THE EMBO JOURNAL

# Genome-wide cooperation of the EMT-transcription factor ZEB1 with YAP and AP-1 in breast cancer

Nora Feldker, Fulvia Ferrazzi, Harald Schuhwerk, Sebastian A. Widholz, Kerstin Guenther, Isabell Frisch, Kathrin Jakob, Julia Kleemann, Dania Riegel, Ulrike Bönisch, Soeren Lukassen, Rebecca L. Eccles, Christian Schmidl, Marc P. Stemmler, Thomas Brabletz and Simone Brabletz
**DOI: embj.2019103209**

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## Review Timeline:


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|---|---|
| Submission Date: | 13th Aug 19 |
| Editorial Decision: | 25th Sep 19 |
| Revision Received: | 11th Oct 19 |
| Editorial Decision: | 11th Dec 19 |
| Revision Received: | 24th Apr 20 |
| Editorial Decision: | 5th Jun 20 |
| Revision Received: | 17th Jun 20 |
| Accepted: | 18th Jun 20 |

*Editor: Daniel Klimmeck*


## 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 25th Sep 2019

Dear Dr. Brabletz,

Thank you for submitting your manuscript EMBOJ-2019-103209 to the EMBO Journal for consideration. My apologies again for the delay in getting back to you due to protracted referee input and detailed discussions in the team. Three referees have been assigned to your manuscript, and we have received reports from all of them, which I copy below. In light of the referees' input together with our internal discussions here in the team, I am afraid we decided that we cannot offer publication in The EMBO Journal. However, we do encourage you to transfer your study to our sister journal Life Science Alliance.

As you will see, the referees appreciate that the analysis extends previous work. However they also raise major concerns with the analysis that I am afraid preclude publication here. While referee #3 is more positive, both referees #1 and #2 are critical, stating a number of major issues related insufficient support for the claims made, generality of the work and pathophysiological relevance of the concept proposed. Also, they point out that causalities in cooperation between ZEB and Jun beyond co-occupancy, as well as proof of functional relevance and cellular downstream details remain open. In addition, generality of the findings is questioned and the exploration of the embryonic phenotype considered not rigorous enough. In addition the referees state a number of additional issues related to statistical analysis, data display and inconsistencies between findings as well as missing controls.

Given the overall negative opinions from good experts on the field, and considering our policy at the journal to generally allow for only one single major round of revision, our conclusions from the reports is, I am afraid that we cannot offer to publish your study in The EMBO Journal.

That being said, we recognize that your findings will be of value to the field. As to your preference at submission, I have thus discussed your work with Andrea Leibfried, executive editor of Life Science Alliance, and would therefore like to propose a transfer of your manuscript and referee reports to the new open-access journal Life Science Alliance. Life Science Alliance is launched as a partnership between EMBO Press, Rockefeller Press, and Cold Spring Harbor Laboratory Press, and publishes work that is of high value to the respective communities across all areas in the life sciences. I am happy to say that Andrea would like to publish your work, pending further minor revision. Andrea states 'We would expect a point-by-point response to all concerns raised and accordingly re-wording of the manuscript, re-analysis of the data already at hand, addition of statistical analysis and changes in data representation. The following experimental revisions should get included, too: rev#1, points 6, 7, 8, 11 (pt 11 is also requested by rev#3 and commented upon by rev#2 in his/her point 11). rev#3, two points mentioned in review.'. You can directly discuss the revision with Andrea by contacting her at a.leibfried@life-science-alliance.org .

