EMBO reports

Splicing factor *ESRP1* controls ER-positive breast cancer by altering metabolic pathways

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

1st Editorial Decision 12 April 2018

Thank you for the submission 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, all referees think the manuscript is of interest, but requires a major revision to allow publication in EMBO reports. All three referees have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. As the reports are below, I will not detail them here. We feel, however, that in particular the points of referee #1 need attention (that partially overlap with the concerns by the other referees):
- to monitor ESPR1 expression and knock down efficiency on the protein level
- to monitor whether hormone sensitivity was restored by the knockdowns
- to provide evidence that EMT processes have been changed by depleting ESPR1
- to provide confirmatory data for the RNA-seq analyses
- to analyse the metabolic changes statistically

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 and/or in a 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 or rejection 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; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

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, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature.

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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. See:
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- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

Please also note that we now mandate that all corresponding authors list an ORCID digital identifier that is linked to their EMBO reports account.

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.

REFEREE REPORTS

Referee #1:

This paper produces novel findings on the role of epithelial splicing regulatory proteins 1 and 2, RNA binding proteins that are known to control epithelial to mesenchyme transition in mediating

resistance to hormone therapy in breast cancer cell line models. They show from existing online datasets that ESRP1 is associated with a poor prognosis in ER positive patients but they do not analyse whether the patients were actually treated with endocrine therapy or not, they just show a worse prognosis.

The change of ESRP1 in 2 cell lines that are fulvestrant resistant and tamoxifen resistant is shown on the RNA level. However, these are not analysed at protein level. Similarly, the knockdown is not validated at protein level either. There are clear effects on colony growth as a result of this knockdown, although no changes in cell cycle or apoptosis. The suppression of growth in vivo is highly convincing.

It would be of interest to know whether hormone sensitivity was restored by the knockdowns. There is no evidence of an EMT process being changed by ESRP1 in these experiments, so no strong evidence is produced as to how ESPR1 might function. The detailed analysis of the epithelial splicing programme shows many are changed in both cell lines by the knockdown.

The EMT splicing signature is studied, a previously published one, but as there is no effect on the EMT of endpoint, this does not seem to be relevant. Going back to human tumour databases, they can show evidence for some of the genes in the EMT splicing programme are also regulated in ESRP1 upregulated cancers, although, as they state clearly, it is not sufficient to induce an EMT phenotype in this breast cancer subtype, so there is no real explanation as to how this ESPR1 knockdown functions.

However, they do try to go on to investigate further pathways and investigate the genes in common between the two resistant cell types. They noted clusters with fatty acid metabolism and lipid metabolism and oxireductase processes, amongst others.

They then analysed using standard techniques, with the XF Extracellular Flux analyser, major energy producing pathways of mitochondria and glycolysis. They claim that the rates were changed in figures 7C and D and 7E, but there is no statistical analysis in the text, nor in the figure or supplementary figures.

Figure 1, it would be much easier to follow if the groups of patients in EFGH were defined as in the A ,B, C and D figures. Also, the number of patients in each arm to have some idea of reliability of the data, for example, the figure 1H shows a clear split after 4 years, but it is not significant probably, it could be small numbers and variation of statistics. Numbers would be helpful to understand this.

Figure 2A, it is not clear what is being compared with what to obtain the statistical significance. The bar should be shown between the comparisons.

Figure 2E and F, knockdown clearly effects the colony growth of the resistant cell lines, but what about the effect on the control cell lines. If they are effected equally, this is not a selective effect of ESR0P1 knockdown.

Figures 2G and H, why was supplemental oestrogen given for these cells, are they are still oestrogen sensitive, although tamoxifen resistant? Controls should be done of the parent cell line also.

Figure 3C, it is well recognised there is a partial EMT transition possible and more markers should be studied. Additionally, it does looks like CDH1 is actually increased in the ESRP1 knockdown, as does SLUG and ZEB2 in the 9C2, whereas they go down in 9C3, so I agree that there is no consistent change, but it needs to be a bit more thorough as there are clearly some effects.

Figure 7, C and D should have statistical comparison shown on the graphs.

Overall, therefore, there is no confirmatory data other than using RNA sequence, normally one would expect to have done some splicing assays or more detailed evaluation of transcripts, specifically by PCR, so that one could try and confirm the RNA sequencing data, which is known to have about a 20% error rate. There are no protein assessments, there is no expected biological effect on EMT and the metabolic changes are unconvincing and not statistically analysed.

Referee #2:

ER+breast cancer represents the most common form of the disease and is amenable to endocrine therapy. However, about 20% of these cancers will recur, with resistance to endocrine therapies and thus necessitating the development of new treatment approaches. The authors investigated the role of ESRP1 and ESRP2, factors that control Epithelial to Mesenchymal Transition (EMT) splicing in breast cancer recurrence.

Analysis of publically available data showed that high levels of ESRP1 are associated with poor prognosis in ER-positive (ER+) breast tumors, which prompted the authors to carry out stable knock-down experiments in relevant (ER+) breast cancer cell line models that are resistant to tamoxifen and fulvestrant and compared to a sensitive cell MCF-7.
RNA-seq of the knockdown cell vs controls showed not only differentially expressed, but also differentially spliced transcripts, a proportion of which also carried ESPR1 binding motifs either downstream of an intron inclusion or upstream of intron exclusion event. A number of these genes are indeed part of an EMT signature. The authors also show that the knockdown of ESPR1 has an effect on colony formation ability and cell growth in vitro and in vivo. Further analysis of the splicing data and comparison to publically available datasets confirmed the presence a proportion of the splicing isoforms in the ER+ TCGA dataset. Finally, the authors perform experiments using the ESPR1 knock-down cells to show that the knock down has an effect in glycolysis and fatty acid oxidation, representing a novel link between ESPR1 and metabolism.

The work provides valuable insights into the role of ESPR1 in EMT and drug resistance and disease progression but does not provide a mechanism of action or any direct link between splicing/isoform expression and the observed differential gene expression data.

Points:

1. The link between splicing and the effects of ESPR1 on tumor growth and cell metabolism are not clear. Which transcripts are spliced and directly involved in these processes? Or which regulator thereof? Do we have prime candidates?
2.ARHGEF11 is one of the prime ESPR1 associated splicing targets: What is the putative functional effect of exon 39 inclusion? How does it relate to the data showing that exon 38 inclusion (as apparent in Figure 6 B) is linked to migration and growth in breast cancer cells (Oncotarget. 2017 Nov 3; 8(54): 92157-92170)? What exactly is the effect of exon 39 inclusion on the AS sequence?
3. In Figure 6 we have a summary of validated (publically available data) isoforms of five transcripts and for two of them we the AS sequence and region of interest information. The authors need to clearly include the AS sequence of the isoforms and the predicted effects on functional domains.
4. Metabolic substrate flux analysis: The authors to include glucose uptake and lactate production data in order allow broader comparisons to other published work.
5. An ESPR1 overexpression experiment should be included in order to confirm its opposite effect on glycolysis for example.
6. A clear summary on any splicing events affecting transcripts for proteins related to glycolysis or fatty acid oxidation should be presented, possibly in supplementary data.

Minor points:
1. For the TCGA splice-seq analysis can the authors provide more details on how they performed the analysis and a bit more detail on their results. For example, can they report in how many of the 100 Luminal A ESPR1high and 100 Luminal B ESPR1low samples the reported splicing events are found. Are the reported splicing events (in the text and in Figure 6) more frequently found in one of the two groups or maybe are the splicing values different in one group relative to the other.

2. In the paragraph "Novel functional role of ESRP1 knockdown on endocrine resistant breast cancer; impact of ESRP1 on metabolic pathways" the differentially expressed genes between the control and the knockdown cell lines based on microarray data should be present in the differentially expressed genes found in the RNA-seq data for the same conditions. The RNA-seq data were

presented in the first paragraphs of the manuscript. Can the authors comment on how many of the presented significant genes from the microarray platform are found in the significantly deregulated gene lists from the RNA-seq data.

3. In Figure 4B-E, in the radar plots can the authors report what the indicated numbers (in the hypothetical axis) correspond to.

