in the Introduction, and "Although Prom1 is a cancer stem cell marker located in plasma membrane detergent-resistant lipid rafts, the cellular functions of Prom1 have remained elusive ..." in the Discussion. They should cite two recent publications showing the role of this protein in the formation of microvilli and primary cilium and membrane dynamics (Thamm K et al., Traffic, 2019; Singer D et al., EMBO J 2019).

3. In the Introduction, please replace "membrane extrusions" by membrane protrusions.

4. Key publications showing the expression of Prom1 in cancer stem cells or cancer initiating cells should be cited, e.g., Singh SK et al., Cancer Res. 2003 and O'Brien CA et al., Nature 2007, in the Introduction (i.e. after "... brain, colon, ovarian and liver tumors").

5. Similarly, citations should be added after "... Although Prom1 is expressed at high levels in various epithelial cells in the brain, kidney digestive track and liver, ...". Florek M et al., Cell Tissue Res. 2005, Karbanová J et al., J Histochem Cytochem, 2007, Immervoll H et al., BCM Cancer 2008 or proper reviews dealing with this subject should be provided.

6. The association of Prom1 with detergent-resistant membrane complexes as well as its binding to membrane cholesterol should be presented in the Introduction. This can be the link between the second and third paragraphs.

7. Uncropped immunoblots should be provided. What is the molecular weight of Prom1? Why, in some blots, are multiple Prom1 bands detected (Fig. 4L) while in others there is only one (Figs 4C and S2)? Do they correspond to different Prom1 splice variants or different glycosylation profile? Which splice variant of Prom1 is used in this study (see Fargeas CA et al., Tissue Antigens 2007)?

8. After backcrossing Prom1-/- mice with C57BL/6N mice for 5 generations as indicated in Materials and Methods section, did the investigators genotype the progenies? Moreover, the authors should describe the characteristics of Prom1 knockout mice (Prom1tm(cre/ERT2)Gilb) (Zhu L et al., Nature 2009) that they used even it was obtained commercially. Have they observed any expression in the liver/hepatocytes of the reporter β-galactosidase gene which is under control of the Prom1 promotor/enhancer? This information might help them to determine which liver cell types express Prom1 (see major point above).

9. Several Prom1-/- mouse lines have been generated in the last decade, and numerous individuals are carrying mutations in PROM1 gene. Have any phenotype in liver and consequences been reported? Considering the cellular redistribution of actin in Prom1-/- cells described here (Fig. 4K), one would expect a physiological impact. Please comment.

10. As the authors concluded: " ...Prom1 and radixin may be excellent target proteins for lowering the blood glucose level in patients with diabetes", they should comment on the publication showing that Prom1-/- mice have a significant high glucose levels and were obese (Karim BO et al., Front Oncol. 2014).

11. In Table S1, clones or antibody specificities should be indicated, especially that of the anti-Prom1 antibody used for IFA. Company names are insufficient.

12. Experimental procedures are often insufficiently described and Figure Legends appear less than minimal (e.g. immunostaining), it is therefore difficult to assess the validity of the data presented. Abbreviations should also be explained (e.g., whole cell lysate; WCL).

Dear Dr. Tiebe,

Title: " Prominin-1-Radixin Axis controls hepatic gluconeogenesis by regulating PKA activity"

EMBOR-2019-49416-T

Authors: Lee et al.

The above manuscript has been revised in accordance with the reviewer's comments. We have addressed all of reviewers' comments and suggestions, and we believe that the changes have improved the manuscript. Specific modifications include:

Referee #1:

In the present manuscript the authors have explored the role of the membrane protein prominin-1 that resides in lipid rafts in hepatocytes and liver. In an elegant series of experiments, they first demonstrate that prominin-1 is necessary for hepatic control of gluconeogenesis by glucagon and adrenergic stimuli. They go on to show that the molecular mechanism for this dependency involves prominin-1 binding of the ERM protein radixin and its function as an A-kinase anchoring protein to localize PKA.

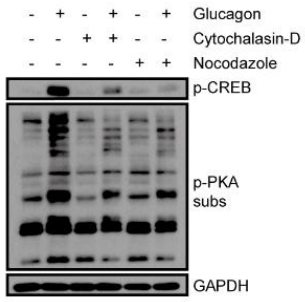
Major comment:

The authors present strong evidence for the requirement of a prominin-1/radixin-anchored pool of PKA to mediate the regulatory effects of glucagon and adrenalin to provide the necessary spatiotemporal control of glucagon signaling. They also discuss a recent series of papers from the Scott laboratory demonstrating that the PKA holoenzyme does not even dissociate when bound to an AKAP yielding a short dynamic range. On this background, it appears somewhat illogical that the substrate the authors present for the prominin-1/radixin-anchored pool of PKA and that would regulate gluconeogenesis is CREB that is phosphorylated in the cytoplasm and subsequently needs to shuttle to the nucleus to mediate its effects as the authors hypothesize. Alternatively, it may be that there is a chain of events leading to CREB phosphorylation and an intermediate substrate between the anchored PKA and CREB. PKA is not the only kinase that may phosphorylate CREB and it could be a kinase cascade effect. It would strengthen the work if the authors were to identify a spatially restricted substrate for the anchored complex and/or a mechanism for signaling to the nucleus. In the absence of such findings, the authors should at least discuss different hypotheses for how the signal may be propagated from the membrane proximal spatially restricted complex to regulate gluconeogenesis more widely than the present discussion.

Regarding the referee #1's the latter hypothesis, serine 133 site of CREB can be phosphorylated by several kinases in different signaling events: CaMKIV in Ca2+ signaling [1], RSK1-3 and MSK1/2 [2-4] in growth factor or stress-induced signaling, and PKA in cAMP-dependent manner. It will be interesting to determine either the unknown kinase that phosphorylates CREB upon cAMP-elicited PKA activation or the previously mentioned kinases responsible for crosstalk between cAMP signaling and other signaling events. However, that would be a daunting study to be dealt within the scope of our study. Therefore, we focused on the former hypothesis, shuttling of either CREB or the free catalytic subunit of PKA.

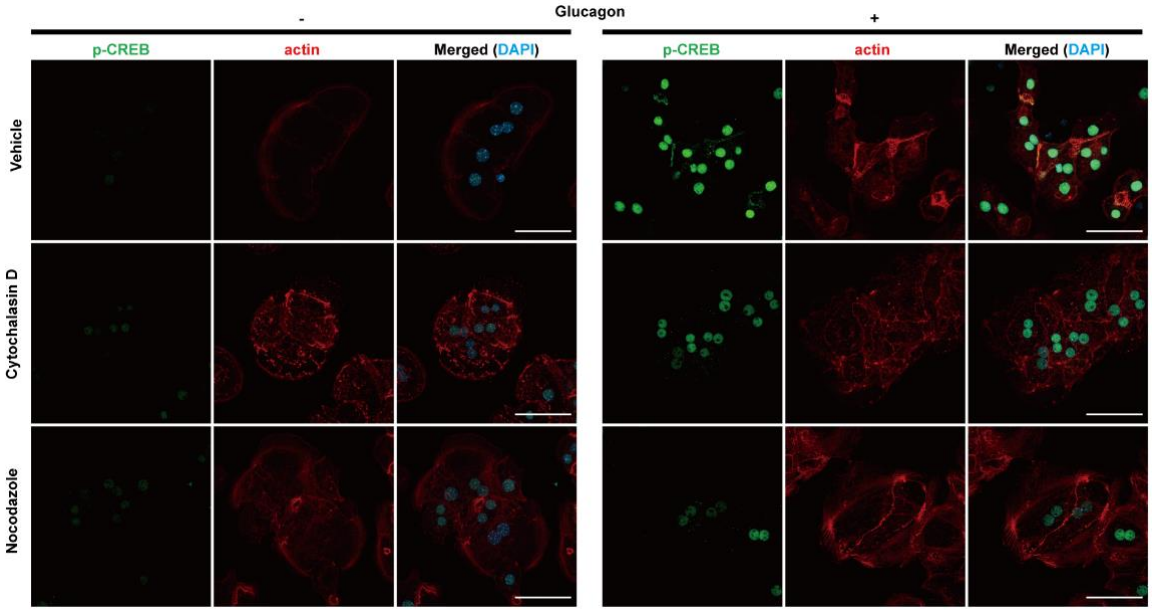
In classical model regarding cAMP-dependent PKA activation, an increased cAMP concentration releases the catalytic subunits from the holoenzyme [5-7] and the free catalytic subunit of PKA diffuses throughout the cell and into the nucleus with the associated CREB phosphorylation [8-10]. Then, the subcellular localization and substrate specificity of PKA are rendered by its interaction with AKAP [11] (A kinase anchoring proteins). On the contrary, Smith et al. have reported that PKA holoenzyme-AKAP79 complex remain bound and proximal to anchoring sites in the presence of physiological concentration of cAMP [12]. One question remains to be answered; where and how CREB is phosphorylated by space-restricted PKA catalytic subunits.