I realize that this negative decision will be disappointing, but I am afraid that the reports at hand did not allow for a different conclusion in this case. I hope you will view the possibility of a transfer favourably. If this is the case, please use the link below to transfer the manuscript directly.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Editor
The EMBO Journal

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Referee #1:

Cell state changes, and particularly those associated with features linked to cellular plasticity, such as EMT, are fundamentally important elements of tumor development, progression, and metastasis. Thus, studies that examine the molecular mechanisms that regulate such cell state changes are of high interest. In Feldker et al., the authors investigate the transcriptional control of Zeb1 in order to further elucidate how this transcription factor functions to regulate cellular state changes in breast cancer. The authors use ChIP-seq, ATAC-seq, and luciferase assays to identify target sites associated with Zeb1, to identify regulatory or co-occupied transcription factors associated with these sites, and to study the transcriptional dynamics active at these sites. The authors describe an interaction between Zeb1 and the AP1 and Hippo pathway factors that regulate the transcriptional activity of these molecules, providing interesting insight into how differential transcriptional behavior associated with Zeb1 may be regulated. The authors deduce from their data that Zeb1 acts as a repressor when bound alone to its target site, while it acts as an activator when bound in conjunction with AP1/HIPPO factors in breast cancer, or other factors in glioblastoma.

While the data provide some interesting insight into the function and regulation of Zeb1 and Zeb1-target genes, there are significant issues that need to be addressed before this manuscript would be suitable for publication:

1) Statistical analyses for the Venn diagrams (1C, 1E) are missing.
2) The authors do not detect LEF/TCF motifs within the ZEB1 peaks in the breast cancer cells, and draw attention to the fact that such motifs were found in glioblastoma cancer cells, leading them to suggest that ZEB1 interacts with YAP/AP1 in breast cancer and TCF/LEF in glioblastoma. These findings are significantly overstated. First, this is based on analysis of a single breast cancer cell line. Second, breast cancer cell lines are a model system that cannot directly be equated with breast cancer, and certainly this one cell line bears little resemblance to the diverse spectrum of diseases collectively called breast cancer. Third, there multiple other differences besides just a BC vs. a GBM cell line, such as culture conditions (suspension vs. adherent) and culture media that may account for differential activity of Wnt signaling and impact TCF/LEF and Zeb1 function. This aspect of the paper either needs significantly more experimental attention, or should be removed from the abstract and main results.
3) The authors showed coincidence between JUN and ZEB1 peak summits and infer direct interaction between Zeb1 and Jun at these sites. However, this conclusion is not justified in the absence of a direct experiment to demonstrate this for the following reason. The authors show that 32% of the ZEB1 peaks do not have a high affinity ZEB1 motif and JUN ChIP-seq peaks only overlap with about 1/3 ZEB1 peaks. Further, ZEB1 physically interracts with JUN and FOSL1. Therefore, it is necessary to show that within JUN & ZEB1 overlapping peaks, such peaks are enriched for both JUN and ZEB1 motifs, and both proteins are actually interacting in those sites before claiming the cooperation between ZEB1 and JUN in chromatin binding.

4) ZEB1 only peaks are enriched near promoters while ZEB1/YAP/AP1 peaks are enriched in the distal regions (presumably enhancers). However, when the author tried to associate these two different groups of ZEB1 peaks to either repression or activation, only the promoter proximal ZEB1/YAP/AP1 peaks (not the majority of these jointly bound peaks) were used. Thus, these data do not support the conclusion that "ZEB1 in cooperation with YAP and AP-1 factors seems to act mainly as a transcriptional activator."
5) Other studies to confirm this point involved the use of transient assays with co-transfected proteins and luciferase expression. It is well known that the DNA in such transient studies is not chromatinized in the same way as endogenous genes. This might explain the huge fold induction observed, but only the very modest effects on "repression." More convincing would have been direct analysis of transcripts produced from endogenous genes engineered by CRISPR to contain or lack YAP/AP1 sites and to analyze expression mediated by expression of endogenous proteins expressed at physiologic levels..
6) Antibody controls are missing for Figure 2A. A straightforward control would be to create KO clones with CRISPR, and there should be no signal.
7) An additional control for Figure 2B would be to attempt the in situ proximity ligation assay between Zeb1 and a factor that it should not interact with (NLS-GFP may be one example), to confirm that the signal is truly showing specific interactions within the nucleus, and not just random interactions between two highly expressed nuclear factors.
8) For the CO-IP experiments, shouldn't the IP lane produce significantly more protein than the input, which is not the case with Zeb1? The presence of pulldown in the IgG lanes raises concerns about specificity and whether these are real interactions or just artifacts.
9) With the ChIP-seq and ATAC-seq data, the authors can make precise editing of target sites of interest with CRISPR to evaluate the consequences on gene expression of manipulating Zeb1 binding sites in different contexts. This would be more convincing than the artificial luciferase assays.
10) While the authors perform some genetic perturbations of Zeb1 and the other factors, an experiment that would add more depth to these studies would be to extrinsically modulate these factors where possible. As the Hippo pathway can be regulated by extrinsic cues, the transcriptional activation of Zeb1-YAP loci should be interrogated in culture conditions favoring differential Hippo activation.
11) In Figure 7 the authors attempt to link their findings in MB231 cells to human breast tumors. Unfortunately, to be convincing, the authors would need to demonstrate that similar mechanisms are at play within these breast cancers. Do the authors see that Zeb1 only tumors show a transcriptomic profile with elevated features linked to the Zeb1-only ChIP-seq sites? Do Zeb1+FOSL1 tumors show a transcriptomic profile with features linked to the Zeb1+API ChIP-seq sites?
Other issues:
1. No supplemental figures and tables were included in this submission.