4. In Figure 5 why some exons and internal/external junctions are colored green and some are colored red.

5. In the legend of figure 6 the sentence: "A splice graph of the gene's exons is shaded based on expression level and shows the selected splice event outlined in red. (B) TCGA Splicegraphs for the EMT signature genes in ESRP1high versus ESRP1low tumors." maybe is less confusing if it is reorganized as "(B) TCGA Splicegraphs for the EMT signature genes in ESRP1high versus ESRP1low tumors. A splice graph of the gene's exons is shaded based on expression level and shows the selected splice event outlined in red."

6. In Figure 7C and 7D can the authors make the figures a bit more organized. For example, in figure 7C the green and blue boxes in the legend are presented 2 times one as 2-control/2C3 and the other as Control/Experimental. Also, can the authors provide more details for the different conditions that are indicated. For example, what do baseline, stressed, glycolysis and glycolytic reserve represent. Not all readers are familiar with the presented concepts. Maybe more details in the legend will be helpful.

7. Figure 5 needs more detailed explanation and is hard to read in detail, as the resolution is not high enough.

Referee #3:

In the current manuscript by Gökmen-Polar and colleagues, the authors addressed how endocrine-resistant ER+ breast cancer is driving disease recurrence by utilizing regulation of differential splicing. They identified that high ESRP1 expression, unlike ESRP2, is correlated with poor survival, particularly in ER+ breast cancer patients. To confirm this finding they further analyzed the role of ESRP1 in MCF7 derived cells which have been selected for drug-resistance. Knockdown of ESRP1 resulted in reduction of colony forming capacity and decreased tumor growth upon xenografting. Interestingly, in contrast to previous reports, in ER+ cancer cell lines ESRP1 is not involved in regulating an EMT program. Specific EMT-specific genes are not consistently changing upon ESRP1 loss. Similarly, also global gene expression was not deregulated in specific EMT-related gene signatures. In contrast the authors found that ESRP1 is regulating differential splicing of a variety of transcripts and knockdown of ESRP1 results in deregulation of many genes involved in fatty acid metabolism. This regulation induced a shift in glycolysis and the glycolytic reserve of the cells, which is suggested as putative target for novel therapy approaches.
With different experimental approaches and state of the art methodology the authors dissected the role of ESRP1 in ER+ breast cancer. They identified a novel aspect of ESRP1 function that specifically in ER+ breast cancer cells is regulating fatty acid metabolism which increases tumor relapse, invasion and metastasis. In ER+ cells ESRP1 is presumably not promoting an epithelial cell phenotype with reduced invasion and metastasis as was observed in other reports. This novel finding is very interesting and supports the notion that differential splicing is regulated in a complex manner, largely dependent on the cell type and cell of origin in cancer.

Nevertheless, I have a few concerns that need to be addressed before the manuscript can be accepted for publication:

1. ESPR1 expression was never analyzed on protein level. How is the expression changing during established endocrine-resistance, is it properly depleted upon knockdown? In line with this: are ESRP1 and 2 always coregulated, what is the level in the analyzed cell lines? Would a double knockdown have a more drastic effect especially on metbolic changes?
2. Although the results have been verified in TCGA samples, to confirm the findings, key

experiments should be carried out with another cell line, especially since all analyzed cell lines are derived from MCF7. In addition, a cell line with low ESRP1 levels should be included to overexpress ESRP1 and observing opposite effects
3. In MCF7 cells it was shown that ESRP1 knockdown results in EMT and upregulation of ZEB1 and ESRP1-loss prevents MCF10A cells to undergo TGFb-induced EMT (Preca BT, 2015, IJC). How do the authors explain this controversy? Did they analyze the effect of ESRP1 knockdown in the parental and non-resistant MCF7 cell line as well with different results?
4. Fig. 7C-D: The middle panel of the left graphs in C and D is not labeled, it is not clear what is shown? Apparently, the detected changes in ECAR and OCR are not significant. So are they of biological relevance? In particular for 9-control and 9c3 no difference is observed or is only marginal. How do the authors explain this?
5. Fig. 7E and p. 12: The authors claim to observe "decreased expression of FASN and Stearoyl-CoA desaturase 1 (SCD1) in knockdown cells". However, I do not see this in the figure, especially not for LCC9 cells and it is unclear how the authors came to that conclusion. Quantification of the blots should also be provided!
6. Fig. 1E: There is an unexpected switch in colors of the column : why is 'ESRP1 high' now shown in red? This is confusing!
7. Fig. 2: The qRT-PCR data are shown in an unusual way which makes it difficult to follow. I suggest to display them as relative expression levels/fold changes.
8. Fig. 2E-F: Something is wrong with the labeling in this figure: the legend indicates that 'control' is shown in blue, which shows reduced colony formation capacity. I guess the color labeling is switched.
9. Fig. 3E: I am a bit puzzled about the finding about the cell differentiation in 2D culture. How can they form more glandular-like structures in 2D? If there are phenotypic differences that change from a more mesenchymal to a more epithelial appearance, the cells need to be plated in much lower densities. The cells in the figures shown here are 90-100% confluent!
10. How are the well-described targets of differential splicing of ESRP1 regulated in this cellular system, like CD44, p120 or FGFR1? Are they not differentially spliced which may indicate a cell-type specific function of ESRP1?

1st Revision - authors' response 24 August 2018

Response to Reviewers:

Referee #1:

This paper produces novel findings on the role of epithelial splicing regulatory proteins 1 and 2, RNA binding proteins that are known to control epithelial to mesenchyme transition in mediating resistance to hormone therapy in breast cancer cell line models. They show from existing online datasets that ESRP1 is associated with a poor prognosis in ER positive patients but they do not analyse whether the patients were actually treated with endocrine therapy or not, they just show a worse prognosis.
We thank to the reviewer for this point. We revised the figure and added the information of ESRP1 levels with clinical outcome (overall survival) treated with tamoxifen or chemotherapy. The following statement is included in the manuscript.

Pages 5-6- lines 113-125
We further assessed the correlation of ESRP1 expression with overall survival in tamoxifen-treated patients using the same platform at BreastMark database. High expression of ESRP1 was associated with shorter overall survival in ER+ tumors treated with tamoxifen [(Hazard ratio (HR) = 5.021 (2.434 - 10.36) Score (logrank) test = 23.55 on 1 df, p=1.218e-06 (n= 210, number of events= 49); **Fig 1C**)]. On the other hand, the overall survival was independent of ESRP1 expression in patients treated with chemotherapy alone (Hazard ratio (HR) = 1.599 (0.6773 - 3.773) Score (logrank) test = 1.17 on 1 df, p=0.28 (n= 129, number of events= 21); **Fig 1D**]. The effect of ESRP1 levels in patients treated with combination of tamoxifen and chemotherapy was not significant (data not shown), probably due to the small numbers in the cohort.
Analysis of The Cancer Genome Atlas (TCGA)[12] cohort also revealed that high ESRP1 expression was associated with significantly shorter overall survival in ER+ breast cancer patients

(n= 100, number of events= 17;P=0.00011; **Fig 1E**), but not in ER- cases (P=0.19; **Fig 1F**). The limitation of the TCGA dataset was that the treatment status was not available for all cases.

The change of ESRP1 in 2 cell lines that are fulvestrant resistant and tamoxifen resistant is shown on the RNA level. However, these are not analysed at protein level. Similarly, the knockdown is not validated at protein level either. There are clear effects on colony growth as a result of this knockdown, although no changes in cell cycle or apoptosis. The suppression of growth in vivo is highly convincing.
Thanks for raising this point. We have included protein levels of ESRP1 in tamoxifen (2-control) and fulvestrant (9-control)-resistant cell lines as well as in ESRP1 knockdown cell lines (2C3 and 9C2, respectively). Using Western blot, we have successfully shown that ESRP1 levels have been significantly reduced in knockdown cells (**Fig 2B**).