Biochemical analysis shows that the catalytic subunit of PKA can be released from the intact AKAP79:PKA holoenzyme in the presence of supraphysiological concentration of cAMP [12]. Such supraphysiological concentration may be achieved within discrete microdomains with high concentration of cAMP [13-15]. Then, the catalytic subunit of PKA can be released and diffuses through the cell and into the nucleus, resulting in the phosphorylation of CREB.



Rebuttal Figure 1 Cytoskeleton disassembly decreased glucagon-induced phosphorylation of PKA substrates.

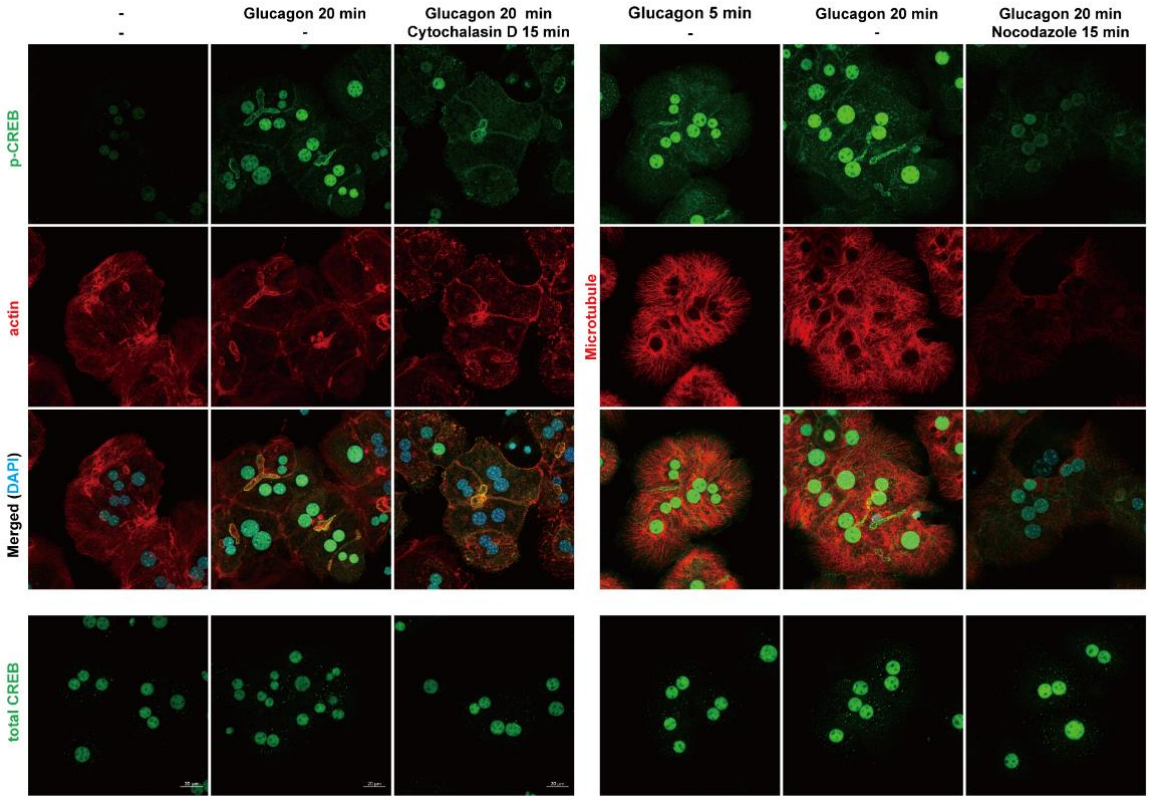
We examined the localization of phosphorylated CREB upon glucagon treatment after cytoskeleton disruptor treatments. We reasoned that targeting actin cytoskeleton by cytochalasin D or microtubule network by nocodazole would interfere with glucagon-elicited PKA signaling via disrupting local distcrete microdomains of prominin-1-radixin-PKA holoenzymes-actin or the trafficking of PKA catalytic subunit or substrates, respectively. Cytoskeleton disassembly resulted in the reduced glucagon-induced phosphorylation of CREB and PKA substrates (Rebuttal Figure 1) and the dramatically decreased nuclear localization of phosphorylated CREB (Rebuttal Figure 2) without affecting cAMP concentration [16, 17] and total CREB level (Rebuttal Figure 3). Next, we tested the effect of cytoskeleton disassembly after glucagon treatment. If CREB were phosphorylated in the cytoplasm and subsequently shuttled to the nucleus, blocking its trafficking by cytoskeleton disruptor would result in accumulation of the phosphorylated CREB near PKA at the plasma membrane. On the other hand, if the free catalytic subunit of PKA shuttled to the nucleus to phosphorylate CREB, there would be overall reduction of the phosphorylated CREB after cytochalasin



Rebuttal Figure 2 Cytoskeleton disassembly decreased glucagon-induced phosphorylation of CREB and nuclear localization

D or nocodazole treatment. Cytoskeleton disassembly indeed decreased overall phosphorylated CREB (Rebuttal Figure 3). Although not conclusive, our results suggested that PKA catalytic subunit is dissociated from the holoenzyme complex at the distinct cAMP microdomains and shuttles into the nucleus for CREB phosphorylation.

Because we believe that, although very important and interesting, this observations requires further follow-ups, we have presented the results in this response letter and discussed our model in the Discussion section.



Rebuttal Figure 3 Cytoskeleton disassembly after glucagon treatment decreased the phosphorylation of CREB and nuclear localization

Minor comments:

1) Page 5, line 10: what does Fig EV1B mean?

It should have been Fig S1B. Editorial mistake has been corrected.

2) Page 9, line 6 from bottom: not clear that Fig S4D is the right figure reference here?

Bicistronic expression of GFP after shRNA confirmed that adenoviral particles transduced hepatocytes mostly, which could be determined by the morphological characteristics of GFP-positive cells such as two nuclei in a cell (previously Fig S4D, now Figure EV4A). It was not clearly explained in the first manuscript. Now, the manuscript has been modified accordingly:

- Changes made

1) Page 9. Line 6 from the bottom Fig S4D -> Page 10, line 6 from the bottom, Figure EV4A.

2) “bicistronic expression of GFP” has been inserted to explain clearly.

3) Fig 1B vs 1E, why is the regulation seen in hepatocytes in the resting situation, but only upon fasting in liver?

Fig 1B (now Fig 1L) showed the relative expression of *G6pc* and *Pck1* in hepatocytes in “Serum-free” condition on which NGS results (now Fig 1K) was based on. We aimed to confirm the results from NGS using quantitative PCR.

The key hepatic gluconeogenic genes *G6pc* and *Pck1* are down-regulated during feeding, and stimulated after fasting (Fig 2B, [18, 19]). In Fig 1E (now Fig 2A), we showed that the stimulation of gluconeogenic genes during fasting was dysregulated in the livers of Prom1 KO mice.

- Changes made

1) Fig 1B and E -> Fig 1L and Fig 2A

4) Fig 1F, pCREB panel, fasting WT vs KO - what is the % down regulation here? In 3 experiments? And why does the effect appear much more pronounced in Fig 2B?

Fasting-induced CREB phosphorylation was decreased by 24 % in *Prom1*$^{-/-}$ mouse livers (Fig 2B and Fig EV1D), as measured quantitatively. Three mice were used for each experimental group.

Fasting induced about 2-fold increase in blood glucagon level (Fig 2D and [20]) which is equivalent to 8 X 10^{-11} mol/L, while in Fig 2B (now Fig 2G), hepatocytes were treated with 1 X 10^{-8} mol/L of glucagon which is a supraphysiological concentration. That was the reason for the more prominent effect seen in Fig 2B (now Fig 2G).

5) Fig 2D: are the differences untreated and across treatments in KO animals significant/not significant? I.e. when comparing in KO untreated vs glucagon -/+ IBMX.

The difference in PKA activities between untreated and glucagon-treated KO hepatocytes was not significant, while the difference between untreated and treated was statistically significant when IBMX was added. Calculated significance levels were indicated on the figure (Fig 2I).

- Changes made

1) Fig 2D -> Fig 2I

6) Fig S1A: what is the difference between the left and right columns of IF pictures? WT vs KO?

Previous Fig S1A showed hepatic expression of Prom1 in mouse liver tissue and primary hepatocytes. It should have been labeled WT and KO for the left and right columns, respectively. However, in responding to the referee #2’s comments, we have investigated the hepatic expression of Prom1 in primary hepatocytes using correlative light and electron microscopy. In the current revised manuscript, Fig 1A-I and Fig 1J substitute Fig S1A.

7) Fig S2A and S2C: as in Fig 2D - would be good also to know whether KO differences as significant or not across treatments.

Calculated significance levels between KO samples were now indicated on the figure (Figure EV2A and EV2C).

