EMBO reports

Calcium signaling induces a partial EMT

Robert Norgard, Jason Pitarresi, Ravikanth Maddipati, Nicole Aiello-Couzo, David Balli, Jinyang Li, Taiji Yamazoe, Max Wengyn, Ian Millstein, Ian Folkert, Derick Rosario-Berrios, Il-Kyu Kim, Jared Bassett, Riley Payne, Corbett Berry, Xiaodong Feng, Kathryn Sun, Michele Cioffi, Priyanka Chakraborty, Mohit Jolly, Silvio Gutkind, David L. Lyden, Bruce Freedman, J. Kevin Foskett, Anil Rustgi, and Ben Stanger
DOI: 10.15252/embr.202051872

Corresponding author(s): Ben Stanger (bstanger@upenn.edu)

Review Timeline:

Submission Date:	11th Oct 20
Editorial Decision:	2nd Nov 20
Revision Received:	7th Feb 21
Editorial Decision:	19th Mar 21
Revision Received:	15th May 21
Accepted:	21st Jun 21

Editor: Achim Breiling

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 2nd Nov 2020

Dear Dr. Stanger,

Thank you for the transfer of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your 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 of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

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 file labeled Appendix. The Appendix should have page numbers and needs

to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

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.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in the respective section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

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 & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets 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. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) 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://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please also note our new reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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

Achim Breiling
Editor
EMBO Reports

Referee #1:

Understanding the mechanisms regulating EMT may have important implications for our understanding of cellular behavior during embryonic development, cancer progression, metastatic dissemination and therapy resistance. In this work the authors found that increase in the calcium influx induces a decrease in cell surface E-Cadherin and increase in Vimentin expression in murine pancreatic cancer cell lines, as well as in the human lung, breast pancreatic cancer cell lines. The authors further show that sustained mobilization of Calcium and subsequent activation calmodulin promote decrease of surface E-cadherin.
The topic of the manuscript is interesting, and the manuscript is well written. However, there are

several key points in this manuscript that remain unclear and some of the claims of the study are not fully supported by the data. The precise mechanisms by which an increase in calcium influx induces partial EMT phenotype remain unclear. In addition, the phenotype of partial EMT remains poorly characterized. If the authors address our concerns and provide more mechanistic insights that would increase the novelty of this study, the manuscript could be suitable for publication in EMBO reports.
These questions are detailed below.

1. The authors claim that calcium mobilization promote partial EMT. However, this claim in based on the decrease of surface E-cadherin and no proper characterization of the expression of epithelial and mesenchymal markers that would support the presence of partial EMT state (in which cells simultaneously express epithelial and mesenchymal markers). The same mouse model was previously used by the same group in 2018. However, a more extensive characterization of EMT programs would be required to better substantiate what are the transcriptional and not transcrpritional programs regulated by the increase in calcium influx.
2. The same concern is also true for human cell lines, where only on the decrease of cell surface E-cadherin and the increase in Vimentin expression is used to define partial EMT. Which epithelial programs are repressed? Which mesenchymal programs are upregulated besides Vimentin?
3. The finding that Calcium influx in cancer cells can decrease extracellular and cytoplasmic domains of E-cadherin is not novel. Calcium influx, induced my mechanical scraping of cells or ionomycin treatment was shown to promote metalloprotease-mediated E-cadherin cleavage and subsequent degradation of the cytoplasmic domain (Ito et al, Oncogene 1999). In this work, the authors showed that the E-cadherin degradation of E-cadherin by calcium influx results in b-catenin translocation from cell-cell contacts to cytoplasm, that could explain, at least partially, the alteration in migration, invasion and upregulation of mesenchymal markers. Surprisingly, this work is not cited by the authors. Much more detailed analysis of the mechanisms leading to surface E-cadherin downregulation and upregulation of mesenchymal markers should be performed to present substantial novelty. Is beta-catenin also activated in the case of pancreatic cancer cell lines and human cell lines investigated by the authors?
4. The authors induced GPCR-Gaq signaling and observed decrease in surface E-cadherin. It is not clear, however, why this signaling pathway and no other mechanisms could be involved. It is not even shown that this pathway is upregulated in the model that the authors use in this study. In addition, a number of calcium channels have been described to be upregulated in cancer cells and promote tumor progression and invasion. Would be important to provide a more systematic approach of the pathways involved in the increased in calcium influx found here.
5. The authors claim that calcium influx induces increased transcription of mesenchymal genes. However, it is not clear whether this is a direct effect of increased calcium influx or whether the loss of adherens junctions that occur as the consequence of decreased surface E-cadherin, induce signaling that triggers EMT.
6. Is this partial EMT state stable? What will happen if these cells are treated with TGFbeta? And upon subcutaneous grafting?
7. The authors perform an analysis of exosomes to define EMT related proteins. However, it is not clear why the EMT-related proteins should be secreted in exosomes and why the exosome analysis and not the total proteome of the tumor cells were performed?
8. Finally, the authors show that increased calcium influx acts via Calmodulin and not Calcineurin. This is potentially an interesting observation, but it would be interesting to investigate further which types of CaM dependent kinases could be activated and be responsible for the partial EMT phenotype.

Referee #2:

These authors recently described a partial-EMT program operating in vivo by carcinoma cells, which lose their epithelial state through a poorly understood post-translational mechanism. Hybrid or partial EMT states are particularly interesting as, while they are thought to be able to more easily navigate barriers to metastatic spread and possess greater metastatic competence, they are poorly understood. In particular, the mechanism that drive cells to adopt a partial state, rather than a complete transformation towards 'totally mesenchymal' state, is not known at all.

Here they use an autochthonous model of pancreatic ductal adenocarcinoma, in which they previously showed that P-EMT is a common feature of stochastically-arising pancreatic tumours - and identify a role of calcium signalling in inducing a stable p-EMT. They show that sustained Ca2+ mobilization via GPCR signalling induces -EMT and that this requires Calmodulin, but not Calcineurin. The paper is very well written and clear, and their conclusions are strongly supported by the data, and opens a new, very interesting avenue for research into EMT with potential relevance for both embryonic and cancer related EMT.