Referee #2:

In this study, the authors perform CHIP-seq analysis of Zeb1 on MDA-MB-231 cells to explore its binding sites in this cell line. The introduction sets up the rationale for the study: uncover an explanation for the seemingly controversial role of Zeb1 in cancer metastasis/invasive program. This is however rather misleading and confusing. The description of the literature surrounding the controversy of EMT transcription factors in distinct cancer types is inaccurate and superficial (and

perhaps unnecessary given the limited scope of the present study on Zeb1). The authors present a confusing picture of the distinct functions attributed to EMT program. For example, deletion of certain transcription factors (not Zeb1) showed impact on therapy resistance but not metastatic dissemination (Zheng 2015). Deletion of Zeb1 in pancreatic cancer suppressed both primary tumor growth and metastasis (Krebs 2017). Zeb1 deletion in breast cancer mouse models in vivo has not been realized, and it is unclear what EMT phenotype will be affected (chemoresistance, metastasis, or both). A revision of the introduction may be needed to bring into better focus the study question and clarify the interpretation of the main conclusions. The study presented does not place the data in the context of breast cancer EMT outcome: there is no clear indication as to what is the functional EMT outcome of the distinct Zeb1 transcription regulation shown here, no indication as to what are the genes repressed/activated and their distinct roles in migration, stemness, metabolism, resistance to apoptosis, etc. As detailed below, the design of the experiment is limited to a single cell line. This should be addressed experimentally. It is noted in the abstract, introduction, discussion, and concluding model figure, that a tissue specific feature has arose from their study, implicating distinct Zeb1 transcriptional partners in breast cancer vs. glioblastoma (published dataset, obtained using three glioma cell lines). This is presented as a main conclusion from the study, but in fact has hardly no representation in the manuscript. The authors should consider performing their own analysis on distinct cell types to strengthen this conclusion.