It would be of interest to know whether hormone sensitivity was restored by the knockdowns. There is no evidence of an EMT process being changed by ESRP1 in these experiments, so no strong evidence is produced as to how ESPR1 might function. The detailed analysis of the epithelial splicing programme shows many are changed in both cell lines by the knockdown.
We have included figures showing that knockdown of ESRP1 in tamoxifen-resistant cells (2-control) increases the sensitivity of cells to tamoxifen treatment **(Fig 2C**). 9-control cells are fulvestrant and cross-resistant to tamoxifen. In these cells, we do see a combinatorial effect of fulvestrant (ICI-182780) more significantly than tamoxifen.The following statement is included in the manuscript.

Page 7- lines 157-163.
To further analyze the impact of endocrine therapy on *ESRP1* knockdown cells, we determined the relative cell density in the presence and absence of β-estradiol (E2), Tamoxifen (TAM) and Fulvestrant (ICI-182780). These studies showed that the *ESRP1* knockdown in 2-control alone results in significant ($p<0.0001$) reduction in cell growth with further decrease in response to E2 and TAM or the combination of these two agents (**Fig 2C**) suggesting that knockdown of ESRP1 in tamoxifen-resistant cells (2-control) increases the sensitivity of cells to tamoxifen treatment. Similar results were seen in 9-control knockdown cells in response to fulvestrant ($p<0.0001$) and to a lesser degree with tamoxifen ($p<0.01$) (**Fig 2C**).

The EMT splicing signature is studied, a previously published one, but as there is no effect on the EMT of endpoint, this does not seem to be relevant. Going back to human tumour databases, they can show evidence for some of the genes in the EMT splicing programme are also regulated in ESRP1 upregulated cancers, although, as they state clearly, it is not sufficient to induce an EMT phenotype in this breast cancer subtype, so there is no real explanation as to how this ESPR1 knockdown functions.
In view of the reviewer's comment, we have expanded the protein analysis for EMT-transcription factors (EMT-TFs) (**Figures 3D-E**).

Page 8- lines 177-187.
One of the hallmarks of EMT is the loss of E-cadherin (encoded by CDH1) [23]. E-cadherin protein levels, representative of epithelialness, increased in both ESRP1 knockdown cells (**Fig 3C-D)**. Vimentin was absent in these models. SLUG (SNAI2), and ZEB2 levels were down in 2C3, but up in 9C2 cells. SNAIL (SNAI1) and ZEB1, both inducers of EMT and repressors of CDH1, decreased in both 2C3 and 9C2 knockdowns. Claudin-1 levels, another regulator of EMT, remained unchanged in response to ESRP1 knockdown. ZO-1 is not expressed in these models (data not shown). We also confirmed these results in another cell line knockdown model (T47D-control and T47D-ESRP1 knockdown) and in ESRP1 overexpression model (MCF-7-control and MCF-7-ESRP1)**(Fig 3E).**
These data shows that knockdown of EMT does not induce key EMT players in ER+ breast cancer models.

However, they do try to go on to investigate further pathways and investigate the genes in common between the two resistant cell types. They noted clusters with fatty acid metabolism and lipid metabolism and oxireductase processes, amongst others. They then analysed using standard techniques, with the XF Extracellular Flux analyser, major energy producing pathways of mitochondria and glycolysis. They claim that the rates were changed in figures 7C and D and 7E, but there is no statistical analysis in the text, nor in the figure or supplementary figures.

We have revised the figure and included **Fig EV3** that is representative of 3 independent assays with statistical analysis in the text. We also included the following explanation in the results section.

Pages 14-15, lines 318-329.
Using the XF Extracellular Flux analyzer, we measured the two major energy producing pathways of the cell-mitochondrial respiration through oxygen consumption rate (OCR) and glycolysis through extracellular acidification rate (ECAR) of ESRP1 knockdown cells compared to their control resistant cells in real-time. These analyses demonstrated that ESRP1 knockdown did not significantly alter the glycolysis rate (ECAR) in both tamoxifen-resistant and fulvestrant-resistant models (**Fig EV3A-D**).

To compare the Oxygen Consumption Rate (OCR) between control and ESRP1 knockdown cells, we measured baseline respiration and spare respiration capacity which is defined as the difference between the basal and maximum respiration for energy production through oxidative phosphorylation. ESRP1 knockdown increased the basal respiration and spare respiration capacity in tamoxifen-resistant cells significantly ($p<0.0001$-Mann-Whitney test), but not fulvestrant-resistant cells (**Fig EV3-E**).

Figure 1, it would be much easier to follow if the groups of patients in EFGH were defined as in the A ,B, C and D figures. Also, the number of patients in each arm to have some idea of 0reliability of the data, for example, the figure 1H shows a clear split after 4 years, but it is not significant probably, it could be small numbers and variation of statistics. Numbers would be helpful to understand this.

Thank you very much for the reviewer for this comment. We added the number of patients and events for each groups. In addition, the colors are matched throughout figures (red is high ESRP1, black is low ESRP1). ESRP2 values were presented in **Fig EV1**). To further clarify the status of ESRP1 expression in regards to treatment, we analyzed the overall survival in tamoxifen or chemo-treated patients.

Pages 5-6, lines 113-125

We further assessed the correlation of ESRP1 expression with overall survival in tamoxifen-treated patients using the same platform at BreastMark database. High expression of ESRP1 was associated with shorter overall survival in ER+ tumors treated with tamoxifen [(Hazard ratio (HR) = 5.021 (2.434 - 10.36) Score (logrank) test = 23.55 on 1 df, p=1.218e-06 (n= 210, number of events= 49); **Fig 1C**)]. On the other hand, the overall survival was independent of ESRP1 expression in patients treated with chemotherapy alone (Hazard ratio (HR) = 1.599 (0.6773 - 3.773) Score (logrank) test = 1.17 on 1 df, p=0.28 (n= 129, number of events= 21); **Fig 1D**]. The effect of ESRP1 levels in patients treated with combination of tamoxifen and chemotherapy was not significant (data not shown), probably due to the small numbers in the cohort.
Analysis of The Cancer Genome Atlas (TCGA)[12] cohort also revealed that high ESRP1 expression was associated with significantly shorter overall survival in ER+ breast cancer patients (n= 100, number of events= 17;*P*=0.00011; **Fig 1E),** but not in ER- cases (P=0.19; **Fig 1F).** The limitation of the TCGA dataset was that the treatment status was not available for all cases.

Figure 2A, it is not clear what is being compared with what to obtain the statistical significance. The bar should be shown between the comparisons.

We have included the bars for the significant comparisons in the figures. Both clones of ESRP1 knockdown decreased the ESRP1 mRNA expression significantly when compared to their control counterparts, (LCC2 empty vector-2-control and LCC9 empty vector-9-control).

Page 7- lines 147-151
The knockdown resulted in dramatic decrease in both mRNA and protein levels in these cell lines (ESRP1 knockdown in LCC2 cells- clones 2C1 and 2C3; ESRP1 knockdown in LCC9 cells-clones 9C2 *P*= 0.0001 and 9C3 *P*= 0.0021; **Fig 2A-B**) compared to their control counterparts (LCC2 empty vector-2-control and LCC9 empty vector-9-control).

Figure 2E and F, knockdown clearly effects the colony growth of the resistant cell lines, but what about the effect on the control cell lines. If they are effected equally, this is not a selective effect of ESR0P1 knockdown.
We are in complete agreement with the reviewer and the experiments were performed with parental cells transfected with empty vector controls. The effect on control cells are not the same as the knockdown cells (2C3 and 9C2) as shown in the figure. Figure 2E legend was mislabeled. We corrected it. Figures 2E-F corresponds to **Fig 2D** in the revised Figure 2.

Figures 2G and H, why was supplemental oestrogen given for these cells, are they are still oestrogen sensitive, although tamoxifen resistant? Controls should be done of the parent cell line also.
The experiments were performed with parental cells transfected with empty vector controls. Supplemental oestrogen is given to measure the impact of the oestrogen in control cells, knockdown and in response to treatments. **Figures 2G-H** are renamed as **Fig 2E**.

Figure 3C, it is well recognized there is a partial EMT transition possible and more markers should be studied. Additionally, it does looks like CDH1 is actually increased in the ESRP1 knockdown, as does SLUG and ZEB2 in the 9C2, whereas they go down in 9C3, so I agree that there is no consistent change, but it needs to be a bit more thorough as there are clearly some effects.