Minor comments:

1. It is important that the difference between TGFBeta induced EMT and ionomycin induced EMT is fully characterised - to really comprehensively show this is a P-EMT rather than a C-EMT as the role of calcium in C-EMT has previously been described. For this reason I want to be really sure of the results presented in Figure 2
To investigate the p-EMT - they look at induction of vimentin - why is there already a lot of vimentin at T-0 in the TGFbeta panel? Fig 2C - TGFB T- looks v similar to ionomycin T48h if you look at the E-Cad and Vimentin protein levels - how much can we trust that the protein levels seen in inomycin 24-72 is downstream of calcium signalling, as the levels look v similar to TGFbeta at T0

2. Fig 2D - cell movement - is there any statistical difference between ionomycin and TGFB in the cell movement and transwell migration assays? Could you also comment on how the cells move in these assays, as P-EMT has been associated more with collective rather than individual migration

3. Are any GPCR receptors unregulated in the in vivo transcriptome data of C-EMT versus P-EMT? - it would be interesting to include this data, as it would strengthen the case for a role of GPCR signalling in vivo.

Referee #3:

In this very interesting work Norgard and colleagues report about the role of calcium signals and calmodulin in the EMT of pancreatic cancer cells. The authors suggest that elevation of cytosolic calcium causes partial EMT without an involvement of the calcium dependent calcineurin-NFAT axis.
This study is based on a previous publication of the working group (Aiello et al 2018) wherein the authors used RNA-seq analyses to evaluate differences between two EMT cell subtypes, characterized by surface expression of e-cadherin (ECAD. By means of pathway analysis the authors found that in ECAD negative cells genes coding for proteins involved in cellular calcium homeostasis are enriched compared to ECAD positive cells. Based on this finding, the authors

hypothesized and later confirmed that calcium is an important regulator of EMT transition.

I have several comments and suggestions that the authors might want to consider when revising this work:

1. Although it is clear that calcium genes are relevant for EMT, the authors did not provide identity of single hits whose regulation is highest. This is important because for example a gene could be upregulated in ECAD negative cells but its function could be inhibitory and would thus lead to reduced calcium levels in the cytosol. For example, upregulation of the PMCA in the PM would have such an effect. Moreover, the differential expression should also be validated on a protein level (WB and IHC for example) and the functional role of the top hits should be confirmed by gene manipulation (up- or downregulation). This is important in order to relate the current findings with many studies, which reported in the past that Ca2+ channels belonging to the CRAC, TRP and IP3R families control cancer cell invasion and growth.

2. Given that the examined cells express a number of Gq-coupled receptors, the authors should try to identify a more physiological stimulus to induce calcium elevation in their cells. Ionomycin is an ionophore and can thus have many unspecific effects. To this end, the observation that ATP-triggered calcium transient is not causing similar effects as ionomycin or CNO on ECAD surface expression warrants further investigation given that calmodulin would very likely be activated by such cytosolic calcium elevations.

3. Performing the study in murine cancer cells reduces the clinical relevance of the findings. The authors confirmed the main findings in other human cells but calcium signaling networks and gene expression patterns of murine and human cells are rather different.

4. It would be interesting to examine how drug sensitivity would be affected in cells in which the reported calcium-calmodulin axis is inactivated.

5. Why is the n for ECAD negative cells so low in Fig 1D compared with the ECAD positive cells? The huge differences might affect the conclusions.

6. The n values in Fig. 1C, right panel, are missing.

7. The PCA findings discussed on page 10 are shown in Fig. S3E and not in Fig. 3E.

The following is a detailed point-by-point response to the Reviewers' comments (new data or substantive changes to the text listed in **boldface**):

Referee #1: Reviewer comments to the author

Understanding the mechanisms regulating EMT may have important implications for our understanding of cellular behavior during embryonic development, cancer progression, metastatic dissemination and therapy resistance. In this work the authors found that increase in the calcium influx induces a decrease in cell surface E-Cadherin and increase in Vimentin expression in murine pancreatic cancer cell lines, as well as in the human lung, breast pancreatic cancer cell lines. The authors further show that sustained mobilization of Calcium and subsequent activation calmodulin promote decrease of surface E-cadherin.

The topic of the manuscript is interesting, and the manuscript is well written. However, there are several key points in this manuscript that remain unclear and some of the claims of the study are not fully supported by the data. The precise mechanisms by which an increase in calcium influx induces partial EMT phenotype remain unclear. In addition, the phenotype of partial EMT remains poorly characterized. If the authors address our concerns and provide more mechanistic insights that would increase the novelty of this study, the manuscript could be suitable for publication in EMBO reports. These questions are detailed below.

1. The authors claim that calcium mobilization promotes partial EMT. However, this claim in based on the decrease of surface E-cadherin and no proper characterization of the expression of epithelial and mesenchymal markers that would support the presence of partial EMT state (in which cells simultaneously express epithelial and mesenchymal markers). The same mouse model was previously used by the same group in 2018. However, a more extensive characterization of EMT programs would be required to better substantiate what are the transcriptional and not transcriptional programs regulated by the increase in calcium influx.

Response: We appreciate the Reviewer's enthusiasm of the current study which follows from our prior work published in 2018. We agree that a better characterization of the transcriptional and post-transcriptional EMT changes brought about by increases in intracellular calcium would provide helpful insight into this partial EMT program. In addition to the transcriptional and proteomic data included in the original manuscript (Fig. 3A and Fig. S3F), we have now performed flow cytometry experiments in human cells to characterize surface expression of epithelial markers (Claudin-7, Mucin-1, and Tight Junction Protein 1) and RT-qPCR data of these same epithelial markers plus mesenchymal markers (N-cadherin and CD44) in human carcinoma cells (**new Fig. S2H**).