- Changes made

    1) Fig S2A and C -> Figure EV2A and C

Referee #2:

Dear Colleagues,

The manuscript entitled "Prominin-1-Radixin Axis controls hepatic gluconeogenesis by regulating PKA activity" (ID: EMBOR-2019-49416-T) by Lee and colleagues describes the potential implication of a Prom1-radixin axis in the glucagon/β-adrenergic receptor-mediated gluconeogenesis. Based on their data, the authors conclude that Prom1 regulates hepatic PKA signaling via radixin functioning as an A kinase-anchored protein. This manuscript contains a large volume of data that make a potentially interesting study. Numerous new findings (e.g., Prom1-radixin interaction, implication of lipid rafts in these interactions, Prom1-radixin axis as a key signaling pathway that regulates cAMP-mediated PKA activation etc.) are described and inter-connected. Understanding the role of Prom1 is also highly important given that this molecule as a cell surface marker enables the identification of stem and cancer stem cells in various organs. Furthermore, various retinal diseases are associated with mutations in the PROM1 gene. Beyond cells with stem cell properties, Prom1 is expressed in fully differentiated cells in numerous tissues and organs. Consequently, the identification of new Prom1 interacting partner and new pathway associated with it deserve a great attention. Unfortunately, numerous claims throughout the manuscript in its current state lack solid demonstration, which raises various questions that need to be experimentally addressed. The literature cited in the present work should be improved, notably regarding the current knowledge about the expression of Prom1/CD133 in murine and human liver.

Major points

1. The authors stressed in the Introduction that Prom1 is localized in plasma membrane protrusions, and this in various cell types. Where is this protein found in mouse liver and primary hepatocytes? The subcellular localization of Prom1 should be investigated by electron microscopy using primary hepatocytes.

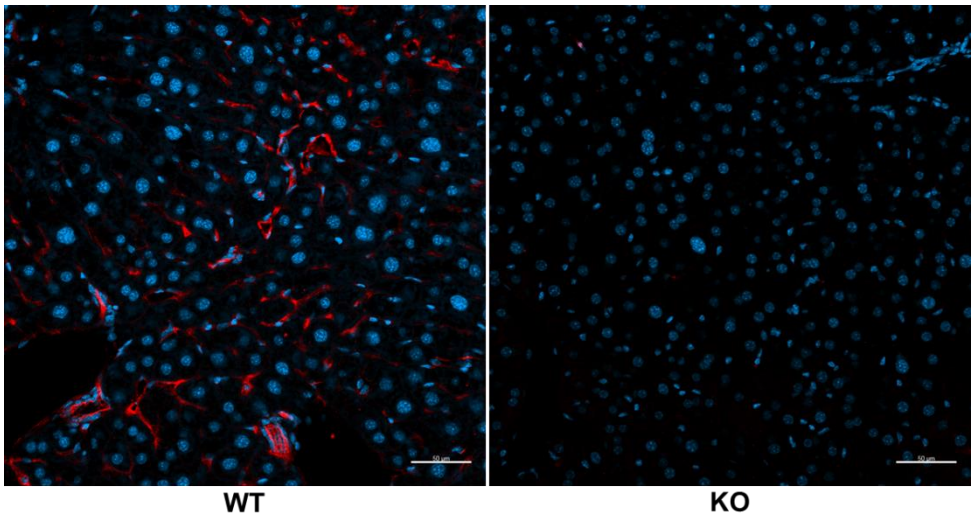
In response to this comment, we have investigated hepatic expression of Prom1 using correlative light and electron microscopy and immunofluorescence staining. For these experiments we have used the 13A4 monoclonal antibody which recognizes mouse Prom1 and has been well characterized and widely used (https://www.labome.com/review/gene/mouse/Prom1-antibody.html). In primary hepatocytes, Prom1 was found in membrane protrusion (Fig 1B and C) and the planar regions of plasma membrane (Fig 1E and F) as reported [21]. We described this observation in the first paragraph of the results section.

2. Do only hepatocytes express it in the liver? Indeed, did any publication report the expression of

Prom1 in human or mouse hepatocytes? These publications should be cited. Yoshikawa S et al. have reported that mature hepatocytes were negative for CD133 (World J Gastroenterol 2009). What exactly are we looking at in Fig. S1? Can we exclude that Prom1 staining (in red) corresponds to vascular sinusoids or other liver cell types, e.g., oval cells or bile duct cells (see Yovchev MI et al., Hepatology 2006; Rountree CB et al., Stem Cells 2007; Shmelkov SV et al., J Clin Invest 2008)? What is the difference between the left and right panels (WT versus Prom1 KO mice) of Fig. S1? Proper cellular- and subcellular-localization are important to understand the role of Prom1 in the liver, and its potential impact on radixin localization. Prom1 has been reported to be associated with the endosomal compartment among other intracellular structures in addition to cellular protrusions.

- We have observed Prom1 expression not only in hepatocytes but also in ductal cells in the liver. In order to examine Prom1 expression pattern in mouse liver, we immunostained frozen liver sections using antibody (13A4) against Prom1 and antibody markers for hepatocytes (K9218 for HNF4α) and cholangiocytes (EP1580Y for CK19). Because of the incompatibility of Prom1 antibody (13A4) with the antigen-retrieval condition for antibody markers for hepatocytes (K9218 for HNF4α) and cholangiocytes (EP1580Y for CK19), Prom1 and cell lineage markers (HNF4α and CK19) were immunostained in two serial tissue-sections (Fig 1J). Prom1 was co-localized with HNF4α in many hepatocytes (►) and with CK19 in epithelium of bile ducts (*). Because Prom1 (CD133) has been used as a marker for hepatic progenitor cells (HPCs) [22], it is also possible that Prom1 may be co-localized with Sca-1 in HPCs.

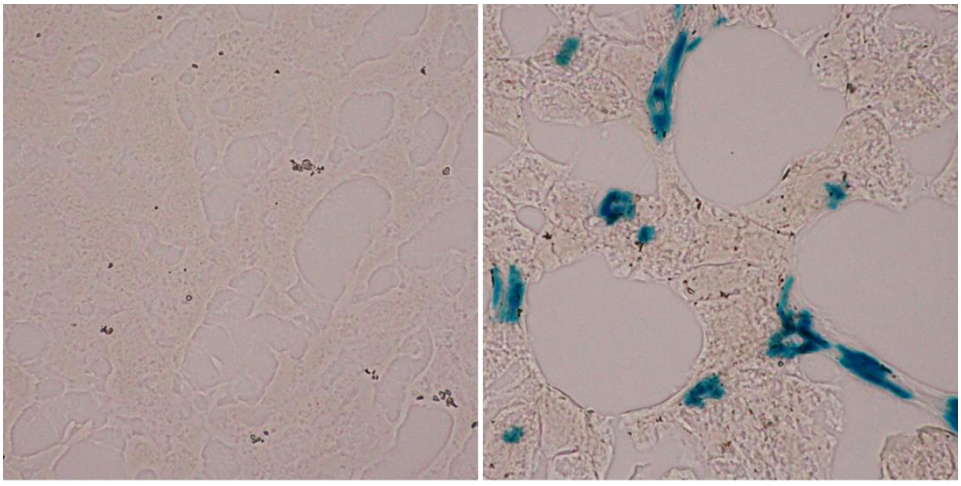
- Yoshikawa et al [23] have reported Prom1 expression in normal liver, while others found Prom1 expression only in neoplastic human liver. Yoshikawa et al also have discussed that the discrepancy between them and previous studies may have been caused by the method of immunostaining, different antibodies and paraffin-embedded vs. frozen specimens. We, too, were able to immunostain Prom1 only in the frozen sections without the antigen-retrieval procedure. Consequently, Prom1 and other cell lineage markers such as HNF4α and CK19 could be stained on two serial sections. Specificity of 13A4 monoclonal antibody for mouse Prom1 has been tested in the livers of WT and KO mice (Rebuttal Figure 4).



Rebuttal Figure 4 Immunofluorescence staining for Prom1 in the livers of WT and KO (*Prom1*$^{-/-}$) mice

- Shmelkov et al [24] and Nguyen et al [25] both have reported hepatic Prom1 expression near bile ducts and bile ductules using two different mouse models, *Prom1*$^{lacZ/+}$ and *Prom1*$^{cre\text{-}ert2\text{-}nlacz}$, respectively. Interestingly, both studies have reported low expression of Prom1 in hepatocytes by detecting β-galactosidase activity. We also observed low hepatocytic

expression of Prom1, when the most common substrate X-gal was used (Rebuttal Figure 5). Recently, Trifinov and colleagues have improved the sensitivity of β-galactosidase detection dramatically by the combination of X-Gal and nitroblue tetrazolium (NBT) as a substrate [26]. Using this method, we were able to observe the expression of Prom1 in hepatocytes as well as the bile ducts (Figure EV1C). These two articles have been cited in the introduction and the procedure for the improved β-galactosidase detection is described in the Materials and Methods section.