1) A critical drawback from the presented study is the generalization made on data obtained from ChIP-seq data of a single breast cancer cell line. The cell line studied, MDA-MB-231, represents a metastatic, mesenchymal-like cell line, thus bringing into question the conclusions made with respect to the role of Zeb1 in inducing acquisition of EMT/mesenchymal features. Does the induction of EMT (by TGFb, for example) in breast cancer cell lines that does not normally express ZEB1 result in a switch from Zeb1-only (repressor) to Zeb-1/Yap/JUN (activator)? The use of a single cell line is sufficient to generalize findings to breast cancer.
2) It is unclear what is shown in Supplementary Figure 1D. It is assumed that the 45.1% activated genes represent the transcripts downregulated in the Zeb1 knockdown cells compared to control cells (please clarify). Can a heat map and pathway analysis be performed on these published data to validate the EMT related signature of Zeb1 knockdown, thereby affirming whether the genes activated are pro-mesenchymal/stem cell/migratory genes while the genes repressed are epithelial genes?
3) Could the author ascertain whether the analysis shown in Figure 1D extend to published ChIP-seq data set of the AP-1 factor JUN? What fraction of gene promoters in the current analysis shown in Figure 1D overlap with analysis from correlation with JUN ChIP-seq data?
4) Can the author clarify the discrepancy in the p-values shown in Figure 1A and Supplementary Table 4. The rank listing shows AP-1 at number 10 in the top 25 enriched motifs, but is listed as rank 2 in Figure 1A and in the text. Please include an explanation of the curated table shown in Figure 1A.
5) Consider providing a circle plot to show the Zeb1, YAP, AP-1 (JUN, FOSL1), and LEF/TCF peak intensity for all chromosomes to support the claim made regarding Zeb1 acting 'genome wide', and to aid in the visualization of Zeb1/AP-1 in breast cancer vs. Zeb1/LEF/TCF in glioblastoma.
6) Figure 2D: Can the authors confirm that modulation in Zeb1 protein content impact JUN pull down? In other words, would the suppression of Zeb1 in results in loss of JUN pull down, or would high expression of Zeb1 (via induction of EMT) in breast cancer cells would enhance JUN pull down? Would this be observed in glioblastoma cells? Consider a pull down assay for ZEB1 in the MCF7 lines with E-box mutants.
7) Figure 3C-D: what are the genes (pathways at least) repressed vs. activated in the Zeb1 only vs. Zeb1/Yap/JUN promoter peaks? It is unclear whether the distinction between the targets for Zeb1

only and the targets of Zeb1/Yap/JUN are functionally relevant and associated with distinct EMT gene expression outcome.
8) Please justify the rationale for transfecting 4x the amount of ZEB1 as YAP/Jun/FOSL1 in these assays in the methods.
9) Figure 4 attempts to make a connection between the Zeb1 only vs. Zeb1/Yap/JUN functional targets, but compares cells without induction of EMT, which is presented in the introduction as a pro-migratory and resistance/stem cell program. There is a disconnect between the data presented and the functional contribution of these observations (pro-metastatic features of EMT program controlled by Zeb1 in breast cancer cells). Is LLGL2 associated with breast cancer metastasis? What about ANKRD1 and DOCK9? Are those specifically associated with metastatic dissemination/migration/stemness? Are there any genes/candidates that would distinguish the distinct functional outcome of EMT of breast cancer cells and are differentially regulated by Zeb1 only vs. Zeb-1/Yap/JUN?
10) The data in Figure 4 suggest that Zeb1-only targets are transcriptionally repressed by Zeb1, whereas Zeb1/Yap/JUN enhance transcription on targets (Figure 1C). Can this be generalized and functionally linked to pro-metastatic EMT program? If not, is it rather associated with a resistance/pro-survival program? The experiment in Figure 4 should be interpreted in the context of inducing the invasive EMT program the authors introduce.
11) It is unclear what the meaning of the data shown in Figure 7 is with respect to the main conclusions of this manuscript. The authors cannot distinguish between Zeb1 only vs. Zeb1/Yap/JUN regulated targets in Figure 7D; and there is no clinical data associated with the data shown in Figure 7C. These data should be carefully interpreted.

Referee #3:

Triple-negative breast cancer is an aggressive subgroup of breast cancer with poor prognosis due to a complex genomic landscape.
In the manuscript entitled "Genome-wide cooperation of the EMT-activator ZEB1 with YAP and AP-1 factors in breast cancer" Dr. Brabletz and colleagues describe a genome-wide interaction of ZEB1 (known as pivotal cancer progression stimulator in many tumor types).
The authors already nicely published in 2016 the clinical relevance of ZEB1 in breast cancer, and here they investigated the role of ZEB1 on a genome-wide level in a cancer of epithelial origin, that is aggressive breast cancer. To do that they use the triple negative breast cancer cell line MDA-MB-231. They corroborate the observation that ZEB1 has different mode of action and show convincing evidence for the mode of action of EMT-TFs in cancer.