These studies show that calcium induced EMT results in a decrease of surface levels of Claudin-7 (CLDN7) and Tight Junction Protein 1 (TJP1). This decrease occurred without changes in mRNA, similar to the behavior of E-cadherin. In addition, CD44 was robustly upregulated, but the prototypical E- to N-cadherin switch was not observed. These

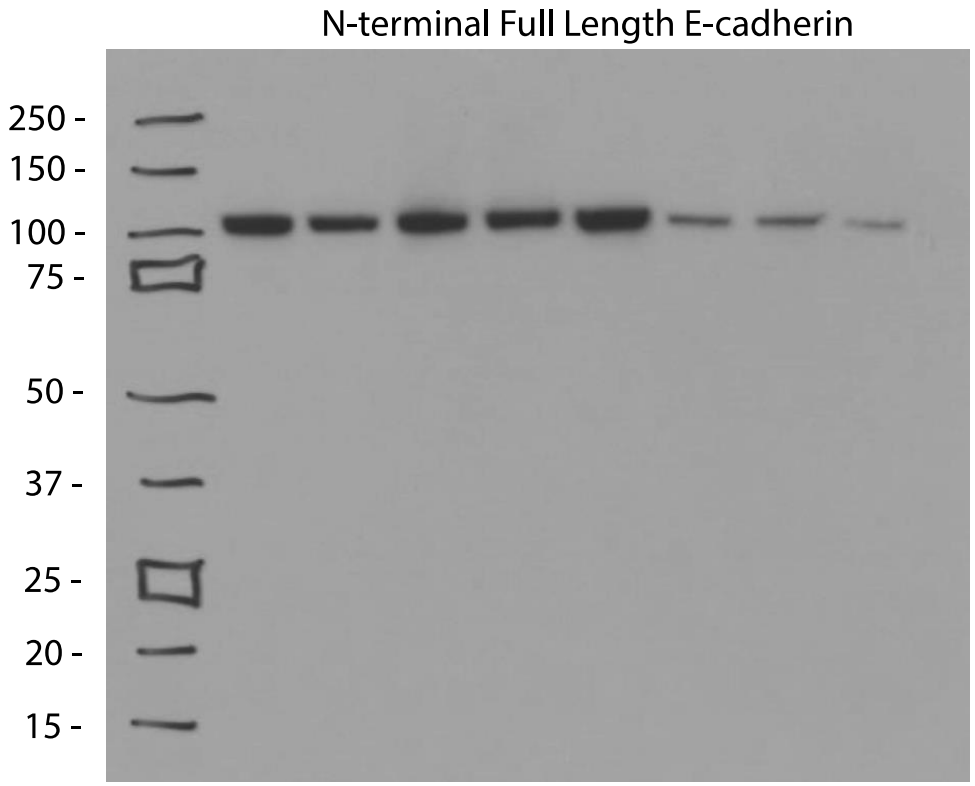
results match the RNA-sequencing data performed in mouse cells in Fig. 3A. This new data provide further evidence that calcium-induced P-EMT is truly a hybrid state characterized by the co-expression of epithelial and mesenchymal genes (e.g. *Ecad)/Muc1* and *Cd44*/*Ncad*). We speculate that this hybrid state confers greater plasticity—an idea we explore more in response #6.

2. The same concern is also true for human cell lines, where only on the decrease of cell surface E-cadherin and the increase in Vimentin expression is used to define partial EMT. Which epithelial programs are repressed? Which mesenchymal programs are upregulated besides Vimentin?

Response: In the revision, we have now further characterized calcium induced EMT in human cells. As shown in **new Fig. S2H**, we have performed flow cytometry experiments to characterize surface expression of epithelial markers (Claudin-7, Mucin-1, and Tight Junction Protein 1) and mesenchymal markers (N-cadherin and CD44). In addition, we performed RT-qPCR data of these same epithelial markers and mesenchymal markers in human carcinoma cells.

3. The finding that Calcium influx in cancer cells can decrease extracellular and cytoplasmic domains of E-cadherin is not novel. Calcium influx, induced my mechanical scraping of cells or ionomycin treatment was shown to promote metalloprotease-mediated E-cadherin cleavage and subsequent degradation of the cytoplasmic domain (Ito et al, Oncogene 1999). In this work, the authors showed that the E-cadherin degradation of E-cadherin by calcium influx results in b-catenin translocation from cell-cell contacts to cytoplasm, that could explain, at least partially, the alteration in migration, invasion and upregulation of mesenchymal markers. Surprisingly, this work is not cited by the authors. Much more detailed analysis of the mechanisms leading to surface E-cadherin downregulation and upregulation of mesenchymal markers should be performed to present substantial novelty. Is beta-catenin also activated in the case of pancreatic cancer cell lines and human cell lines investigated by the authors?

Response: We apologize for not citing this important reference, which is now included in the revised manuscript. Indeed, this article helped stimulate our interest in calcium signaling. In our initial studies for the potential mechanism underlying P-EMT, we considered E-cadherin cleavage as a potential mechanism. However, the N-terminal antibody used in our study (for flow cytometry, western blot, and IF) detects full length E-cadherin. We have included the raw western blot below. Only full length E-cadherin (~130 KDa) was detected and second band or ~80KDa band was detected. In addition, we observed no decrease in the full-length product by WCL on western blot, concluding that cleavage was likely not happening in this context (Fig. 2C). Therefore, we considered other possibilities.



In addition, we investigated the behavior of beta-catenin during P-EMT. We have previously shown (Aiello et al. 2018) that during P-EMT (in autochthonous KPCY tumors) beta catenin relocalizes from the membrane to endocytic vesicles. Now, we have performed cellular fractionation experiments following treatment with ionomycin. As shown in a **new figure S2D** we saw no evidence for differences in β-catenin activation (nuclear accumulation) between the groups, leading us to conclude that it is not the mechanism mediating Ca2+-induced partial EMT. Therefore, we turned to other potential regulators such as CAMK2, discussed in response #8 below.

4. The authors induced GPCR-Gaq signaling and observed decrease in surface E-cadherin. It is not clear, however, why this signaling pathway and no other mechanisms could be involved. It is not even shown that this pathway is upregulated in the model that the authors use in this study. In addition, a number of calcium channels have been described to be upregulated in cancer cells and promote tumor progression and

invasion. Would be important to provide a more systematic approach of the pathways involved in the increased in calcium influx found here.

Response: We apologize for the confusion. We do not claim that GPCR-Gαq activation is the sole mechanism by which calcium flux can occur. Rather, our study provides evidence using a validated DREADD assay that GPCR signaling may be one physiologically relevant stimulus. Consistent with this conclusion, we now show that the Gαq subunit is upregulated in P-EMT tumors *in vivo* (included in a **new Fig. S4A**). To our knowledge, there is no signature to define GPCR-Gαq pathway activation functionally other than calcium signaling itself, which is the signature that led us to consider calcium flux as a regulator of P-EMT in the first place. We have included these limitations in the Discussion (**page 15-16**).