We agree with the reviewer and expanded our analysis with EMT-TFs to further clarify the effect of *ESRP1* knockdown in EMT (see **Fig 3C**). We also included additional data in ESRP1 knockdown (T47D) and overexpression (MCF-7) models (see **Fig 3D**).

Page 8, lines 177-187
One of the hallmarks of EMT is the loss of E-cadherin (encoded by CDH1) [23]. E-cadherin protein levels, representative of epithelialness, increased in both ESRP1 knockdown cells (**Fig 3C-D**). Vimentin was absent in these models. SLUG (SNAI2), and ZEB2 levels were down in 2C3, but up in 9C2 cells. SNAIL (SNAI1) and ZEB1, both inducers of EMT and repressors of CDH1, decreased in both 2C3 and 9C2 knockdowns. Claudin-1 levels, another regulator of EMT, remained unchanged in response to ESRP1 knockdown. ZO-1 is not expressed in these models (data not shown). We also confirmed these results in another cell line knockdown model (T47D-control and T47D-ESRP1 knockdown) and in ESRP1 overexpression model (MCF-7-control and MCF-7-ESRP1)(**Fig 3E**). These data shows that knockdown of EMT does not induce key EMT players in ER+ breast cancer models.

Figure 7, C and D should have statistical comparison shown on the graphs.
We agree with the reviewer and revised the figure by plotting 3 independent assays. The statistical analysis and variance based on 3 independent assays are now shown with error bars. (**Fig EV3**).

Overall, therefore, there is no confirmatory data other than using RNA sequence, normally one would expect to have done some splicing assays or more detailed evaluation of transcripts, specifically by PCR, so that one could try and confirm the RNA sequencing data, which is known to have about a 20% error rate. There are no protein assessments, there is no expected biological effect on EMT and the metabolic changes are unconvincing and not statistically analysed.
We agree with the reviewer about the significant error rate with RNA-seq. For this reason, we have validated the data with a probe-based technology which is associated with very low error rates.

Referee #2:

ER+breast cancer represents the most common form of the disease and is amenable to endocrine therapy. However, about 20% of these cancers will recur, with resistance to endocrine therapies and thus necessitating the development of new treatment approaches. The authors investigated the role of ESRP1 and ESRP2, factors that control Epithelial to Mesenchymal Transition (EMT) splicing in breast cancer recurrence.
Analysis of publically available data showed that high levels of ESRP1 are associated with poor prognosis in ER-positive (ER+) breast tumors, which prompted the authors to carry out stable knock-down experiments in relevant (ER+) breast cancer cell line models that are resistant to tamoxifen and fulvestrant and compared to a sensitive cell MCF-7.

RNA-seq of the knockdown cell vs controls showed not only differentially expressed, but also differentially spliced transcripts, a proportion of which also carried ESPR1 binding motifs either downstream of an intron inclusion or upstream of intron exclusion event. A number of these genes are indeed part of an EMT signature. The authors also show that the knockdown of ESPR1 has an effect on colony formation ability and cell growth in vitro and in vivo. Further analysis of the splicing data and comparison to publically available datasets confirmed the presence a proportion of the splicing isoforms in the ER+ TCGA dataset. Finally, the authors perform experiments using the ESPR1 knock-down cells to show that the knock down has an effect in glycolysis and fatty acid oxidation, representing a novel link between ESPR1 and metabolism.
The work provides valuable insights into the role of ESPR1 in EMT and drug resistance and disease progression but does not provide a mechanism of action or any direct link between splicing/isoform expression and the observed differential gene expression data.

Points:

1. The link between splicing and the effects of ESPR1 on tumor growth and cell metabolism are not clear. Which transcripts are spliced and directly involved in these processes? Or which regulator thereof? Do we have prime candidates?
We thank the reviewer on this comment and included a table (**Table 2)** to provide prime candidates for *ESRP1* regulation. Additional **Appendix Figures S5-S6** are added for the key genes showing their altered alternative splicing events in response to *ESRP1* knockdown.

Page 13, lines 298-313
We next compared the expression levels of key fatty acid metabolism/ lipid metabolism and oxireductase processes at the gene, alternative splicing and protein level. FASN, SCD and PHGDH have altered at the alternative splicing and protein level in ESRP1 knockdown cells (**Table 2 and Appendix Figures S5-S6**). The same exon inclusions based on the splicing index levels was significant in both tamoxifen and fulvestrant resistant models (see locations in **Table 2**). At the protein level, decreased expression of FASN and Stearoyl-CoA desaturase 1 (SCD1) in knockdown cells was observed particularly in tamoxifen-resistant (2C3 vs 2-control) model rather than fulvestrant-resistant model (**Fig 7C**). These results may be specific to acquired tamoxifen-resistant cells, as FASN and SCD1 protein levels remained the same in response to ESRP1 knockdown in T47D breast cancer cell line (**Appendix Fig S1A**). On the other hand, PHGDH levels decreased in all models including tamoxifen and fulvestrant resistant cells as well as in T47D model with prominent blockade of expression in tamoxifen-resistant cells. Results from overexpression of ESRP1 in MCF-7 model suggested that overexpression of ESRP1 may not be enough to result in altered expression of these metabolic genes, as acquired resistant cell lines to tamoxifen may have additional characteristics and *ESRP1* knockdown results in decrease of these genes, in particular, in these cells.

2.ARHGEF11 is one of the prime ESPR1 associated splicing targets: What is the putative functional effect of exon 39 inclusion? How does it relate to the data showing that exon 38 inclusion (as apparent in Figure 6 B) is linked to migration and growth in breast cancer cells (Oncotarget. 2017 Nov 3; 8(54): 92157-92170)? What exactly is the effect of exon 39 inclusion on the AS sequence?
The location identified in our data is identical to that described in the prior Oncotarget paper. The functional significance of this exon's presence or absence is not the focus of the current study but will be investigated in further studies.

3. In Figure 6 we have a summary of validated (publically available data) isoforms of five transcripts and for two of them we the AS sequence and region of interest information. The authors need to clearly include the AS sequence of the isoforms and the predicted effects on functional domains.
We have included the location of each cassette exon events (see new Figure 5) and determined whether any major protein domains are affected. Except ARHGEF11, the locations are not associated with known domains.

4. Metabolic substrate flux analysis: The authors to include glucose uptake and lactate production data in order allow broader comparisons to other published work.

Data has now been added as supplemental information (**Appendix Fig 7A-B**).

5. An ESPR1 overexpression experiment should be included in order to confirm its opposite effect on glycolysis for example.

Data has now been added as supplemental information (**Appendix Fig 7A-B).**

6. A clear summary on any splicing events affecting transcripts for proteins related to glycolysis or fatty acid oxidation should be presented, possibly in supplementary data.

We have included the splicing events for the key genes and summarized in **Table 2.** Additional **Appendix Figures S5-S6** are added for the key genes showing their altered alternative splicing events in response to *ESRP1* knockdown.

Minor points:

1. For the TCGA splice-seq analysis can the authors provide more details on how they performed the analysis and a bit more detail on their results. For example, can they report in how many of the 100 Luminal A ESPR1high and 100 Luminal B ESPR1low samples the reported splicing events are found. Are the reported splicing events (in the text and in Figure 6) more frequently found in one of the two groups or maybe are the splicing values different in one group relative to the other.

   Thanks for this comment. We have included the following explanation to figure legend 6.

   Page 40-lines 967-971.

   The analysis of splice events was performed using the following filter criteria (Min Gene RPKM>=2, |dPSI|>=0.1, p-value<=0.02, Min Group Obs %>=.85);RPKM-reads per kilobase of transcript per million aligned reads), |dPSI|-absolute changes in percent splicing (dPSI, ΔΨ). The events are presented in Suppl. Table 10 as differential expression of splice events between ESRP1low and ESRP1high cases.