Nevertheless, considering the multifaceted activity di ZEB1 in epithelial and non-epithelial tumors, and the translational importance of these findings, the authors should explore more deeply the findings presented in Figure 7.

• It is worthy to investigate the expression and correlation level of ZEB1 and FOSL1 in in silico available data set (TGCA for instance) to validate the results in a bigger cohort.
• Moreover, would be interesting to investigate the correlation of ZEB1 mRNA expression with the expression of YAP, FOSL1 and JUN in human primary culture.

**Thomas Brabletz, Point by point reply to the decision**

Dear Dr. Brabletz,

Thank you for submitting your manuscript EMBOJ-2019-103209 to the EMBO Journal for consideration. My apologies again for the delay in getting back to you due to protracted referee input and detailed discussions in the team. Three referees have been assigned to your manuscript, and we have received reports from all of them, which I copy below. In light of the referees' input together with our internal discussions here in the team, I am afraid we decided that we cannot offer publication in The EMBO Journal. However, we do encourage you to transfer your study to our sister journal Life Science Alliance.

As you will see, the referees appreciate that the analysis extends previous work. However they also raise major concerns with the analysis that I am afraid preclude publication here. While referee #3 is more positive, both referees #1 and #2 are critical, stating a number of major issues related insufficient support for the claims made, generality of the work and pathophysiological relevance of the concept proposed. We agree to both reviewers that the claims made and the generality of the work is a bit overstated in the manuscript and will weaken this aspect within the whole manuscript, and instead focus more on the importance of our findings for breast cancer. We are nevertheless convinced about the relevance for the pathophysiology of (breast) cancer!
Also, they point out that causalities in cooperation between ZEB and Jun beyond co-occupancy, as well as proof of functional relevance and cellular downstream details remain open. We can adress all these points within a reasonable time frame of a revision.
In addition, generality of the findings is questioned and the exploration of the embryonic phenotype considered not rigorous enough See above for the generality issue. We did not discuss an embryonic phenotype?
In addition the referees state a number of additional issues related to statistical analysis, data display and inconsistencies between findings as well as missing controls. We feel that these critics are a bit overstated. These are minor points , which can all be easily addressed within a few days.
Given the overall negative opinions from good experts on the field, and considering our policy at the journal to generally allow for only one single major round of revision, our conclusions from the reports is, I am afraid that we cannot offer to publish your study in The EMBO Journal. We do not feel that referees 1 and 2 are overall negative. We successfully addressed reviews which were much more negative in the past! Most of the specific concerns can be addressed or resolved by discussion. The more negative statements at the beginning of their reviews are in our view often due to misunderstandings (see point by point rebuttal below, particularly to reviewer 1), which can be resolved. **Indeed this shows to us, that we should better explain some conclusions. We are absolutely convinced that we can address all of the concerns in one single round of revision.**

That being said, we recognize that your findings will be of value to the field. As to your preference at submission, I have thus discussed your work with Andrea Leibfried, executive editor of Life Science Alliance, and would therefore like to propose a transfer of your manuscript and referee reports to the new open-access journal Life Science Alliance. Life Science Alliance is launched as a partnership between EMBO Press, Rockefeller Press, and Cold Spring Harbor Laboratory Press, and publishes work that is of high value to the respective communities across all areas in the life sciences. I am happy to say that Andrea would like to publish your work, pending further minor revision. Andrea states 'We would expect a point-by-point response to all concerns raised and accordingly re-wording of the manuscript, re-analysis of the data already at hand, addition of statistical analysis and changes in data representation. The following experimental revisions should get included, too: rev#1, points 6, 7, 8, 11 (pt 11 is also requested by rev#3 and commented upon by rev#2 in his/her point 11). rev#3, two points mentioned in review.'. You can directly discuss

the revision with Andrea by contacting her at a.leibfried@life-science-alliance.org .