5. The authors claim that calcium influx induces increased transcription of mesenchymal genes. However, it is not clear whether this is a direct effect of increased calcium influx or whether the loss of adherens junctions that occur as the consequence of decreased surface E-cadherin, induce signaling that triggers EMT.

Response: We thank the reviewer for this insightful comment. Unfortunately, decoupling calcium signaling from E-cadherin loss is experimentally difficult. However, previous work by other labs has demonstrated that loss of adherens junction proteins (specifically E-cadherin) can result in an increase in invasion and metastasis. For example, a prior study by the Weinberg group (Onder et al., 2011), showed that E-cad loss leads to the induction of EMT-associated genes (**new Fig S6A**, data re-analyzed by us). Thus, we agree that loss of adherens junctions may contribute to the upregulation of mesenchymal genes associated with calcium influx. This point has been noted in the revised Discussion (**page 16**).

6. Is this partial EMT state stable? What will happen if these cells are treated with TGFbeta? And upon subcutaneous grafting?

Response: To assess the stability of the phenotype, we performed a plasticity experiment by monitoring surface E-cadherin during and after EMT induction. In this experiment murine PDA tumor cells were treated with either ionomycin or TGFβ for seven days and then released for an additional seven days. As shown in a **new Fig. 2D**, treatment with either agent led to a significant reduction in surface E-cadherin levels (by flow cytometry). Upon removal of ionomycin, surface E-cadherin levels quickly rebounded to pre-treatment levels (even surpassing the DMSO control on the first day). By contrast, surface E-cadherin returned to pre-treatment levels much more gradually after release from TGFβ withdrawal. These results indicate differences in the kinetics of plasticity associated with these two inducers. This point is discussed in the revised manuscript (**page 16**).

We treated cells with both ionomycin and TGFβ (**new Fig S2C**). We found that TGFβ is a dominating force as we have previously seen in other studies in our lab (Aiello, et al. 2018., and Yuan, et al. 2020).

Unfortunately, we are unable to do these experiments in subcutaneous grafts due to the high degree of plasticity of the cells (also previously noted in Aiello, et al. 2018). Upon ingrafting, these cell lines readily switch between E and M states, as we can no longer stimulate them with our EMT-inducing ligands, such as ionomycin. We are currently performing follow up experiments further characterizing Camk2b knockout/over-expression systems *in vivo*, to be explored in future manuscripts. While we hope to define genetically tractable means of inducing *stable* partial-EMT states, this is beyond the scope of the current work.

7. The authors perform an analysis of exosomes to define EMT related proteins. However, it is not clear why the EMT-related proteins should be secreted in exosomes and why the exosome analysis and not the total proteome of the tumor cells were performed?

Response: As noted in the text, we elected to perform the exosome analysis as a more global window into changes in the cellular proteome associated with these two EMT programs. We agree that a more comprehensive examination of the cellular proteome might have yielded additional information. However, because whole cell proteomics would have added an additional level of technical complexity, we opted for this simpler approach.

8. Finally, the authors show that increased calcium influx acts via Calmodulin and not Calcineurin. This is potentially an interesting observation, but it would be interesting to investigate further which types of CaM dependent kinases could be activated and be responsible for the partial EMT phenotype.

Response: We appreciate this question and have worked hard to understand downstream events. We now include loss-of-function data (**new Fig. S4D-G and Fig. 4E**) that identify CaM kinase 2 beta (Camk2b) as an essential downstream mediator of P-EMT.

Referee #2: Reviewer comments to the author

These authors recently described a partial-EMT program operating in vivo by carcinoma cells, which lose their epithelial state through a poorly understood post-translational mechanism. Hybrid or partial EMT states are particularly interesting as, while they are thought to be able to more easily navigate barriers to metastatic spread and possess greater metastatic competence, they are poorly understood. In particular, the mechanism that drive cells to adopt a partial state, rather than a complete transformation towards 'totally mesenchymal' state, is not known at all.

Here they use an autochthonous model of pancreatic ductal adenocarcinoma, in which they previously showed that P-EMT is a common feature of stochastically-arising pancreatic tumors - and identify a role of calcium signaling in inducing a stable p-EMT. They show that sustained Ca2+ mobilization via GPCR signaling induces -EMT and that

this requires Calmodulin, but not Calcineurin. The paper is very well written and clear, and their conclusions are strongly supported by the data, and opens a new, very interesting avenue for research into EMT with potential relevance for both embryonic and cancer related EMT.

Minor comments:

1. It is important that the difference between TGFBeta induced EMT and ionomycin induced EMT is fully characterized - to really comprehensively show this is a P-EMT rather than a C-EMT as the role of calcium in C-EMT has previously been described. For this reason, I want to be really sure of the results presented in Figure 2 To investigate the p-EMT - they look at induction of vimentin - why is there already a lot of vimentin at T-0 in the TGFbeta panel? Fig 2C - TGFB T- looks v similar to ionomycin T48h if you look at the E-Cad and Vimentin protein levels - how much can we trust that the protein levels seen in ionomycin 24-72 is downstream of calcium signaling, as the levels look v similar to TGFbeta at T0

Response: We agree that a better characterization of the molecular changes accompanying ionomycin-induced plasticity would further our understanding of the differences between C-EMT and P-EMT. Therefore, we have expanded our study to include additional epithelial (Claudin-7, Mucin-1, and Tight Junction Protein 1) and mesenchymal markers (N-cadherin and CD44) at both the protein and RNA level. As shown in a **new Fig. S2H**, these data highlight the simultaneous expression of both epithelial (ECAD/MUC1) and mesenchymal (CD44, NCAD) markers in association with the calcium-induced P-EMT phenotype.

We appreciate the Reviewer's careful evaluation of our western blots. The cell lines being used are highly aggressive murine pancreatic tumor cells that are primed to undergo P-EMT *in vivo* (Aiello et al. 2018). Therefore, within the culture there are cells that contain low levels of vimentin as seen in the western blot in T0. While we cannot explain precisely why there is more vimentin in T0 for TGFβ besides the fact that these are non-clonal cell lines, we have repeatedly seen that Vimentin mRNA is increased upon addition of ionomycin in both mouse (RNA sequencing data in Fig. 3A) and in human cell lines (Fig. S2G).