Rebuttal Figure 5 β-galactosidase detection using X-Gal as a substrate

- Previous Fig S1A showed hepatic expression of Prom1 in mouse liver tissue and primary hepatocytes. It should have been labeled WT and KO for the left and right columns, respectively. However, in responding to major point #2, we have investigated the hepatic expression of Prom1 in primary hepatocytes using correlative light and electron microscopy and frozen liver sections using immunofluorescence staining. In current revised manuscript, Fig 1A-I and Fig 1J substitute Fig S1A.

- It is reported that Prom1 on the plasma membrane is either released to membrane vesicles as ectosomes [27] or internalized to be recycled and released as exosomes or to be degraded after the fusion with lysosomes [28]. Therefore, the function of Prom1 may vary depending on its intracellular localization. In this manuscript, we focus on Prom1 function on the plasma membrane.

3. In addition to the data provided by proximity ligation assay (Prom1-radixin), a co-localization of Prom1 and radixin or actin should be documented by double-immunofluorescence microscopy (e.g., as in Fig. 4K). Does the radixin level change upon Prom1 knockdown?

- Because the antigen-retrieval condition for radixin antibody is incompatible with Prom1 antibody (clone: 13A4), we immunostained Prom1 and radixin in two frozen sections separately using actin staining as a reference in order to determine the co-localization of Prom1, radixin and actin. We found that both Prom1 and radixin were co-localized with actin in cell-cell contact sites called the canalicular membranes (Figure EV4D). This observation suggested that Prom1 and radixin may be co-localized at the cortices near the canalicular membranes. This result has been included as Figure EV4D.

- Prom1 knockdown did not affect radixin expression level (Fig 4C and Figure EV3D).

4. Did the authors also observe a redistribution of actin upon silencing of Prom1 or radixin by means of sh technology? This would strengthen the data obtained with Prom1-/- cells. Does radixin influence the localization of Prom1? Was any morphological alteration detected in Prom1-deficient cells? Does Prom1 interact directly with actin or is radixin required? These questions can be addressed using radixin-deficient cells and Prom1 immunofluorescence and immunoprecipitation. In Fig. 4J, actin immunoblot should be presented. The interaction of Prom1 and actin was proposed (Yang Z et al., J Clin Invest 2008).

- After silencing Prom1 or radixin, we immunostained radixin and labeled actin with phalloidin. Indeed, Prom1 or radixin knockdown redistributed actin and canalicular structures were disappeared from the plasma membrane (Figure EV4E).

- Our model suggests that the protein complex may be arranged in the order of Prom1, radixin, and actin from the membrane to actin cortices. Silencing radixin did not affect Prom1 localization, supporting our model (Fig 4D).

- We have observed routinely that primary hepatocytes from *Prom1*$^{-/-}$ mice were slightly larger than cells from WT mice. This observation may have resulted from the redistribution of cortical actin due to Prom1 deficiency. We tested the difference in cell size using FACS, and demonstrated that *Prom1*$^{-/-}$ hepatocytes were indeed larger than WT hepatocytes. This observation is now added to the revised manuscript in the Results section (Figure EV1).

- As Yang et al [29] have reported, Prom1 and actin were co-immunoprecipitated, but in radixin-dependent manner. This result is now added in Fig 5E and described in the revised manuscript.

5. Since most investigators are using non-ionic Triton X-100 for the isolation of lipid rafts, the rational for using Brij 35 as detergent should be explained. Brij 35 was described to poorly solubilize both endoplasmic reticulum and plasma membrane forms of Prom1 and only the latter were associated with lipid rafts. This suggests that Prom1-associated Brij 35 insoluble complexes are not necessarily related to lipid raft (Roper K et al., Nat Cell Biol. 2000). Experiments showing that cholesterol depletion affects the association of Prom1 and radixin with lipid rafts and their subcellular localization would demonstrate the role of lipid rafts in the phenomena described here and hence strengthen the manuscript. A membrane protein marker of non-lipid raft domains should be presented in Fig. 4L. The data regarding flotillin as a lipid raft marker should be mentioned in the main text.

- We had chosen Brij 35 as a detergent to isolate Prom1-containing lipid rafts most efficiently based on the study by Roper et al [30]. In the accompanying study, they show that 91 % of Brij 35-insoluble pellet was plasma membrane form of Prom1, one of the highest yield along with Tween 20. However, as referee #2 has pointed out in minor point #7, these raft fractions contained ER forms of Prom1 (previous Fig 4L). Although the presence of ER forms in the fractions did not affect our interpretation, we carried out the same experiment with Lubrol WX in order not to confuse readers, and improved the quality of results with plasma membrane form of Prom1 as a major band (now Fig 5G).

- As referee #2 suggested, cholesterol depletion by methyl-β-cyclodextrin resulted in the loss of Prom1 and radixin from the lipid rafts. We have now included this result in the manuscript (Fig 5H).

- Immunoblots of the sucrose-gradient fractions for transferrin receptor as a non-raft marker is

added (Fig 5G and H) and the description regarding flotillin being a raft marker is included in the manuscript (Page 12).

Other points

1. The rationale for studying Prom1 in gluconeogenesis in the liver is ill exposed in the Introduction. The authors need to justify with adequate referencing their choice that appears abrupt at the moment.

The last paragraph in the Introduction has been added in order to address this comment. In fact, we stumbled upon Prom1 function in gluconeogenesis by analyzing NGS data sets.

2. The authors mentioned "... indicating that Prom1 might be necessary for the formation of membrane extrusions (Zacchigna et al., 2009)" in the Introduction, and "Although Prom1 is a cancer stem cell marker located in plasma membrane detergent-resistant lipid rafts, the cellular functions of Prom1 have remained elusive ..." in the Discussion. They should cite two recent publications showing the role of this protein in the formation of microvilli and primary cilium and membrane dynamics (Thamm K et al., Traffic, 2019; Singer D et al., EMBO J 2019).

Two recent advances in understanding Prom1 functions has been cited in the Introduction and Discussion as referee #2 suggested.

3. In the Introduction, please replace "membrane extrusions" by membrane protrusions.

“Extrusions” has been corrected to “protrusions”.

4. Key publications showing the expression of Prom1 in cancer stem cells or cancer initiating cells should be cited, e.g., Singh SK et al., Cancer Res. 2003 and O'Brien CA et al., Nature 2007, in the Introduction (i.e. after "... brain, colon, ovarian and liver tumors").

Those publications were cited accordingly.

5. Similarly, citations should be added after "... Although Prom1 is expressed at high levels in various epithelial cells in the brain, kidney digestive track and liver, ...". Florek M et al., Cell Tissue Res. 2005, Karbanová J et al., J Histochem Cytochem, 2007, Immervoll H et al., BCM Cancer 2008 or proper reviews dealing with this subject should be provided.

A review article that discusses the molecular and cellular features of prominin-1 has been cited.

6. The association of Prom1 with detergent-resistant membrane complexes as well as its binding to membrane cholesterol should be presented in the Introduction. This can be the link between the second and third paragraphs.

The association of Prom1 with detergent-resistant membrane rafts and its binding to cholesterol has been included now at the beginning of the third paragraph in order to segue between the second and third paragraph of the Introduction.

7. Uncropped immunoblots should be provided. What is the molecular weight of Prom1? Why, in some blots, are multiple Prom1 bands detected (Fig. 4L) while in others there is only one (Figs 4C and S2)? Do they correspond to different Prom1 splice variants or different glycosylation profile? Which splice variant of Prom1 is used in this study (see Fargeas CA et al., Tissue Antigens 2007)?

- In compliance with EMBO reports' policies, we included uncropped images for immunoblottings and microscopy. Please see SourceData files.
- As addressed in the author's response for major point #5, blotting band for Prom1 at lower molecular weight in previous Fig 4L corresponded to ER form of Prom1 [30] which only appeared when cells were lysed with Brij 35. That band disappeared when Lubrol WX was used instead (Fig 5G and H).
- In this study, s1 variant of human Prom1 ([31], NM_001145847.1) was used for overexpression experiments (Fig 5D and E and Fig 6A-C). Splicing variant of human Prom1 was described in Appendix Table S4.

8. After backcrossing Prom1-/- mice with C57BL/6N mice for 5 generations as indicated in Materials and Methods section, did the investigators genotype the progenies? Moreover, the authors should describe the characteristics of Prom1 knockout mice (Prom1tm(cre/ERT2)Gilb) (Zhu L et al., Nature 2009) that they used even it was obtained commercially. Have they observed any expression in the liver/hepatocytes of the reporter β-galactosidase gene which is under control of the Prom1 promotor/enhancer? This information might help them to determine which liver cell types express Prom1 (see major point above).