I realize that this negative decision will be disappointing, but I am afraid that the reports at hand did not allow for a different conclusion in this case. I hope you will view the possibility of a transfer favourably. If this is the case, please use the link below to transfer the manuscript directly.
**We would like to ask for a revision in EMBO J, because we do not feel that the reviews were so bad, and we can address all concerns in one revision round.**

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Editor
The EMBO Journal

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Referee #1:

Cell state changes, and particularly those associated with features linked to cellular plasticity, such as EMT, are fundamentally important elements of tumor development, progression, and metastasis. Thus, studies that examine the molecular mechanisms that regulate such cell state changes are of high interest. In Feldker et al., the authors investigate the transcriptional control of Zeb1 in order to further elucidate how this transcription factor functions to regulate cellular state changes in breast cancer. The authors use ChIP-seq, ATAC-seq, and luciferase assays to identify target sites associated with Zeb1, to identify regulatory or co-occupied transcription factors associated with these sites, and to study the transcriptional dynamics active at these sites. The authors describe an interaction between Zeb1 and the AP1 and Hippo pathway factors that regulate the transcriptional activity of these molecules, providing interesting insight into how differential transcriptional behavior associated with Zeb1 may be regulated. The authors deduce from their data that Zeb1 acts as a repressor when bound alone to its target site, while it acts as an activator when bound in conjunction with AP1/HIPPO factors in breast cancer, or other factors in glioblastoma.

While the data provide some interesting insight into the function and regulation of Zeb1 and Zeb1-target genes, there are significant issues that need to be addressed before this manuscript would be suitable for publication:
We do not see an overall negative review till here, but an encouragement to address the following points before publication.

1) Statistical analyses for the Venn diagrams (1C, 1E) are missing. **Will be done**.

2) The authors do not detect LEF/TCF motifs within the ZEB1 peaks in the breast cancer cells, and draw attention to the fact that such motifs were found in glioblastoma cancer cells, leading them to suggest that ZEB1 interacts with YAP/AP1 in breast cancer and TCF/LEF in glioblastoma. These findings are significantly overstated. We accept this critics about the comparison between breast cancer and glioma and would **weaken this conclusion all over the manuscript.** First, this is based on analysis of a single breast cancer cell line. This critics is a bit overstated (see also same point of reviewer 2). Everybody working with the factor ZEB1 knows, how difficult it is to handle this factor. In particular good antibodies for ChIP are not available. We started ZEB1 ChIP-Seq with three Zeb1 expressing breast cancer cell line and finally got reliable results (according to our cooperation partner at the MPI for

Epigenetics in Freiburg) only for MDA-MB 231. The critic is also a bit unfair, because in other referenced papers accepted in high-ranked journals, ChIP-Seq was also only done for one cell line (e.g. Zanconato et al , Nat Cell Biol using MDA-MB231, and even Rosmaninho et al. (also published in EMBO J !) only used one glioblastoma line for ZEB1 ChIP-Seq. The statement of reviewer 2 to this paper (claiming ZEB1 ChIP Seq in three glioblastoma cell lines) is not true! Moreover they did not even compare their ZEB1 ChIP Seq to a LEF1 ChIP Seq, while we have analysed ChIP Seqs for all three factors ZEB1,YAP and JUN!. **Thus we hope that this point is not seen different to the other mentioned publications! We accept part of the critics and will validate important results in additional cell lines.**
Second, breast cancer cell lines are a model system that cannot directly be equated with breast cancer, and certainly this one cell line bears little resemblance to the diverse spectrum of diseases collectively called breast cancer. We are of course aware that breast cancer is not breast cancer and already stated this throughout the manuscript. In fact we clearly showed that our findings are valid for and characterize the most aggressive breast cancer type, the claudin-low subtype!
Third, there multiple other differences besides just a BC vs. a GBM cell line, such as culture conditions (suspension vs. adherent) and culture media that may account for differential activity of Wnt signaling and impact TCF/LEF and Zeb1 function. This aspect of the paper either needs significantly more experimental attention, or should be removed from the abstract and main results. We are of course aware of the importance of cell culture conditions. However 1. both cell types (MDA-MB231 and the glioblastoma line are adherent), 2. in the mentioned glioblastoma paper (Rosmaninho, EMBO J) Wnt-signaling was not involved, and 3. importantly, MDA-MB 231 express a high endogenous level of LEF1 in the culture conditions we used for ZEB1 ChIP Seq, but nevertheless the LEF1-motif was not detected at ZEB1 peaks, unlike shown in glioblastoma! We will demonstrate this fact in a revised version. **Nevertheless we accept parts of the critics and will weaken the statements concerning comparison of breast cancer vs. glioblastoma.**