2. Fig 2D - cell movement - is there any statistical difference between ionomycin and TGFB in the cell movement and transwell migration assays? Could you also comment on how the cells move in these assays, as P-EMT has been associated more with collective rather than individual migration

Response: We have now included a statistical analysis which supports a difference between the degree of movement induced by ionomycin vs. TGFβ (Fig. 2E). Cell movement, when assayed by live imaging in 2-dimensional space, follows a general pattern of single cell migration (regardless of EMT type or stimulus). Assessment of collective vs. single cell migration can be better assessed by 3D and in vivo experiments. This will be performed in the future but falls outside of the scope of the current manuscript.

3. Are any GPCR receptors unregulated in the in vivo transcriptome data of C-EMT versus P-EMT? - it would be interesting to include this data, as it would strengthen the case for a role of GPCR signaling in vivo.

Response: Fig. S5 shows all GPCRs that are differentially expressed between C-EMT and P-EMT.

Referee #3: Reviewer comments to the author

In this very interesting work Norgard and colleagues report about the role of calcium signals and calmodulin in the EMT of pancreatic cancer cells. The authors suggest that elevation of cytosolic calcium causes partial EMT without an involvement of the calcium dependent calcineurin-NFAT axis.

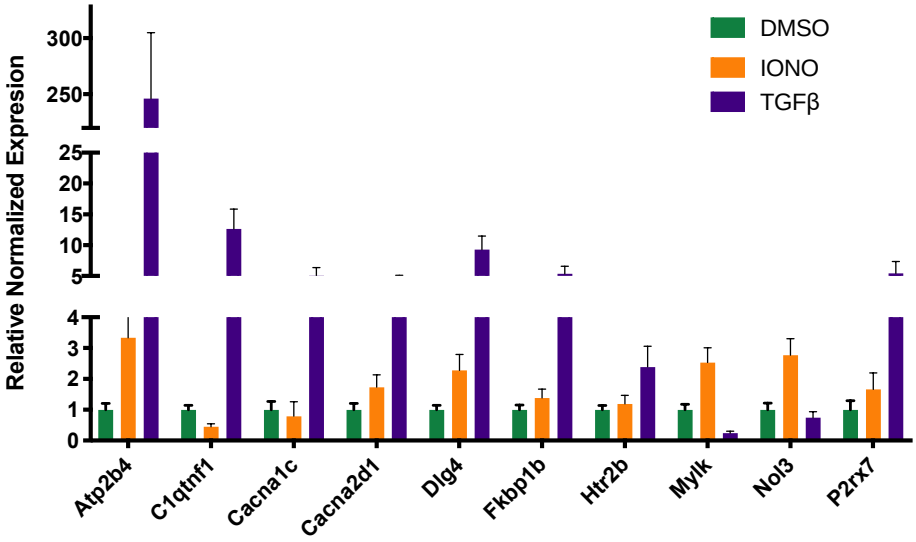
This study is based on a previous publication of the working group (Aiello et al 2018) wherein the authors used RNA-seq analyses to evaluate differences between two EMT cell subtypes, characterized by surface expression of e-cadherin (ECAD. By means of pathway analysis the authors found that in ECAD negative cells genes coding for proteins involved in cellular calcium homeostasis are enriched compared to ECAD positive cells. Based on this finding, the authors hypothesized and later confirmed that calcium is an important regulator of EMT transition.

I have several comments and suggestions that the authors might want to consider when revising this work:

1. Although it is clear that calcium genes are relevant for EMT, the authors did not provide identity of single hits whose regulation is highest. This is important because for example a gene could be upregulated in ECAD negative cells but its function could be inhibitory and would thus lead to reduced calcium levels in the cytosol. For example, upregulation of the PMCA in the PM would have such an effect. Moreover, the differential expression should also be validated on a protein level (WB and IHC for example) and the functional role of the top hits should be confirmed by gene manipulation (up- or downregulation). This is important in order to relate the current findings with many studies, which reported in the past that Ca2+ channels belonging to the CRAC, TRP and IP3R families control cancer cell invasion and growth.

Response: We appreciate the desire to confirm sequencing results. However, in this instance, we mined previous data from our publication in 2018, and regretfully no longer have sufficient material to confirm our *in vivo* experiments. We have now selected the top calcium regulated genes from GSEA and confirmed them by RT-qPCR after treatment with ionomycin. These data are provided for the Reviewer (below) and can be included as supplementary data if desired. Significantly, most of these genes were also induced upon TGFβ treatment, making it less likely they are involved in a P-EMT-specific mechanism. These data support the notion that an upregulation of these

channels may regulate cancer cell invasion and growth. While we cannot specifically link the P-EMT program to any specific calcium channel, we believe that our new data on Camk2b (**new Fig. 4E, Fig. S4E-F**) provides additional mechanistic insights into P-EMT.



2. Given that the examined cells express a number of Gq-coupled receptors, the authors should try to identify a more physiological stimulus to induce calcium elevation in their cells. Ionomycin is an ionophore and can thus have many unspecific effects. To this end, the observation that ATP-triggered calcium transient is not causing similar effects as ionomycin or CNO on ECAD surface expression warrants further investigation given that calmodulin would very likely be activated by such cytosolic calcium elevations.

Response: The identification of a more physiological stimulus is also a question of great interest to us. In our previous Fura-2 experiments, we tried several ligands (Trypsin, Acetylcholine, TGFβ, ATP, and EGF) to elicit *transient* calcium responses. Of these, only ATP elicited a calcium response (included in the manuscript, Fig. 4A) which, as noted by the Reviewer, was not capable of elucidating a P-EMT. By contrast, sustained elevations in intracellular calcium were achieved by ionomycin treatment and prolonged CNO exposure in the DREADD experiments, and these resulted in a P-EMT. (Of note, transient exposure to CNO elicited a transient calcium signal and no EMT response). Collectively, these results strongly suggest that the calcium-associated P-EMT program requires a sustained increase in intracellular calcium (and sustained calmodulin/Camk2b activity).