   2. In the paragraph "Novel functional role of ESRP1 knockdown on endocrine resistant breast cancer; impact of ESRP1 on metabolic pathways" the differentially expressed genes between the control and the knockdown cell lines based on microarray data should be present in the differentially expressed genes found in the RNA-seq data for the same conditions. The RNA-seq data were presented in the first paragraphs of the manuscript. Can the authors comment on how many of the presented significant genes from the microarray platform are found in the significantly deregulated gene lists from the RNA-seq data.
   The lists of RNA-seq, and HTA are presented in **the Supplementary Tables S1-S4** for RNA-seq and **Supplementary Tables S6-S9** for HTA, respectively. In addition, we added the additional information based on the summary of these tables.

   Pages 29, lines 420-433
   Although the listed metabolic genes were not significantly altered at RNA-seq data, we have validated the changes at the mRNA (HTA analysis) and the protein levels (Western blot analysis) in response to *ESRP1* knockdown in particular in tamoxifen-resistant cells (**Table 2 and Appendix Figures S5 and S6**). Other significant genes including *CD44* and *CTNND1* are present and altered in RNA-seq data. However, the lack of both key EMT-TFs and morphological EMT phenotype suggest that the role of *ESRP1* in these models are independent of EMT. The differences between RNA-seq and HTA analysis may be due to the differences of the two platforms. The depth of RNA-seq (30 million) may not be enough to get the same results. Indeed, Nazarov et al.[48] reported that the stochastic variability was higher for the sequencing data than for microarrays due to lack of reads for short and low abundant genes. This usually reduces the number of differentially expressed genes and genes with predictive potential for RNA-seq compared to microarray data. HTA 2.0 is a probe-based technology (10 probes per exon and 4 probes per exon-exon splice junction) and is independent on the depth bias of RNA-seq.

3. In Figure 4B-E, in the radar plots can the authors report what the indicated numbers (in the hypothetical axis) correspond to.
The plots are based on gene expression levels or splice index as appropriate. We have included the **Supplementary Table 5** to provide granular details on the basis of which the plots has been drawn.

4. In Figure 5 why some exons and internal/external junctions are colored green and some are colored red.
Red color indicates the inclusion of an exon, whereas green color represents skipping of an exon. We included this information in the corresponding figure legend.

5. In the legend of figure 6 the sentence: "A splice graph of the gene's exons is shaded based on expression level and shows the selected splice event outlined in red. (B) TCGA Splicegraphs for the EMT signature genes in ESRP1high versus ESRP1low tumors." maybe is less confusing if it is reorganized as "(B) TCGA Splicegraphs for the EMT signature genes in ESRP1high versus ESRP1low tumors. A splice graph of the gene's exons is shaded based on expression level and shows the selected splice event outlined in red."
We thank the reviewer for pointing this out. The figure legend has been modified as suggested.

6. In Figure 7C and 7D can the authors make the figures a bit more organized. For example, in figure 7C the green and blue boxes in the legend are presented 2 times one as 2-control/2C3 and the other as Control/Experimental. Also, can the authors provide more details for the different conditions that are indicated. For example, what do baseline, stressed, glycolysis and glycolytic reserve represent. Not all readers are familiar with the presented concepts. Maybe more details in the legend will be helpful.

We thank the reviewer for this comment. We have combined the data from 3 independent assays and provided additional information in updated **Figure EV3**.

7. Figure 5 needs more detailed explanation and is hard to read in detail, as the resolution is not high enough.

We agree with the reviewer that this image condenses an enormous amount of data. In addition to highlighting the exon, we have included the locations for each cassette and exon inclusion/ skipping events. The details of the figure can also be obtained from the **Supplementary Tables 8-9**.

Referee #3:

In the current manuscript by Gökmen-Polar and colleagues, the authors addressed how endocrine-resistant ER+ breast cancer is driving disease recurrence by utilizing regulation of differential splicing. They identified that high ESRP1 expression, unlike ESRP2, is correlated with poor survival, particularly in ER+ breast cancer patients. To confirm this finding they further analyzed the role of ESRP1 in MCF7 derived cells which have been selected for drug-resistance. Knockdown of ESRP1 resulted in reduction of colony forming capacity and decreased tumor growth upon xenografting. Interestingly, in contrast to previous reports, in ER+ cancer cell lines ESRP1 is not involved in regulating an EMT program. Specific EMT-specific genes are not consistently changing upon ESRP1 loss. Similarly, also global gene expression was not deregulated in specific EMT-related gene signatures. In contrast the authors found that ESRP1 is regulating differential splicing of a variety of transcripts and knockdown of ESRP1 results in deregulation of many genes involved in fatty acid metabolism. This regulation induced a shift in glycolysis and the glycolytic reserve of the cells, which is suggested as putative target for novel therapy approaches.
With different experimental approaches and state of the art methodology the authors dissected the role of ESRP1 in ER+ breast cancer. They identified a novel aspect of ESRP1 function that specifically in ER+ breast cancer cells is regulating fatty acid metabolism which increases tumor relapse, invasion and metastasis. In ER+ cells ESRP1 is presumably not promoting an epithelial cell

phenotype with reduced invasion and metastasis as was observed in other reports. This novel finding is very interesting and supports the notion that differential splicing is regulated in a complex manner, largely dependent on the cell type and cell of origin in cancer.

Nevertheless, I have a few concerns that need to be addressed before the manuscript can be accepted for publication:
1. ESPR1 expression was never analyzed on protein level. How is the expression changing during established endocrine-resistance, is it properly depleted upon knockdown? In line with this: are ESRP1 and 2 always coregulated, what is the level in the analyzed cell lines? Would a double knockdown have a more drastic effect especially on metbolic changes?
We have performed Western blotting using ESRP1 antibody and revised **Fig 2**. Protein data for both 2C3 and 9C2 knockdowns are shown in **Fig 2B**.

2. Although the results have been verified in TCGA samples, to confirm the findings, key experiments should be carried out with another cell line, especially since all analyzed cell lines are derived from MCF7. In addition, a cell line with low ESRP1 levels should be included to overexpress ESRP1 and observing opposite effects
We have performed ESRP1 knockdown in T47D cells (T47D-kESRP1 compared to T47D-control cells). We also overexpressed ESRP1 in MCF7 cells, which has a lower endogenous expression of ESRP1

Pages 7, lines 152-156.

Knockdown was also performed in T47D cells, which have high levels of ESRP1compared to MCF-7 cells. Overexpression of ESRP1 have been performed in MCF-7 cells, which have lower levels of endogeneous *ESRP1* level**. Appendix Fig S1A-B** demonstrates the mRNA and protein level expression for both models.

3. In MCF7 cells it was shown that ESRP1 knockdown results in EMT and upregulation of ZEB1 and ESRP1-loss prevents MCF10A cells to undergo TGFb-induced EMT (Preca BT, 2015, IJC). How do the authors explain this controversy? Did they analyze the effect of ESRP1 knockdown in the parental and non-resistant MCF7 cell line as well with different results?
We thank the reviewer for this comment and included the paragraph below.

Page 9, lines 190-201

Preca et al [24] reported that ZEB1 overexpression in MCF10A downregulates ESRP1 and switches cells to CD44s, suggesting the importance of ZEB1 for EMT phenotype. In addition, ER+ and luminal breast tumors mostly retain the CD44 variable exon[25]. High expression of CD44s has also been shown to be essential for cells to undergo epithelial-to mesenchymal transition[26]

To further understand the impact of *ESRP1* knockdown on the CD44 splice variants in our models, we assessed the CD44s versus CD44v switch in response to ESRP1 knockdown using qRT-PCR (**Appendix Fig S4**). We have observed that CD44s isoform is significantly dominant in fulvestrant knockdown (9C2), but the switch from CD44v2 to CD44s was not significant in 2C3-ESRP1 knockdown in tamoxifen-model. In 9C2-ESRP1 knockdown cells, ZEB1 was down compared to 9-control cells. In 2C3 and 2-control, very low level ZEB1 was present and was not induced in response to ESRP1 knockdown. These data clearly shows that ESRP1's role in our models is different than Preca et al. and is independent of EMT.