- For littermate-controlled experimental setups to reduce background variations, we performed genotyping after backcrossing (Appendix Figure S1).
- As reported by Zhu et al [32], we did not find any apparent phenotype of Prom1 KO mice. Primary hepatocytes from Prom1 KO mice were slightly larger than WT hepatocytes possibly due to the redistribution of actin cytoskeleton (Figure EV1A).
- As addressed in the author's response for major point #2, we performed β-galactosidase staining using the improved method, taking advantage of Prom1 knockout mice (*Prom1*$^{cre\text{-}ert2\text{-}nlacz}$) (Figure EV1C).
- The revised manuscript includes the description of this result in the second paragraph of the Results section.

9. Several Prom1-/- mouse lines have been generated in the last decade, and numerous individuals are carrying mutations in PROM1 gene. Have any phenotype in liver and consequences been reported? Considering the cellular redistribution of actin in Prom1-/- cells described here (Fig. 4K), one would expect a physiological impact. Please comment.

Three unique *Prom1*$^{-/-}$ mouse lines have been generated to study Prom1 functions in various organs [32-34]. Those studies reported no specific phenotypes or defects other than blindness. Interestingly, Nishide et al [33] reports that elimination of Prom1-expressing cells causes body weight loss in mice. In addition, individuals with a frame-shift mutation [35], a non-sense mutation [36], or a missense mutation [29] in *PROM1* locus show retinal degeneration or sometimes polytactyly, but no

phenotype in liver has been reported. Zacchigna et al [34] suggested that lack of phenotype in Prom1-deficient mice may have come from the expression of Prom2 with the redundant functions and that *Prom1*$^{-/-}$ mice in stressed conditions may reveal additional roles. Interestingly, retina and liver in which loss of Prom1 show phenotypes have no Prom2 expression [37]. This has been discussed in the Discussion section.

10. As the authors concluded: " ...Prom1 and radixin may be excellent target proteins for lowering the blood glucose level in patients with diabetes", they should comment on the publication showing that Prom1-/- mice have a significant high glucose levels and were obese (Karim BO et al., Front Oncol. 2014).

This discrepancy may have come from the fact that different mouse strain has been used for backcrossing of *Prom1*$^{cre\text{-}ert2\text{-}nlacz}$ in two studies, C57BL/6N and 129SvEv. We would like to point out that the fasting blood glucose level of WT mice seemed a little bit too high (158 mg/dl) to be considered as a normal. Also, we demonstrated the decreased blood glucose level after fasting in two different models, Prom1 knockout or radixin knockdown mice. We have discussed this contradictory observations in the Discussion section.

11. In Table S1, clones or antibody specificities should be indicated, especially that of the anti-Prom1 antibody used for IFA. Company names are insufficient.

Detailed information regarding antibodies used in this study has been updated in Appendix Table S1.

12. Experimental procedures are often insufficiently described and Figure Legends appear less than minimal (e.g. immunostaining), it is therefore difficult to assess the validity of the data presented. Abbreviations should also be explained (e.g., whole cell lysate; WCL).

We have added the detailed experimental procedures in Methods section.

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1st Revision - Editorial Decision 29th Apr 2020

Dear Dr. Ko,

I would like to mention one additional point. Referee 2 in his/her minor point 1 mentions that a preprint should not be cited. Please cite the papers that referee 2 recommends. However, as per our guidelines you can also cite manuscripts posted on recognized preprint servers as follows:

Author NAME1, Author NAME2, (YEAR) article title. bioRxiv doi: 1234/002.dfj123 [PREPRINT]

Please see https://www.embopress.org/page/journal/14693178/authorguide#referencesformat for further info.

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Thank you for submitting your revised manuscript. It has now been seen by both of the original referees.

As you can see, the referees find that the study is significantly improved during revision. However, there are a couple of outstanding concerns still to be addressed as noted below:

• Referee 2 finds that prominin-1/radixin co-localization data are currently not compelling. We agree with the referee that this needs to be addressed experimentally. Please let me know if you would like to discuss this point further.
• Referee 1 noted in his/her initial evaluation that regulation of gluconeogenesis in the nucleus by localized changes in cAMP by prominin-1/radixin is hard to imagine, given the cell membrane localization of prominin-1/radixin. I can see that this concern was not completely resolved after revision. Therefore please discuss this caveat openly in the text.
• Please address the remaining minor concerns of both referees.
• 'Author Contributions' should be separated from Acknowledgements and get its own section. We noted that Jun-Seok Kim is missing.
• 'Conflict of Interest' should be separated from Acknowledgements section and get its own section following Author Contributions.
• We noted that a callout for Appendix Table S1 is missing from the text.
• We realized with thanks that you provided source data. They need to be uploaded one file per figure for technical reasons.
• We noticed that there is a list of abbreviations on p. 32. Please integrate abbreviations directly into the manuscript text and to remove the list.
• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb - as well as 2-5 one-sentence bullet points that summarize the key findings of the paper.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your

minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

I have read the rebuttal letter and studied the revisions made in the paper. My major criticism of the first version of the manuscript was that there is a breach of logic in the concept presented by the authors as they present beautiful evidence for an anchored PKA complex responding to glucagon signalling. Yet the main substrate and down-stream effector is CREB which the authors indicate shuttle between the cytoplasm and nucleus and meets the anchored complex, but where either CREB or PKA catalytic subunit travels over longer distances inside the cell. I suggested that there could be a different substrate for PKA or a kinase cascade with another kinase directly phosphorylating CREB. The authors however have taken the route of arguing that CREB is a direct substrate that shuttles in and out of the nucleus and that either goes to the anchored sites where the Prominin-1-Radixin complex holds PKA or that PKA is completely dissociated due to very high local cAMP levels (?). They present some experiments where all the cytoskeleton is disrupted or where nuclear shutting is blocked, both of which are quite crude approaches that have major and multiple effects inside the cells. The authors have not put these experiments in the paper as they feel they evidence is too preliminary. I think that argues that the topic is not addressed in full at present. Maybe the effect should be problem also with anchoring disruptor peptides such at Ht31 and/or RIAD or with radian mutated in the PKA binding site to prove that the effect is PKA-anhoring dependent. Furthermore, could the effect be replicated with a PKA catalytic subunit targeted to the nucleus and/or a PKI inhibitor with an NLS signal? The CREB/PKA system as described at present does not seem to require and anchored cytoplasmic locale for its effect.

Referee #2:

The revised manuscript entitled "Prominin-1-Radixin Axis controls hepatic gluconeogenesis by regulating PKA activity" (ID: EMBOR-2019-49416V2) by Lee and colleagues has been improved and numerous new experiments have been conducted following my comments/suggestions. Notably, the authors have addressed my concerns about the subcellular localization of prominin-1 and its expression in the liver. Experiences with lipid rafts have been substantially improved. However, several minor issues related to the new text and figures remain to be clarified, without requiring further experience. However, the co-localization of prominin-1 and radixin MUST be addressed experimentally as it is the main focus of this study, according to its title. The subcellular localization of prominin 1 translocating from the plasma membrane to the endocytic compartments, and even into the nucleus, needs to be addressed/discussed. The authors should develop these points further in the Discussion.

Minor points

1. The authors cited in the Introduction a manuscript (Ref. 24) deposited in BioRxiv, which is not peer review per se. Please cite published articles. For instance, they could cite Karbanová J et al. (2008) J Histochem Cytochem, 56: 997, where the authors describe the expression of human prominin-1 in the liver using specific anti-prominin-1 antibodies, which is more relevant than monitoring the reporter lacZ product under the control of endogenous prom1 promoters in a knock-in model (Ref. 21).

2. Since references 21 and 22 were published more than ten years ago, I suggest deleting "Recently" at the beginning of the sentence (last paragraph of the introduction).

3. At the beginning of the Results section, they should refer to Weigmann A et al. (1997) PNAS, 94: 12425 for the antibody 13A4. As this antibody was used to identify mouse prominin-1, it would strengthen the current study.

4. In the rebuttal letter the authors mentioned that the hepatocytic expression of prominin-1 is low. This observation should be stressed in the manuscript.

5. I am confused with the three following statements in the main text:
- Because of the incompatibility of antibody markers for hepatocytes (K9218 for HNF4α) and cholangiocytes (EP1580Y for CK19) with the antigen-retrival condition for Prom1 antibody, we immunostained Prom1 or HNF4α/CK19 on separate serial sections.
- We also determined the co-localization of Prom1, radixin and actin by indirect comparison using actin as a reference, because the antigen-retrieval condition for radixin antibody is incompatible with the Prom1 antibody (clone: 13A4).
- For CK-19 and HNF4α immunocytochemistry, antigen-retrieval was performed on thin cryosections,

What are these antigen-retrieval conditions and for which antibodies is this technique necessary? These are important questions given that the authors were unable to demonstrate co-expression of the proteins of interest. This should be clarified in the text. Can't the authors use alternative antibodies? Numerous anti-prominin-1 antibodies are commercially available.