In an effort to identify other physiological stimuli capable of eliciting a P-EMT, we scanned the list of differentially expressed Gαq-GPCRs (Fig. S5) and tested several candidates (GPCRs with known ligands that were commercially available). While some of these experiments gave promising results, the findings were not reproducible between cell lines, suggesting that one GPCR may be sufficient in one setting while

another may be in a different context. Importantly, all of these GPCRs ultimately lead to downstream Gαq activation. These studies are what led us to suggest in the manuscript that there may not be a single GPCR (or other stimulus) prompting a sustained increase in intracellular calcium, leading to P-EMT, but rather that several stimuli may act in concert to raise calcium levels above some threshold for a sufficient period of time. This concept is raised on page 15-16 of the Discussion.

In our revised manuscript, we have performed a plasticity experiment where we expose cells to ionomycin for 7 days and then withdraw the drug for 7 days (Fig. 2D). Cells revert to their pre-treatment epithelial state within a day of ionomycin withdrawal, providing further evidence of the importance of sustained elevations of intracellular calcium in P-EMT and providing an explanation of why transient increases induced by ATP might be insufficient.

3. Performing the study in murine cancer cells reduces the clinical relevance of the findings. The authors confirmed the main findings in other human cells but calcium signaling networks and gene expression patterns of murine and human cells are rather different.

Response: We agree and have extended our results in human carcinoma lines and are presented in a **new Fig. S2H**

4. It would be interesting to examine how drug sensitivity would be affected in cells in which the reported calcium-calmodulin axis is inactivated.

Response: While we agree this is an interesting question, we believe such studies would need to be done in a comprehensive manner to be interpretable (e.g. how many and which drugs should be examined). While we do intend to perform such experiments in the future, we believe they are beyond the scope of the current report.

5. Why is the n for ECAD negative cells so low in Fig 1D compared with the ECAD positive cells? The huge differences might affect the conclusions.

Response: As noted in this and other publications, cellular plasticity occurs spontaneously in culture (i.e. cells are in an equilibrium between the epithelial and mesenchymal state). In the cell line used for Figure 1, approximately 5% of culture is mesenchymal at steady state, thus accounting for the much smaller fraction of ECAD negative cells shown. However, we have confidence in the robustness of the result, as the data are highly significant, and the experiment has been repeated several times across different cell lines as indicated.

6. The n values in Fig. 1C, right panel, are missing.

Response: To avoid confusion, we have moved the n values to the figure legend for both Fig. 1C and 1D.

7. The PCA findings discussed on page 10 are shown in Fig. S3E and not in Fig. 3E.

Response: This has been corrected.

1st Revision - Editorial Decision 19th Mar 2021

Dear Dr. Stanger,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, referee #2 now supports the publication of your study in EMBO reports, whereas referees #1 and #3 have remaining concerns or suggestions to improve the manuscript we ask you to address in a final revised manuscript. After referee cross-commenting, we do not think it is necessary to provide additional mechanistic insight as requested by referee #3. Please address the remaining points by referee #1 either with further experimental data or in a detailed point-by-point response.

I also have these editorial requests I ask you to address:

- We plan to publish your manuscript in the Report format. For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details please refer to our guide to authors: http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

- Please remove the statement on word count and content from the title page and move the COI statement and the author contributions down next to the acknowledgements. Please order the sections like this: Title page - Abstract - Introduction - Results and Discussion - Materials and Methods - DAS (data availability section) - Acknowledgements - Author contributions - Conflict of interest - References - Figure legends - Expanded View Figure legends - Expanded View Table legends.

- Please combine the 'abstract' and the paragraph 'significance' into one paragraph named 'abstract' with not more than 175 words.

- Please name the data availability paragraph 'Data Availability' and add the respective URL link to the GEO dataset.

- Please make sure that all the funding information is entered into the online submission system and is complete and similar to the one in the manuscript text file.

- Please name the three EV tables 'Table EVx' and add a legend for them after the EV figure legends. Please also change their callouts in the manuscript text.

- Please name the three movie files 'Movie EVx' and change their call-out in the manuscript text. Please ZIP these files together with the respective legend as a text file and upload the combines ZIP file, one for each movie. Please remove their legends from the article file.

- For the microscopic images, please add scale bars of similar style and thickness to all the microscopic images (presently Figs. 2A and EV4G are missing scale bars), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- As they are significantly cropped, could you provide the source data for the few Western blots

shown in the manuscript (including the EV figures)? The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

- Per journal policy, we do not allow 'data not shown' (see pages 6 and 9 of your manuscript). All data referred to in the paper should be displayed in the main or Expanded View figures, or an Appendix. Thus, please add these data, or (if these are not essential) remove the 'data not shown'. See:
http://www.embopress.org/page/journal/14693178/authorguide#unpublisheddata

- Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (se also below). Please provide error bars and statistical testing where applicable.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

In the revised version of the manuscript the authors addressed some of the initial concerns. However, several questions remain incompletely answered. If these questions are properly addressed, the manuscript should be considered for publication in EMBO reports :
1. The first question was referring to the mouse cells (" The authors claim that calcium mobilization promotes partial EMT. However, this claim in based on the decrease of surface E-cadherin and no proper characterization of the expression of epithelial and mesenchymal markers that would support the presence of partial EMT state (in which cells simultaneously express epithelial and mesenchymal markers). The same mouse model was previously used by the same group in 2018. However, a more extensive characterization of EMT programs would be required to better