4. Fig. 7C-D: The middle panel of the left graphs in C and D is not labeled, it is not clear what is shown? Apparently, the detected changes in ECAR and OCR are not significant. So are they of biological relevance? In particular for 9-control and 9c3 no difference is observed or is only marginal. How do the authors explain this?
We have updated the ECAR and OCR data and the combined results of 3 independent assays are displayed in **Fig EV3**). We agree with the reviewer that the effect is specific to 2C3 cells, *ESRP1* knockdown did not significantly alter the glycolysis rate (ECAR) in both tamoxifen-resistant and fulvestrant-resistant models (**Fig EV3**). ESRP1 knockdown increased the basal respiration and spare respiration capacity in tamoxifen-resistant cells, but not fulvestrant resistant cells. . WE included the following paragraph.

Page 14-15, lines 318-329

Using the XF Extracellular Flux analyzer, we measured the two major energy producing pathways of the cell-mitochondrial respiration through oxygen consumption rate (OCR) and glycolysis through extracellular acidification rate (ECAR) of ESRP1 knockdown cells compared to their control resistant cells in real-time. These analyses demonstrated that ESRP1 knockdown did not significantly alter the glycolysis rate (ECAR) in both tamoxifen-resistant and fulvestrant-resistant models (**Fig EV3A-D**).
To compare the Oxygen Consumption Rate (OCR) between control and ESRP1 knockdown cells, we measured baseline respiration and spare respiration capacity which is defined as the difference between the basal and maximum respiration for energy production through oxidative phosphorylation. ESRP1 knockdown increased the basal respiration and spare respiration capacity in tamoxifen-resistant cells significantly ($p<0.0001$-Mann-Whitney test), but not fulvestrant-resistant cells (**Fig EV3-E**).

5. Fig. 7E and p. 12: The authors claim to observe "decreased expression of FASN and Stearoyl-CoA desaturase 1 (SCD1) in knockdown cells". However, I do not see this in the figure, especially not for LCC9 cells and it is unclear how the authors came to that conclusion. Quantification of the blots should also be provided!
We have updated the figure (**Fig 7C**). We agree with the reviewer that the decrease of FASN and SCD is specific to LCC2 knockdown, but not LCC9 knockdown. Therefore, these results suggests that the effect of ESRP1 knockdown is important for tamoxifen resistant cells rather than fulvestrant resistant cells. We have revised the results and discussion in the manuscript.

6. Fig. 1E: There is an unexpected switch in colors of the column: why is 'ESRP1 high' now shown in red? This is confusing!
We agree with the reviewer and the oversight is regretted. The figure is now extensively modified and the coloring pattern corrected.

7. Fig. 2: The qRT-PCR data are shown in an unusual way which makes it difficult to follow. I suggest to display them as relative expression levels/fold changes.
We regraphed the data to make clearer. We prefer to display the data in terms of delta-CT rather than fold change.
8. Fig. 2E-F: Something is wrong with the labeling in this figure: the legend indicates that 'control' is shown in blue, which shows reduced colony formation capacity. I guess the color labeling is switched.
We thank the reviewer and corrected the mislabeling in the figure.

9. Fig. 3E: I am a bit puzzled about the finding about the cell differentiation in 2D culture. How can they form more glandular-like structures in 2D? If there are phenotypic differences that change from a more mesenchymal to a more epithelial appearance, the cells need to be plated in much lower densities. The cells in the figures shown here are 90-100% confluent!

As a US- board certified pathologist and the Fellow of the Royal College of Pathologists, the senior author can assure the reviewer that the change in morphology is not a function of cell density. ESRP1 has been described as a pro-EMT factor, hence we have conducted this experiment as multiple cell densities and in different types of chambers. The results are very consistent. They also are supported by all the data provided in the manuscript including gene expression and western blots for EMT-TFs. The data is also supported by prior work on EMT in ER+ breast cancer as reported by Taube et al and cited in our manuscript. Of note, Dr. Robert Weinstein is a co-author of the Taube paper.

10. How are the well-described targets of differential splicing of ESRP1 regulated in this cellular system, like CD44, p120 or FGFR1? Are they not differentially spliced which may indicate a cell-type specific function of ESRP1?
We have added a table and appendix figures (**Table 2 and Appendix Figures S5 and S6)** and provided additional data for the well-described targets of differential splicing of *ESRP1* regulated in this cellular system, like CD44, p120 or FGFR1.

2nd Editorial Decision 21 September 2018

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 enclosed below). As you will see, the referees now support the publication of your manuscript in EMBO reports.

Before we can proceed with formal acceptance, I have the following editorial requests that we ask you to address in a final revised version of the manuscript:

- Please change the title to: "Splicing factor ESRP1 controls ER-positive breast cancer by altering metabolic pathways".

- Please provide the abstract written in present tense.

- Please have the entire manuscript proofread by a native speaker.

- Please add the grant support information to the acknowledgements (and remove this paragraph from the title page).

- Please call the running head "running title", and change it to "ESRP1 in ER-positive breast cancer".

- Please restrict the key words to five.

- Please format the references in EMBO reports style. Please use 'et al' if there are more than ten authors (but the first ten should be shown). See:
http://embor.embopress.org/authorguide#referencesformat

- Regarding data quantification and statistics please carefully check that the number "n" for how many independent experiments (biological replicates) were performed is clearly specified in the respective figure legends, as well as the test used to calculate p-values. Please provide statistical testing where applicable, and add a paragraph describing the statistical testing used throughout the manuscript. See also:
http://embor.embopress.org/authorguide#statisticalanalysis

- Could statistical testing be performed for the diagrams shown in Fig. 2D, 2E, S1A, S4 and S7?

- Please add scale bars to the microscopic images in Figs. 3A/B, and define their length in the respective legend.

- Please provide the source data (entire blots) for ALL the Western blots (also for those shown in the Appendix, and in EV figures). Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

- The panels for Figures 2D, 2E, 3D and 7A are grainy and of low resolution. Please provide these in better quality.

- If the data shown in the excel files presently called "Table S1-12" are indeed Supplementary Tables, they should be included into the Appendix. If these are source data for figure panels, please provide them as source data files. Indicate to which Figure these belong, combine the data that are connected to one Figure, and send these as one single file (using different tabs). Or should files be datasets?

- Please add a TOC with page numbers to the Appendix. Also Appendix Figures should only have one page. Please provide Figure S2 and S7 on one page. Figures S5 and S6 are very messy. Can these data be presented in a more comprehensive way? Or should these be datasets?

- Please provide Appendix Figure S2B in better quality, and without overlapping labelling.

- Please provide an ORCID for the corresponding author Yesim Gokmen-Polar, and link it to his author account.

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 (comments), we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

When submitting your revised manuscript, we will require:
- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures) - of those changed.
- the revised Appendix

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 about 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 or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Fully answered my queries

Referee #2:

The authors have addressed the major and minor points raised in the review. As a result, additional and revised, information and explanations are included in the main text. The figures have also been revised and improved as requested, while additional supporting information and documentation is now included as supplementary information. As a result the manuscript is clearer and has been improved overall.

Referee #3:

The authors extensively revised the manuscript and performed new research. All my points were adequately addressed and I can now truly recommend the manuscript for publication in EMBO reports.

2nd Revision - authors' response 17 October 2018

Response to Queries:

- Please change the title to: "Splicing factor ESRP1 controls ER-positive breast cancer by altering metabolic pathways".
Modified as suggested.

- Please provide the abstract written in present tense. –
Modified as suggested.

- Please have the entire manuscript proofread by a native speaker.
proofread as suggested.

- Please add the grant support information to the acknowledgements (and remove this paragraph from the title page).
Modified as suggested.

- Please call the running head "running title", and change it to "ESRP1 in ER-positive breast cancer".
Modified as suggested.

- Please restrict the key words to five.
Modified as suggested.

- Please format the references in EMBO reports style. Please use 'et al' if there are more than ten authors (but the first ten should be shown). See:
http://embor.embopress.org/authorguide#referencesformat
Formatted as suggested.