The authors must show the co-localization of prominin-1 and radixin by double-immunofluorescence. The title of the manuscript is Prominin-1-Radixin axis controls ...!

6. The authors indicated: "Prom1-/- mouse used in this study to determine Prom1 functions in the liver did not show any apparent phenotype, as reported [22], except Prom1-/- primary hepatocytes were slightly larger than Prom1+/+ hepatocytes." Does the absence of prominin 1 affect the number of microvilli at the apical plasma membrane of hepatocytes?

7. The new data presented in Figure 5G, H are very interesting and reinforce their conclusion on the interaction of prominin-1 and radixin. The authors should mention that prominin-1 and flotillin are associated with distinct lipid rafts, the former being enriched in fractions 8 and 9 of the gradient, as is radixin, while the latter is found mainly in fraction 9. In addition, prominin-1 and radixin are sensitive to cholesterol reduction but flotillin is not.

8. Did the authors observe prominin 1 in the nucleus or cytoplasmic compartments? Numerous studies have reported nuclear localization of prominin 1 (e.g., Singer D et al. (2019) EMBO J 38: e99845; Lee YM and et al. (2018) Anticancer Res, 38: 4819; Chen Y-L et al. (2017) BMC Cancer 17:

474; Nunukova A et al. (2015) Int J Mol Med. 36; 65; Huang M et al. (2015) Dis Markers 2015: 986095). Others have also demonstrated the localization of prominin 1 in the endocytic compartments where it could play a biological role in the signaling pathway. These issues need to be discussed in the context of the current topic.

Other points

In the Introduction (last paragraph); epithelial and not epithelical.

In the Results (first paragraph): antigen-retrieval condition and not antigen-retrival condition.

Dear Dr. Tiebe,

Title: "Prominin-1-Radixin Axis controls hepatic gluconeogenesis by regulating PKA activity"

EMBOR-2019-49416-T

Authors: Lee et al.

We have addressed all of your points and reviewers' comments and suggestions. Please see below for the synopsis blurb and minor changes we have made:

Synopsis

- The transmembrane protein Prominin-1 is expressed in hepatocytes as well as cholangiocytes in the liver.
- Prominin-1 functions as an anchor for radixin which brings PKA holoenzyme to membrane microdomains where local cAMP concentration is increased by glucagon-induced activation of adenylyl cyclase.
- *Prom1* knockout and knockdown result in inhibition of glucagon-elicited CREB phosphorylation and gluconeogenesis in hepatocytes and livers.

Minor changes we have made include:

• Referee 2 finds that prominin-1/radixin co-localization data are currently not compelling. We agree with the referee that this needs to be addressed experimentally. Please let me know if you would like to discuss this point further.

We now have demonstrated co-localization of PROM1 and radixin in human liver samples with which double immunofluorescence staining are possible (Fig 5C).

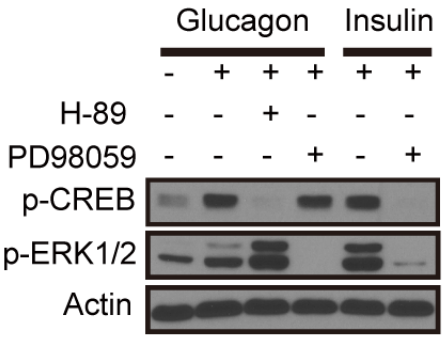
• Referee 1 noted in his/her initial evaluation that regulation of gluconeogenesis in the nucleus by localized changes in cAMP by prominin-1/radixin is hard to imagine, given the cell membrane localization of prominin-1/radixin. I can see that this concern was not completely resolved after revision. Therefore please discuss this caveat openly in the text.

Instead of leaving this caveat as it may and discuss in the text, we showed translocation of PKA catalytic subunit into the nucleus after glucagon stimulation using proximity ligation assay (Fig EV4F and G). In addition, we tested the presence of cascading kinases that may connect Prom1/radixin/PKA holoenzyme at the membrane and CREB in the nucleus (Fig EV4H). We reported this finding in page 14 of the Results section and page 19 of the Discussion section.

Referee #1:

I have read the rebuttal letter and studied the revisions made in the paper. My major criticism of the first version of the manuscript was that there is a breach of logic in the concept presented by the authors as they present beautiful evidence for an anchored PKA complex responding to glucagon signalling. Yet the main substrate and down-stream effector is CREB which the authors indicate shuttle between the cytoplasm and nucleus and meets the anchored complex, but where either CREB or PKA catalytic subunit travels over longer distances inside the cell. I suggested that there could be a different substrate for PKA or a kinase cascade with another kinase directly phosphorylating CREB. The authors however have taken the route of arguing that CREB is a direct substrate that shuttles in and out of the nucleus and that either goes to the anchored sites where the Prominin-1-Radixin complex holds PKA or that PKA is completely dissociated due to very high local cAMP levels (?). They present some experiments where all the cytoskeleton is disrupted or where nuclear shutting is blocked, both of which are quite crude approaches that have major and multiple effects inside the cells. The authors have not put these experiments in the paper as they feel they evidence is too preliminary. I think that argues that the topic is not addressed in full at present. Maybe the effect should be problem also with anchoring disruptor peptides such at Ht31 and/or RIAD or with radian mutated in the PKA binding site to prove that the effect is PKA-anhoring dependent. Furthermore, could the effect be replicated with a PKA catalytic subunit targeted to the nucleus and/or a PKI inhibitor with an NLS signal? The CREB/PKA system as described at present does not seem to require and anchored cytoplasmic locale for its effect

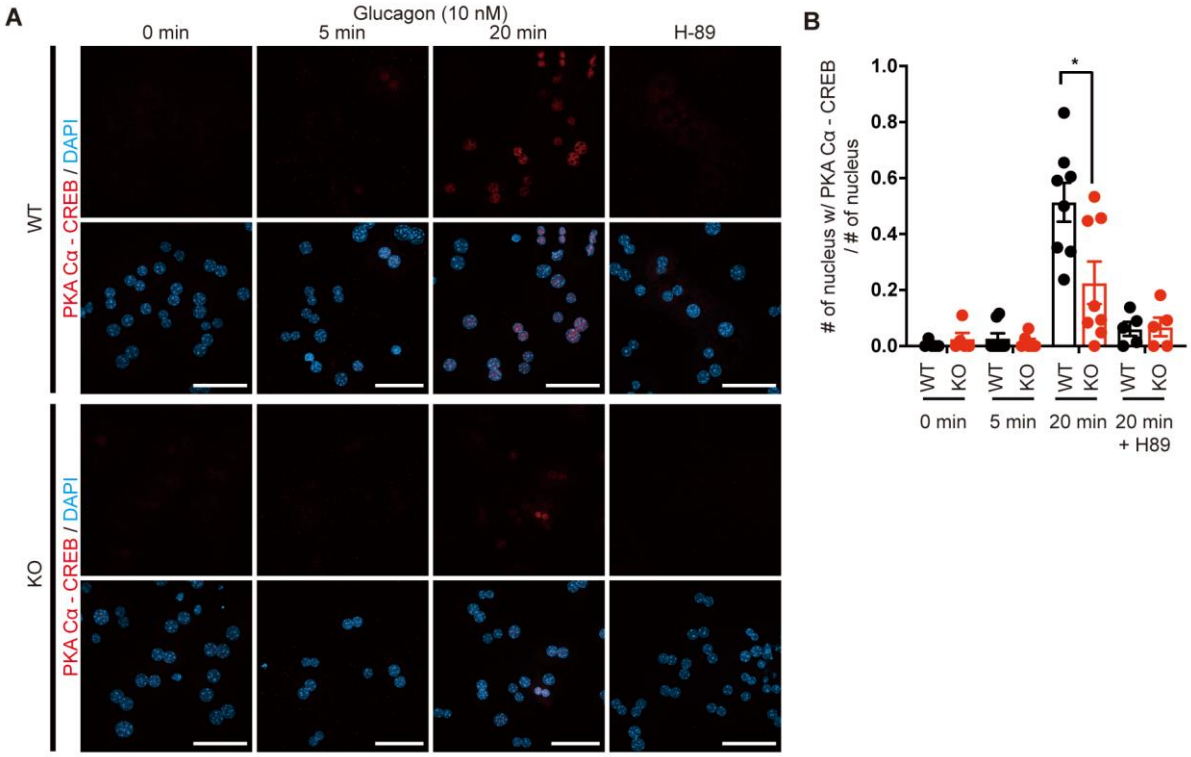
Addressing reviewer's criticism, we have re-considered reviewer's first suggestion; the presence of the unknown cascading kinases that connect Prom1/radixin/PKA holoenzyme at the plasma membrane and CREB in the nucleus. Dalle and colleagues have demonstrated that glucagon induces activation of ERK1/2 which is mediated by PKA, and promotes nuclear translocation of ERK1/2, resulting in CREB phosphorylation in MIN6 cell line and pancreatic islets [1]. Therefore, we examined the role of ERK1/2 in glucagon-induced CREB phosphorylation in primary hepatocytes (Rebuttal Figure 1).