substantiate what are the transcriptional and not transcriptional programs regulated by the increase in calcium influx"), while the second question is referring to human cell lines. The authors perform some additional characterization of human cells lines but no additional characterization was performed for mouse cell lines. The authors make a reference to the Figure 3a and 3f that refer to RNA-seq and exosome analysis (respectively). It would be important to validate by IF or WB the observations found by RNA-seq to properly illustrate the hydrid EMT state (for markers such as Krt15, Vim, Twist1, Snai2, Prrx1, CD44...that seem to be differentially regulated on their RNA-seq data).
2. Decoupling the calcium signaling from E-cadherin loss is indeed difficult. However, the overexpression of E-cadherin in the cell line that would restore the adherens junctions between the cells and treatment with Iono could potentially bring some insights.
3. The authors performed additional experiments to answer to the question 6 ("Is this partial EMT state stable? What will happen if these cells are treated with TGFbeta? And upon subcutaneous grafting?"). The authors now provide the data on the E-cadherin dynamics upon treatment of cells with Iono or Tgfb. However, this question intended to go one step further, and to understand whether the partial EMT state that is generated upon increased Calcium influx is stable or whether further EMT can be induced upon certain conditions. The experiment that would be useful to address this question, would be to compare Iono vs Iono+Tgfb treatment, and to analyze several epithelial and mesenchymal markers (Krt15, Krt19, Muc1, Cdh1, Cldn2, Twist1, Snai2, Prrx1, CD44), not only the evolution of E-cadherin.
4. Finally, the authors perform interesting experiments showing that Camk2b KD prevents the loss of E-cadherin upon Iono treatment. From these data the following question arise: how other epithelial and mesenchymal markers evolve upon CAMK2B KD? How specific this effect is for Camk2b, what happens upon Camk2alpha or Camk2gamma KD?

Referee #2:

The authors have submitted a heavily revised version of their manuscript, containing a substantial amount of data in answer to all reviewers comments, including my own. They have thoroughly addressed my comments, and I strongly support publication in EMBO without further revision.

Referee #3:

In the new revised version, the authors address some, but not all of the points raised by the reviewers. Accordingly, several important questions remain unanswered or not fully clarified. The paper as a whole is improved, however, the newly obtained data is in my opinion not sufficient to fully overcome the critics raised regarding the more detailed examination of 1) the molecular mechanisms and players linking alterations in cytosolic calcium and EMT and 2) the pathophysiological relevance of the study.
Taken together, this remains an interesting study. However, taking into consideration 1) that the role of calcium in EMT has been described before in many studies 2) the lack of physiological stimulus that leads to calcium elevation that can activate the Gq signaling cascade and induce the described cellular phenotype and 3) the fact that the critical molecular players are still not identified i.e. validated, I unfortunately cannot recommend publishing of this work in its present form. In my opinion, additional work, based on the not addressed comments from the first revision is required.

The following is a detailed point-by-point response to the Reviewers' comments (new data or substantive changes to the text listed in **boldface**):

Referee #1: Reviewer comments to the author

In the revised version of the manuscript the authors addressed some of the initial concerns. However, several questions remain incompletely answered. If these questions are properly addressed, the manuscript should be considered for publication in EMBO reports:

1. The first question was referring to the mouse cells (" The authors claim that calcium mobilization promotes partial EMT. However, this claim in based on the decrease of surface E-cadherin and no proper characterization of the expression of epithelial and mesenchymal markers that would support the presence of partial EMT state (in which cells simultaneously express epithelial and mesenchymal markers). The same mouse model was previously used by the same group in 2018. However, a more extensive characterization of EMT programs would be required to better substantiate what are the transcriptional and not transcriptional programs regulated by the increase in calcium influx"), while the second question is referring to human cell lines. The authors perform some additional characterization of human cells lines but no additional characterization was performed for mouse cell lines. The authors make a reference to the Figure 3a and 3f that refer to RNA-seq and exosome analysis (respectively). It would be important to validate by IF or WB the observations found by RNA-seq to properly illustrate the hydrid EMT state (for markers such as Krt15, Vim, Twist1, Snai2, Prrx1, CD44...that seem to be differentially regulated on their RNA-seq data).

Response: We regret the misunderstanding. To extend our RNA-seq and exosome analysis of mouse cells (Figure 3A and EV3F) and flow cytometric analysis of surface protein levels in human cells (Figure EV2H), we performed additional characterization of several epithelial and mesenchymal proteins in ionomycin- and TGFβ- treated murine tumor cells by western blot. **These new data, included as Figure EV3H, validates our conclusion that the epithelial program is preserved at the protein level following ionomycin-induced P-EMT (but not in TGFβ-induced C-EMT).** We also sought to test other markers as suggested by the Reviewer. However, we found that most of the antibodies we purchased failed to exhibit good reactivity against mouse proteins (additional details can be provided on request). Therefore, we are unfortunately unable to provide additional data characterizing the phenotype beyond those included in our new Figure EV3H.

2. Decoupling the calcium signaling from E-cadherin loss is indeed difficult. However, the overexpression of E-cadherin in the cell line that would restore the adherens junctions between the cells and treatment with Iono could potentially bring some insights.

Response: We appreciate the Reviewer's acknowledgement that decoupling calcium signaling from ECAD loss is difficult. Unfortunately, all our tumor cell lines at baseline

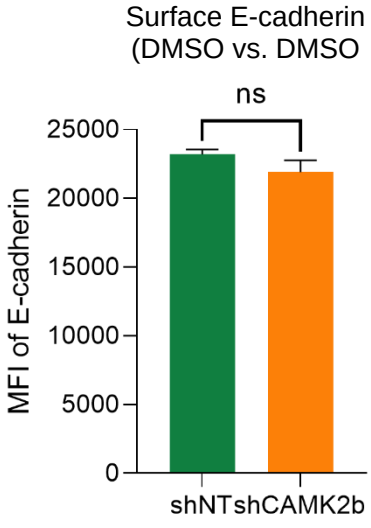
are >95% ECAD high by flow cytometry, and even those that undergo P-EMT still possess abundant ECAD (albeit retained intracellularly). Hence, we respectfully submit that ECAD overexpression in these cells would provide limited additional insight and not change the major conclusions of our study.

3. The authors performed additional experiments to answer to the question 6 ("Is this partial EMT state stable? What will happen if these cells are treated with TGFbeta? And upon subcutaneous grafting?"). The authors now provide the data on the E-cadherin dynamics upon treatment of cells with Iono or Tgfb. However, this question intended to go one step further, and to understand whether the partial EMT state that is generated upon increased Calcium influx is stable or whether further EMT can be induced upon certain conditions. The experiment that would be useful to address this question, would be to compare Iono vs Iono+Tgfb treatment, and to analyze several epithelial and mesenchymal markers (Krt15, Krt19, Muc1, Cdh1, Cldn2, Twist1, Snai2, Prrx1, CD44), not only the evolution of E-cadherin.