- Regarding data quantification and statistics please carefully check that the number "n" for how many independent experiments (biological replicates) were performed is clearly specified in the respective figure legends, as well as the test used to calculate p-values. Please provide statistical testing where applicable, and add a paragraph describing the statistical testing used throughout the manuscript. See also:
http://embor.embopress.org/authorguide#statisticalanalysis
- Could statistical testing be performed for the diagrams shown in Fig. 2D, 2E,/ S1A, S4 and S7?
Statistical information is added where applicable in the figure legends.

- Please add scale bars to the microscopic images in Figs. 3A/B, and define their length in the respective legend.
Added.

- Please provide the source data (entire blots) for ALL the Western blots (also for those shown in the Appendix, and in EV figures). Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.
Provided as suggested.

- The panels for Figures 2D, 2E, 3D and 7A are grainy and of low resolution. Please provide these in better quality.
Better quality figures are provided. Note, the original western blot of Fig 3D is grainy and cannot be modified.

- If the data shown in the excel files presently called "Table S1-12" are indeed Supplementary Tables, they should be included into the Appendix. If these are source data for figure panels, please provide them as source data files. Indicate to which Figure these belong, combine the data that are connected to one Figure, and send these as one single file (using different tabs). Or should files be datasets?
Modified as suggested.

- Please add a TOC with page numbers to the Appendix. Also Appendix Figures should only have one page. Please provide Figure S2 and S7 on one page. Figures S5 and S6 are very messy. Can these data be presented in a more comprehensive way? Or should these be datasets?
Modified as suggested.

- Please provide Appendix Figure S2B in better quality, and without overlapping labelling.
Modified as suggested.

- Please provide an ORCID for the corresponding author Yesim Gokmen-Polar, and link it to his author account.
ORCID No: 0000-0001-9927-4893

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 (comments), we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.
Modified as suggested.

When submitting your revised manuscript, we will require:
- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures) - of those changed.
- the revised Appendix
Modified as suggested.

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 about 400 pixels) that can be used as a visual synopsis on our website.
Synopsis figure and short summary are included.

In checking your manuscript submitted to EMBO Reports it has come to our attention that the following must be addressed before we can begin the editorial process.

1) Please provide an Author Checklist.Please find the link to the checklist below:
http://embor.embopress.org/authorguide

Provided.

2) Please provide all Appendix material in one PDF file. Appendix Tables S1 + S2 may be added to the Appendix pdf, or if you would rather they remain excel files should be made into EV tables (in which case please correct the callouts).

All Appendix material is provided in one PDF file. Appendix Tables S1 + S2 are renamed as Table EV1 and EV2, respectively. We would like to keep the tables in excel files.

3) You say that you have provided Source Data, but none has been uploaded.
Source data files are uploaded.

4) Tables S1-S12: You say that they have modified these files, but they have not been provided. Please explain what these file are; i.e. Source Data, Supplementary Tables?

Those are renamed as Source Data files and EV Tables.

3rd Editorial Decision 2 November 2018

Thank you for the submission of your revised manuscript to our editorial offices. I went now through the manuscript, and there are still editorial requests that need to be addressed in a further

revised version:

- Please have the manuscript carefully proofread by a native speaker. There are still too many grammatical errors that render the manuscript partly difficult to comprehend. We cannot proceed with the paper if this is not improved. Our publisher also offers a manuscript editing service: https://wileyeditingservices.com/en/english-language-editing/

- Please add the entire material & methods information to the main manuscript. There should no methods information in the Appendix.

- Regarding the Appendix, please add page numbers to the Appendix, and to the TOC, and move the figure legends below each figure. What does the information on lines refer to in the TOC? I suggest to remove this.

- It is further not clear what happened to the 12 Appendix Tables (S1-12) you included into the V2 of this manuscript. It seems e.g. the original tables S1 and S2 have now been omitted from the paper. I think these should be added as Datasets. Please do that, using the nomenclature Dataset EVx, and add call-outs to the manuscript text. Please also explain in your final point-by-point response to these editorial requests where exactly the data of the V2-tables S1-12 can now be found in the final manuscript.

- Please add a paragraph to the Methods section that explains the statistics used throughout the manuscript.

- There is a callout to Supplemental Table 10 in the legend of Fig. 6. Please replace this with the correct callout. Finally, please re-check that all the callouts in the manuscript text are correct!

We further noted some inconsistencies regarding the Western blot source data (SD):
- Fig. 3C: The SD for CHD1 does not match to the figure panel (e.g. 2C3 seems to be 9C2 but mirrored). The panels are also swapped comparing to the figure in the V2. Further, for ZEB2 2-control and 2C3 the brightness of the SD is much lower. Please use as unmodified images as possible, with similar contrast/brightness in the figure and the SD.
- Fig. 3E: For GAPDH and Claudin again the contrast/brightness is very different compared to the source data. Please use as unmodified images as possible, with similar contrast/brightness in the figure and the SD. For GAPDH it is even not clear if the SD blot is the same than the one shown in the figure.
- Appendix Figure S1: For GAPDH, SCD1 and PHGDH MCF7 there is again a clear difference to the SD. Please provide as unmodified images as possible, with similar contrast/brightness in the figure and the SD, and check that this is indeed the correct SD.

Please clarify, and reassure that for each WB panel the identical and correct SD is provided. Please provide all SD combined in one PDF file per figure.

When submitting your revised manuscript, we will require:
- a Microsoft Word file (.doc) of the revised and improved manuscript text
- editable TIFF or EPS-formatted figure files (main figures and EV figures) in high resolution (of those with changes and adjusted WB panels).
- the corrected source data
- the revised Appendix
- the 2 datasets

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

3rd Revision - authors' response 4 December 2018

Thank you for the submission of your revised manuscript to our editorial offices. I went now through the manuscript, and there are still editorial requests that need to be addressed a further revised version:

1) Please have the manuscript carefully proofread by a native speaker. There are still too many grammatical errors that render the manuscript partly difficult to comprehend. We cannot proceed with the paper if this is not improved. Our publisher also offers a manuscript editing service:

https://wileyeditingservices.com/en/english-language-editing/
The manuscript is edited using Wiley editing services as recommended.

2) Please add the entire material & methods information to the main manuscript. There should be not methods information in the Appendix.

We have added the entire material & methods to the main manuscript.

3) Regarding the Appendix, please add page number to the Appendix, and to the TOC, and move the figure legends below each figure. What does the information on lines refer to in the TOC? I suggest to remove this.

We have revised the above points per suggestions.

4) It is further not clear what happened to the 12 Appendix Tables (S1-12) you included into the V2 of this manuscript. It seems e.g. the original tables S1 and S2 have now been omitted from the paper. I think these should be added as Datasets. Please do that, using the nomenclature Dataset EVx, and add call-outs to the manuscript text. Please also explain in your final point-by-point response to these editorial requests where exactly the data of the V2-tables S1-12 can now be found in the final manuscript.

Thanks for asking these points. All the 12 Appendix Tables (S1-12) are converted into Source data files and renamed based on the relevant figures per suggestion of the EMBO Reports publication department. They were originally labeled as Supplementary files and uploaded as Appendix files. Since these are excel files, we can't convert them to pdf files required for Appendix files. We therefore converted them to Source data files to keep the excel files. All 12 tables are renamed as below. We separated Supp. Table 5 into two separate tables for further clarity. We have 13 tables now. Please find the original Supplementary file names and revised source data names of these tables below.