CREB phosphorylation.

Glucagon marginally induced ERK1/2 phosphorylation, while insulin significantly induced ERK1/2 phosphorylation. Although PD98059, ERK1/2 inhibitor, completely suppressed insulin-induced CREB phosphorylation (the first lane from the right in Rebuttal Figure 1), it did not affect glucagon-induced CREB phosphorylation (the third lane from the right in Rebuttal Figure 1). Interestingly, H-89, a PKA inhibitor, suppressed glucagon-induced CREB phosphorylation, while it activated ERK1/2 (the third lane from the left in Rebuttal Figure 1, [2]). This observation indicates that at least in primary hepatocyte ERK1/2 is not involved in regulation of glucagon-induced CREB phosphorylation.

Then, how is Prom1/radixin/PKA holoenzyme formation at the plasma membrane transduced to CREB phosphorylation in the nucleus? We followed the location where the interaction of PKA catalytic subunit and CREB upon glucagon treatment took place in the cell using proximity ligation assay (Rebuttal Figure 2).



Rebuttal Figure 2 - **A.** The molecular interaction between catalytic subunit of PKA and CREB in WT (*Prom1*$^{+/+}$) and KO (*Prom1*$^{-/-}$) hepatocytes was determined by a proximity ligation assay using anti-PKA Cα and anti-CREB antibodies. These hepatocytes were stimulated with glucagon (10 nM) for the indicated time or treated with H-89, PKA inhibitor, prior to glucagon stimulation. Scale bar, 50 μm. **B.** The portion of cells with nuclear PKA Cα-CREB interaction (**A**) in more than 100 cells from each group was statistically determined.

PKA catalytic subunit and CREB interaction took place in the nucleus 20 min after glucagon stimulation (the second row from the right in the upper panel of Rebuttal Figure 2A). In KO hepatocytes, the interaction was reduced significantly (the lower panel of Rebuttal Figure 2A). The treatment of H-89, a specific PKA inhibitor suppressed the interaction, which indicated the specificity of this assay (the first row from the right). The portion of nuclear PKA catalytic subunit and CREB interaction in more than 100 cells from each group was determined (Rebuttal Figure 2B). This observation strongly indicates that PKA catalytic subunit is released from the complex of Prom1/radixin/PKA holoenzyme, and translocated into the nucleus. Our observation is consistent with what Gervasi and colleagues have reported regarding the dynamics of PKA activation: in mouse brain slice serotonin-mediated PKA responses were obtained in 30 s at the membrane, in 2.5 min in the cytosol, and in 13 min in the nucleus, indicating slow passive diffusion of the free PKA catalytic subunit into the nucleus [3]. We believe that diffusion of PKA C subunit after glucagon stimulation explains our additional results well. Our findings are described and discussed in the text (page 14 of the Results section and page 19 of the Discussion section, Fig EV4F, G and H).

Referee #2:

The revised manuscript entitled "Prominin-1-Radixin Axis controls hepatic gluconeogenesis by regulating PKA activity" (ID: EMBOR-2019-49416V2) by Lee and colleagues has been improved and numerous new experiments have been conducted following my comments/suggestions. Notably, the

authors have addressed my concerns about the subcellular localization of prominin-1 and its expression in the liver. Experiences with lipid rafts have been substantially improved. However, several minor issues related to the new text and figures remain to be clarified, without requiring further experience. However, the co-localization of prominin-1 and radixin MUST be addressed experimentally as it is the main focus of this study, according to its title. The subcellular localization of prominin 1 translocating from the plasma membrane to the endocytic compartments, and even into the nucleus, needs to be addressed/discussed. The authors should develop these points further in the Discussion.

In order to determine the localization of prominin-1 and radixin in the same tissue sample slide, we have used human liver samples with which antibodies against prominin-1 and radixin are compatible in the immunofluorescence staining procedure. We observed co-localization of PROM1 and radixin at the cell-cell contact sites in human liver (Fig 5C). In addition, PROM1 was found with the vesicles in the cytosolic area of cells.

Minor points

1. The authors cited in the Introduction a manuscript (Ref. 24) deposited in BioRxiv, which is not peer review per se. Please cite published articles. For instance, they could cite Karbanová J et al. (2008) J Histochem Cytochem, 56: 997, where the authors describe the expression of human prominin-1 in the liver using specific anti-prominin-1 antibodies, which is more relevant than monitoring the reporter lacZ product under the control of endogenous prom1 promoters in a knock-in model (Ref. 21).

Two references in which the expression of prominin-1 in the liver has been reported are now included.

2. Since references 21 and 22 were published more than ten years ago, I suggest deleting "Recently" at the beginning of the sentence (last paragraph of the introduction).

Change has been made accordingly.

3. At the beginning of the Results section, they should refer to Weigmann A et al. (1997) PNAS, 94: 12425 for the antibody 13A4. As this antibody was used to identify mouse prominin-1, it would strengthen the current study.

We cited the original article which reports the development of MAb 13A4 where applicable.

4. In the rebuttal letter the authors mentioned that the hepatocytic expression of prominin-1 is low. This observation should be stressed in the manuscript.

We have mentioned the low expression of Prom1 and cited the reference in the last paragraph of the introduction.

5. I am confused with the three following statements in the main text:

- Because of the incompatibility of antibody markers for hepatocytes (K9218 for HNF4α) and cholangiocytes (EP1580Y for CK19) with the antigen-retrival condition for Prom1 antibody, we immunostained Prom1 or HNF4α/CK19 on separate serial sections.

- We also determined the co-localization of Prom1, radixin and actin by indirect comparison using actin as a reference, because the antigen-retrieval condition for radixin antibody is incompatible with the Prom1 antibody (clone: 13A4).

- For CK-19 and HNF4α immunocytochemistry, antigen-retrieval was performed on thin cryosections,

What are these antigen-retrieval conditions and for which antibodies is this technique necessary? These are important questions given that the authors were unable to demonstrate co-expression of the proteins of interest. This should be clarified in the text. Can't the authors use alternative antibodies? Numerous anti-prominin-1 antibodies are commercially available.

For immunofluorescence staining of tissue samples for CK19, HNF4α, radixin, and human PROM1, heat-induced antigen-retrieval using hot-steam in sodium citrate solution (pH = 6.0) was applied to the slides. In such condition, MAb 13A4 for Prom1 could not detect Prom1 in the liver. Instead, re-fixation of the thin sections in 4 % paraformaldehyde in 0.1 M phosphate buffer was used to retrieve Prom1 epitope, in which HNF4α and radixin epitopes could not be retrieved. This was the reason we described as “incompatible” procedure. Also, we have tried most of commercially available antibodies against mouse Prom1, if not all, for immunocytochemistry to no avail. Instead, we have immunostained the human tissue sample with a specific antibody for PROM1 (HB#7) and radixin (C4G7) for double immunostaining of liver samples, and are able to demonstrate the co-localization of PROM1 and radixin in the liver (Fig 5C). The antigen-retrieval technique is now described in the Methods section.

The authors must show the co-localization of prominin-1 and radixin by double-immunofluorescence. The title of the manuscript is Prominin-1-Radixin axis controls ...!

We performed double immunofluorescence staining for Prom1 and radixin with human liver tissue samples and presented in Fig 5C.

6. The authors indicated: "Prom1-/- mouse used in this study to determine Prom1 functions in the liver did not show any apparent phenotype, as reported [22], except Prom1-/- primary hepatocytes were slightly larger than Prom1+/+ hepatocytes." Does the absence of prominin 1 affect the number of microvilli at the apical plasma membrane of hepatocytes?

Using high-resolution microscopy, prominin-1 mutants, and several cell lines, Thamm and colleagues suggest that prominin-1 may be a regulator of membrane protrusion [4]. However, we did not observe the difference between wildtype and *Prom1* knockout hepatocytes in terms of the shape and frequency of microvilli on the membrane (Fig 1E vs. H).

7. The new data presented in Figure 5G, H are very interesting and reinforce their conclusion on the interaction of prominin-1 and radixin. The authors should mention that prominin-1 and flotillin are associated with distinct lipid rafts, the former being enriched in fractions 8 and 9 of the gradient, as is radixin, while the latter is found mainly in fraction 9. In addition, prominin-1 and radixin are sensitive to cholesterol reduction but flotillin is not.

We described this observation in the main text now.

8. Did the authors observe prominin 1 in the nucleus or cytoplasmic compartments? Numerous studies have reported nuclear localization of prominin 1 (e.g., Singer D et al. (2019) EMBO J 38: e99845; Lee YM and et al. (2018) Anticancer Res, 38: 4819; Chen Y-L et al. (2017) BMC Cancer 17: 474; Nunukova A et al. (2015) Int J Mol Med. 36; 65; Huang M et al. (2015) Dis Markers 2015: 986095). Others have also demonstrated the localization of prominin 1 in the endocytic compartments where it could play a biological role in the signaling pathway. These issues need to be discussed in the context of the current topic.