Response: **New data showing the effects of treatment with ionomycin and TGFβ, either alone or in combination, are shown in a new Figure EV3H-I.**

4. Finally, the authors perform interesting experiments showing that Camk2b KD prevents the loss of E-cadherin upon Iono treatment. From these data the following question arise: how other epithelial and mesenchymal markers evolve upon CAMK2B KD? How specific this effect is for Camk2b, what happens upon Camk2alpha or Camk2gamma KD?

Response: In our initial revision of this manuscript, we included new data to show that *Camk2b* KD reduces the ability of cells to undergo ionomycin-driven partial EMT. While this manuscript has been under review, a newly published article showed that Camk2 is an essential component of partial EMT in squamous cell carcinomas (Pastushenko/Mauri et al., 2021 *Nature*; PMID: 33328637). However, we would like to clarify that in our study as well as in the above-mentioned *Nature* paper, *Camk2b* knockdown alone in the absence of ionomycin stimulation was insufficient to prompt a change in epithelial-mesenchymal state. As shown below (provided for the Reviewer and Editor), *Camk2b* loss did not result in enhanced surface ECAD levels on its own.



Importantly, **we only saw changes in *Camk2b*, but not *Camk2alpha* or *Camk2gamma* transcripts in our RNA-seq, and thus focused on *Camk2b*. We have added these data to the revision to clarify this point (Figure EV4E).**

Referee #2: Reviewer comments to the author

The authors have submitted a heavily revised version of their manuscript, containing a substantial amount of data in answer to all reviewers comments, including my own. They have thoroughly addressed my comments, and I strongly support publication in EMBO without further revision.

Response: We thank the Reviewer for these thoughtful comments and for noting our responsiveness to comments from all the Reviewers.

Referee #3: Reviewer comments to the author

In the new revised version, the authors address some, but not all of the points raised by the reviewers. Accordingly, several important questions remain unanswered or not fully clarified. The paper as a whole is improved, however, the newly obtained data is in my opinion not sufficient to fully overcome the critics raised regarding the more detailed examination of 1) the molecular mechanisms and players linking alterations in cytosolic calcium and EMT and 2) the pathophysiological relevance of the study.

Taken together, this remains an interesting study. However, taking into consideration 1) that the role of calcium in EMT has been described before in many studies 2) the lack of physiological stimulus that leads to calcium elevation that can activate the Gq signaling cascade and induce the described cellular phenotype and 3) the fact that the critical molecular players are still not identified i.e. validated, I unfortunately cannot recommend publishing of this work in its present form. In my opinion, additional work, based on the not addressed comments from the first revision is required.

Response: (1) We respectfully disagree that the role of EMT has been described before in many studies. While we agree that calcium has previously been implicated in the EMT process, previous associations were indirect and lacking functional support. If we have overlooked important references in this regard, we would be happy to include them in the manuscript. Moreover, our study demonstrates a specific role for calcium signaling in partial EMT, a hybrid state whose molecular underpinnings are particularly obscure.

(2/3) While it is certainly our long-term goal to identify physiological stimuli that can activate a P-EMT-inducing Gq signaling cascade, such an effort that will likely take years of additional research. As we note in the manuscript, it is possible or even likely that several GPCR ligands may act in concert to bring calcium levels above the threshold needed to initiate P-EMT, which would make the identification or validation of a single activator particularly difficult. In addition, we note that no physiological stimulus has been identified or confirmed for the EMT programs induced by treating cultured cells with TGFβ, which is the most widely studied *in vitro* model of EMT. Nonetheless, in other recently published work from our group (Pitarresi et al., 2021. *Cancer Discovery*), we showed that the secreted factor PTHrP binds to its cognate receptor PTH1R and drives EMT and metastasis in pancreatic cancer. Although we did not go into functional downstream mechanism in that paper, others in the field have demonstrated that binding of PTHrP to PTH1R activates Gq signaling, thus releasing intracellular Ca^{2+} stores. The molecular mechanisms driving EMT by PTHrP-PTH1R will be pursued in other follow-up studies beyond the scope of this manuscript.

In summary, given the paucity of knowledge regarding molecular mechanisms driving partial EMT, and the fact that our hypothesis derives from a physiologically relevant (i.e. autochthonous) cancer model, we believe that the molecular mechanisms of the P-EMT program laid out in our manuscript represent a substantial advance in the field.

2nd Revision - Editorial Decision 21st Jun 2021

Ben Stanger
University of Pennsylvania
Medicine
BRB II/III, Rm 512
421 Curie Boulevard
Philadelphia, PA 19104
United States

Dear Dr. Stanger,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the report from the referee that was asked to re-evaluate your study, you will find at the end of this message. As you will see, the referees now fully supports the publication of your study in EMBO reports.

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

Below, I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: 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."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

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

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2020-51872V3 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Referee #1:

The manuscript is suitable for publication in EMBO reports without further revision.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ben Z. Stanger
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51872-T

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 $\chi 2$ 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?	All experiments were run in at least duplicate with multiple cell lines to ensure proper power and adequate, interpretable results.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Statistical analysis of multiple comparisons was performed using ANOVA with Tukey's multiple comparison test, and comparisons between two groups were performed using Students' unpaired t-test. All statistical analyses were performed with Graphpad Prism 8. Error bars show standard deviation (SD) or standard error of the mean (SEM) as indicated. This is indicated where/when necessary.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Before analyzing data, a normality test is always performed through PRISM software.
Is there an estimate of variation within each group of data?	Yes, either SD or SEM is shown and indicated in the figure legends.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
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

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).	AntibodiesApplicationSourceCat # anti-E-cadherinWestern, IFTakaraM108 anti-GAPDHWesternCell Signaling Technology2118
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Murine PDA cell lines PD7591 (female), PD798 (male), PD483 (female), PD3077 (male), PD454 (male), PD883 (male) were derived from primary KPCY tumors of mixed genetic background. All human cell lines (Capan2, MCF7, HCC827) were obtained originally from ATCC. PD6419 was derived from C57BL/6 KPCY tumors. Cell lines were regularly tested for mycoplasma using MycoAlert Mycoplasma Detection Kit (Lonza).

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All sequencing data has been deposited in the Gene Expression Omnibus (GEO): GSE157892.
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).	All sequencing data has been deposited in the Gene Expression Omnibus (GEO): GSE157892.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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