Fig 4_source data file 1. Differentially regulated genes in ESRP1-LCC2 (2C3) knockdown cells compared to LCC2 control (2-control) resistant cell lines using RNA-seq **(previous Suppl Table S1 or Appendix Table S1).**

Fig 4_Source data file 2. Differentially regulated genes in ESRP1-LCC9 (9C2) knockdown cells compared to LCC9 control (9-control) resistant cell lines using RNA-seq **(previous Suppl Table S2 or Appendix Table S2).**

Fig 4_Source data file 3. Identification of differential alternative splicing events (ASEs) in LCC2-ESRP1 (2C3) knockdown cells compared to LCC2 control (2-control) resistant cell lines using RNA-seq **(previous Suppl Table S3 or Appendix Table S3).**

Fig 4_Source data file 4. Identification of differential alternative splicing events (ASEs) in LCC9-ESRP1 (9C2) knockdown cells compared to LCC9 control (9-control) resistant cell lines using RNA-seq **(previous Suppl Table S4 or Appendix Table S4).**

Fig 4_Source data file 5. Alterations in EMT genes and cassette exons of EMT splicing signature in ESRP1 knockdown cells-LCC2 set **(previous Suppl Table S5 or Appendix Table S5 for LCC2 set).**

Fig 4_Source data file 6. Alterations in EMT genes and cassette exons of EMT splicing signature in ESRP1 knockdown cells-LCC9 set **(previous Suppl Table S5 or Appendix Table S5 for LCC9 set).**
.Fig 5_Source data file 1. Validation of differential alternative splicing events (ASEs) in LCC2-ESRP1 (2C3) knockdown cells compared to LCC2 control (2-control) resistant cell lines using HTA analysis **(previous Suppl Table S8 or Appendix Table S8).**

Fig 5_Source data file 2. Validation of differential alternative splicing events (ASEs) in LCC9-ESRP1 (9C2) knockdown cells compared to LCC9 control (9-control) resistant cell lines using HTA analysis **(previous Suppl Table S9 or Appendix Table S9).**

Fig 6_Source data file 1. Identification of differential alternative splicing events (ASEs) in TCGA BRCA SpliceSeq dataset of ESRP1-high versus ESRP1-low breast tumors **(previous Suppl Table S10 or Appendix Table S10).**

Fig 7_Source data file 1. Differential gene expression in LCC2 (2-control) versus ESRP1 knockdown (2C3) cells using HTA 2.0 analysis **(previous Suppl Table S6 or Appendix Table S6).**
Fig 7_Source data file 2. Differential gene expression in LCC9 (9-control) versus ESRP1 knockdown 9C2 cells using HTA 2.0 analysis **(previous Suppl Table S7 or Appendix Table S7).**
Fig 7_Source data file 3. DAVID Functional Annotation Clusters Downregulated in ESRP1 knockdown cells **(previous Suppl Table S11 or Appendix Table S11).**

Fig 7_Source data file 4. DAVID Functional Annotation Clusters Upregulated in ESRP1 knockdown cells **(previous Suppl Table S12 or Appendix Table S12).**

5) Please add a paragraph to the Methods section that explains the statistics used throughout the manuscript.

We have added the paragraph in regards to the statistical analyses at the end of the Materials & Methods section.

Statistical analysis
In vitro experiments
Two-way ANOVA tests were used for statistical analysis by GraphPad Prism 5.0 and Microsoft Excel. All results are representative of three independent biological replicates and expressed as mean values SD. In all cases, differences were considered to be statistically significant at $P < 0.05$.
Kaplan-Meier curves
BreastMark: Breast Cancer Survival Analysis Tool uses the software CGI (the web server with the R/perl-based algorithm) to calculate the *P* values for the endpoint "overall survival" using log rank test. TCGA-BRCA Kaplan-Meier curves: A log rank test was used to calculate *P* values for the endpoint "overall survival" using the "survival" package in R (R Foundation for Statistical Computing).

6) There is a callout to Supplemental Table 10 in the legend of Fig. 6. Please replace this with the correct callout. Finally, please re-check that all the callouts in the manuscript text are correct!

Corrected and checked as **Fig 6 Source data file 1**

7) We further noted some inconsistencies regarding the Western blot source data (SD):

- Fig. 3C: The SD for CHD1 does not match to the figure panel (e.g. 2C3 seems to be 9C2 but mirrored).

Our original western blot assays are done in the order of the following lanes: 2C2, LCC2, 9C2, and LCC9. However, we presented the data in the order of lanes as 2-control (LCC2), 2C3, 9-control (LCC9) and 9C2 to make the figures easy to follow to the readers. Although the order of the lanes is different, all the data are identical.
A minor discrepancy was noted in the CDH1 data, this has been corrected in the latest version. It does not change any of the interpretation or conclusions.
For SLUG and ZEB2, we have included the less saturated images shown on the right hand side of the raw data figure 3. We use the Amersham Imager 600 to detect the Chemiluminescent signals of the Western blot intensities. We have included two different exposure times and chose the less saturated ones as shown here.

The panels are also swapped comparing to the figure in the V2. Further, for ZEB2 2-control and 2C3 the brightness of the SD is much lower. Please use as unmodified images as possible, with similar contrast/brightness in the figure and the SD.

As mentioned above, we chose the lane order in the figures to make data easy to follow for the reviewers. All the data is correct when reading the lane labels.

- Fig. 3E: For GAPDH and Claudin again the contrast/brightness is very different compared to the source data. Please use as unmodified images as possible, with similar contrast/brightness in the figure and the SD. For GAPDH it is even not clear if the SD blot is the same than the one shown in the figure.
We corrected the figures using the unmodified versions.

- Appendix Figure S1: For GAPDH, SCD1 and PHGDH MCF7 there is again a clear difference to the SD. Please provide as unmodified images as possible, with similar contrast/brightness in the figure and the SD, and check that this is indeed the correct SD.

Please clarify, and reassure that for each WB panel the identical and correct SD is provided. Please provide all SD combined in one PDF file per figure.

Thank you very much for this comment. We corrected the figures and their corresponding GAPDH's based on their original Western blots as seen in the figure.

When submitting your revised manuscript, we will require:
- a Microsoft Word file (.doc) of the revised and improved manuscript text
- editable TIFF or EPS-formatted figure files (main figures and EV figures) in high resolution (of those with changes and adjusted WB panels).
- the corrected source data
- the revised Appendix

- the 2 datasets-as explained above in response #4.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Sunil Badve
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-46078

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 justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ 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?	For all in vitro experiments, three biological replicates were performed allowing the drawing of statistically significant conclusions (evidenced by p values) (see Figure Legends). Data (mean + SD) is calculated using two-way ANOVA based on the three independent assays.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We determined the number of mice for each condition based on the following citation: Workman et al. Guidelines for the welfare and use of animals in cancer research. British Journal of Cancer (2010) 102, 1555 – 1577. Accordingly, pilot tumor growth studies using small numbers of animals (at least 5) are recommended to establish the patterns of local and metastatic growth
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data points are excluded.
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.	For in vivo tumorigenicity assays, mice were randomly assigned to cages (five mice per cage).
For animal studies, include a statement about randomization even if no randomization was used.	We stated that mice were randomly assigned to cages (5 mice per cage).
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.	For animal studies, the investigators were blinded to group allocation during data collection and analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	We stated that "the investigators were blinded to group allocation during data collection and analysis" in the Materials and methods section.
5. For every figure, are statistical tests justified as appropriate?	All statistical tests were justified as appropriate
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We included the statistical methods to assess the assumptions of the test in the figure legends.
Is there an estimate of variation within each group of data?	The standard deviation was calculated for each group.
Is the variance similar between the groups that are being statistically compared?	ANOVA was performed for statistical analysis for cell line models. Log-rank test was used for the overall survival significance (Kaplan-Meier plots).

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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-tun

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/

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).	We described the details of the antibodies used in the Materials and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No authentication was performed on all cell lines. They wer all tested for mycoplasma contamination before the assay are performed.

* 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.	Six-to-eight-week-old female athymic mice (nu/nu) were purchased from Harlan Sprague Dawley, Inidanapolis. All animals were housed in a SPF (Specific-Pathogen Free) facility at the Indiana University. These are described in the Materials and methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We stated that " All animal experiments were done under a protocol approved by the Indiana University Institutional Animal Care and Use Committee (IU IACUC).
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.	This study was in compliance with the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	We stated in the Materials and Methods section as follows: "All protocols were reviewed and approved by the Institutional Review Board of Indiana University. Samples and clinical records were anonymized prior to access by the authors and linked with a numerical identifier. The requirement for informed consent was waived by the IRB.
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.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	There is no restriction on the availability and the use of human data or samples.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable
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.	Not applicable
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.	Not applicable

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	We stated the Data and materials availability in the Appendix Material source files and at https://iu.app.box.com/folder/27394447544
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 data are presented as figures, expanded view figures, and source data and appendix figures/tables.
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).	Not applicable
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.	Not applicable

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.	Not applicable