We have not observed Prom1 in the nucleus of hepatocytes or cholangiocytes in the liver tissues using the same specific Prom1 antibody used in the aforementioned study. Although we have seen Prom1 associated with cytosolic vesicles in mouse liver, at this moment we do not know the possible function of Prom1 in these vesicle structures and it will be a topic for future study. This issue has been

discussed in the text (page 20 of Discussion section).

Other points

In the Introduction (last paragraph); epithelial and not epithelical.

In the Results (first paragraph): antigen-retrieval condition and not antigen-retrival condition.

We corrected the misspelling.

References

1. Dalle S, Longuet C, Costes S, Broca C, Faruque O, Fontes G, Hani EH, Bataille D (2004) Glucagon promotes cAMP-response element-binding protein phosphorylation via activation of ERK1/2 in MIN6 cell line and isolated islets of Langerhans. *J Biol Chem* **279**: 20345-55
2. Davis MI, Ronesi J, Lovinger DM (2003) A predominant role for inhibition of the adenylate cyclase/protein kinase A pathway in ERK activation by cannabinoid receptor 1 in N1E-115 neuroblastoma cells. *J Biol Chem* **278**: 48973-80
3. Gervasi N, Hepp R, Tricoire L, Zhang J, Lambolez B, Paupardin-Tritsch D, Vincent P (2007) Dynamics of Protein Kinase A Signaling at the Membrane, in the Cytosol, and in the Nucleus of Neurons in Mouse Brain Slices. *The Journal of Neuroscience* **27**: 2744-2750
4. Thamm K, Šimaitė D, Karbanová J, Bermúdez V, Reichert D, Morgenstern A, Bornhäuser M, Huttner WB, Wilsch-Bräuninger M, Corbeil D (2019) Prominin-1 (CD133) modulates the architecture and dynamics of microvilli. *Traffic* **20**: 39-60

2nd Revision - Editorial Decision 27th Aug 2020

Dear Prof. Ko,

Thank you for submitting your revised manuscript. We have now received the reports from both of the original referees.

My apologies for this unusual delay in getting back to you, it took longer than anticipated to receive the referee reports.

As you can see, the referees acknowledge that the manuscript has been significantly improved and now support publication. Before I can formally accept the manuscript, please address the remaining minor concerns of the referees by keeping the 'track changes on'.

- Please tone down the conclusions based on the newly added data as in the comments of referee #1, but please keep the data in.

- Please perform the minor changes requested by referee #2.

Thank you again for giving us the opportunity to consider your manuscript. I am looking forward to receiving your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

I have read the re-revised manuscript. I still think the authors provide very nice evidence for an anchored AKAP complex at the cell membrane with Prominin-1 and Radixin and where the latter serves as an AKAP that holds PKA and where the complex mediates effects of glucagon and that regulates phosphorylation of unknown substrate proteins detected by antibodies to phosphorylated PKA phosphorylation sites. I also think the authors present good evidence to indicate that the Prominin-1/Radixin/PKA complex is involved at some level (indirectly?) in signalling to the nucleus and CREB phosphorylation. However, in response to my comments in two rounds of review that there is some breach of logic in the concept when they argue both for a discrete localised signalling complex at the membrane and next that the effector molecule, PKA catalytic subunit, travels slowly over longer distances into the cell, the authors have now taken a route where they argue that this is indeed the case as they see colocalization inside the nucleus of CREB and PKA catalytic subunit by PLA and argue that this nuclear pool of PKA is donated by the Prominin-1/Radixin/PKA complex at the membrane. These new data now incorporated into the ms. raises several new questions: i) Does PKA catalytic subunit (a kinase with a turnover number of 600 phoshophorylation events / minute) really sit together with CREB for so long that this can be detected by PLA or is this an artifact in a dense environment in the nucleus?; ii) If is true that PKA catalytic and CREB sit tightly enough together in the nucleus to be detected by PLA is this really the population of PKA catalytic subunit from the Prominin-1/Radixin complex? Do that population

completely dissociate under physiological concentrations of hormone? Other experiments with live imaging and e.g. FRET sensors would be required to establish that I think. In addition, experiments knockdown and expression of a radixin with mutations in the binding site for PKA could go some way in addressing this. I think the authors here have inadvertently stepped into an open question in the PKA field and that is whether PKA really goes in the nucleus under physiological stimuli that gives local pools of cAMP and if yes, where this pool of PKA comes from. Newer literature indicates that many anchored PKA complexes does not completely dissociate (see papers from Donelson Smith and John Scott on this matter in eLife and Science). Citing older literature that does not appreciate these newer findings does not really help addressing this. I am sorry, but I really think the earlier version of the paper was better in this respect and a revised version that discusses these items but does not drive a firm conclusion not fully substantiated by the data (and possibly with out the nuclear PLA data) would be better suited for publication.

Referee #2:

Comments to Authors

Dear Colleagues,
The revised manuscript entitled "Prominin-1-Radixin Axis controls hepatic gluconeogenesis by regulating PKA activity" (ID: EMBOR-2019-49416V3) by Lee et al. has been significantly improved. I can only suggest adding one reference (Walker T. et al. 2013) in the Discussion that showed upregulation of prominin-2 in prominin-1-deficient animals. This will reinforce the authors' conclusion.

Discussion section:
.... In addition, individuals with a frame-shift mutation (Maw et al, 2000), a non-sense mutation (Zhang et al, 2007) or a missense mutation (Yang et al., 2008) in the PROM1 locus show retinal degeneration or sometimes polytactyly. Zacchigna et al. (Zacchigna et al., 2009) suggested that lack of phenotype in Prom1- deficient mice may have come from the expression of Prom2 with the redundant functions (WALKER 2013).

Prominin-1 allows prospective isolation of neural stem cells from the adult murine hippocampus. Walker TL, Wierick A, Sykes AM, Waldau B, Corbeil D, Carmeliet P, Kempermann G. J Neurosci. 2013 Feb 13;33(7):3010-24. doi: 10.1523/JNEUROSCI.3363-12.2013.

Some minor points are listed below.

The authors have used equilateral triangles to indicate the object of interest (e.g., Figures 1J, 5C), an isosceles triangle would be more appropriate.

Polydactyly instead polytactyly (see Discussion)
Cholangiocyte instead cholangiocyute (Results section)
Preventing instead prevenitng
Plasma membrane instead plsma membrane
Translocates instead tranlocates
Röper et al, 2000 instead Roper et al, 2000
Karbanová et al, 2008 instead Karbanova et al, 2008
Triton X-100 instead tritron X-100 (Methods section; immunofluorescence staining)
Cambridge instead cambridge

The authors have completed all minor editorial requests.

3rd Revision - Editorial Decision 10th Sep 2020

Dear Dr. Ko,

Thank you for submitting your revised manuscript to EMBO Reports. I have now looked at everything and all is fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice study!

Kind regards,

Deniz Senyilmaz Tiebe

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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Corresponding Author Name: Sungsoo Lee and Young-Gyu Ko
Journal Submitted to: EMBO reports
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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 iustified
- 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?	Sample size is described in each figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size is described in each figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We did not exclude any raw data.
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used for the experiments on animals.
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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding of the investigators were used for the experiments on animals.
5. For every figure, are statistical tests justified as appropriate?	Yes, for statistical data in each data, no substantial difference has been observed among the variations.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical analysis between experimental groups was performed using the Student's t-test or unpaired t tests. Data analysis was not blinded. Significance levels are: * P < 0.05; ** P < 0.01; *** P < 0.001; NS, non-significant
Is there an estimate of variation within each group of data?	We have presented ±SEM for each group of data as described in the figure legends.

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http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes, for statistical data in each data, no substantial difference has been observed among the variations.

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).	Antibodies used in this study are listed in Appendix. We gave the species of antibodies used as well as the company we bought them and the catalog number.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* 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.	Prom1 knockout mice (Prom1cre-ert2-nlacz) were purchased from The Jackson Laboratory (Stock NO. 017743, Bar Harbor, ME, USA). The Prom1-/- mice were backcrossed with C57BL/6N mice for five generations. Mice were maintained in a 12 h light–12 h dark cycle, in stable conditions of temperature (22°C), with food and water available ad libitum
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal studies were conducted with the approval of the Korea University Institutional Animal Care and Use Committee and the Korean Animal Protection Law (KUIACUC-2017-14, -2018-6 and -2019-0111).
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.	We confirm compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Dongguk University Hospital Institutional Review Board (Approval number: 110757-201909-HR-06-02)
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.	Informed consent were obtained from patients for the use of biopsy samples in clinical research as well as diagnosis.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	RNA-Seq data are deposited with GEO, Accession GEO ID: GSE144018
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).	N/A
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).	N/A
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.	N/A

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.	No