3) The authors showed coincidence between JUN and ZEB1 peak summits and infer direct interaction between Zeb1 and Jun at these sites. However, this conclusion is not justified in the absence of a direct experiment to demonstrate this for the following reason. The authors show that 32% of the ZEB1 peaks do not have a high affinity ZEB1 motif and JUN ChIP-seq peaks only overlap with about 1/3 ZEB1 peaks. We do not understand this critical point here, because the mentioned numbers (from Fig. 1A, 68% of Zeb1 peaks are at Zeb1 motifs and therefore 32 % not, and Fig. 1c one third of the ZEB1 peaks overlap with Jun peaks) fit (32% of peaks without ZEB1 motif, is equivalent to one third overlap with Jun peaks) and support the hypothesis that ZEB1 at these peaks binds indirectly through Jun/Fos to AP1 sites. Somehow the reviewer misunderstood the figures? **We will make it more clear.** Further, ZEB1 physically interracts with JUN and FOSL1. Therefore, it is necessary to show that within JUN & ZEB1 overlapping peaks, such peaks are enriched for both JUN and ZEB1 motifs and both proteins are actually interacting in those sites. before claiming the cooperation between ZEB1 and JUN in chromatin binding. I fear the reviewer did not understand our conclusion, because the latter is exactly not the case: overlapping peaks for Jun and Zeb1 do not enrich for the classical ZEB1 motif! Therefore, how should ZEB1 enrich at an AP1-site (without a ZEB1-site), if not indirectly through JUN/FOS. We have long ago shown that ZEB1 alone does not directly bind to an AP-1 site. **We will also make this more clear in the manuscript. In addition, we will further validate the interaction experimentally.**

4) ZEB1 only peaks are enriched near promoters while ZEB1/YAP/AP1 peaks are enriched in the distal regions (presumably enhancers). However, when the author tried to associate these two different groups of ZEB1 peaks to either repression or activation, only the promoter proximal ZEB1/YAP/AP1 peaks (not the majority of these jointly bound peaks) were used. Thus, these data do not support the conclusion that "ZEB1 in cooperation with YAP and AP-1 factors seems to act mainly as a transcriptional activator." First, I can refer to **ATAC-Seq in Fig 6B fully supporting our conclusion**: Zeb1/Yap/AP1 peaks enrich in open chromatin

regions (also mainly distal regions!), whereas Zeb1 only peaks are found mainly in closed chromatin. Second, **we performed experiments for both promoter and enhancer (Dock9)** regions. In this study we focused on promoters, because they can clearly be attributed to genes, whose transcripts we have shown to be also depending on Zeb1 expression. We currently carefully analyse enhancer regions, which also includes identification of the respective genes (by defining enhancers with ChromHMM and annote genes with e.g. TAD boundary files, etc.) and further functional validation (by CRISPR modification in chromatin context). This is a long project which just started with a new grant support and goes far beyond inclusion in this manuscript.

5) Other studies to confirm this point involved the use of transient assays with co-transfected proteins and luciferase expression. It is well known that the DNA in such transient studies is not chromatinized in the same way as endogenous genes. This might explain the huge fold induction observed, but only the very modest effects on "repression." More convincing would have been direct analysis of transcripts produced from endogenous genes engineered by CRISPR to contain or lack YAP/AP1 sites and to analyze expression mediated by expression of endogenous proteins expressed at physiologic levels. Reporter assays are standard to measure the effect of transcription factors. Also DNA in transient studies is chromatinized, of course we agree, not fully in the same way. Nevertheless performing CRISPR engeneering to study promoter mutations are not standard experiments for promoter analyses, and were not requested in similar publications (eg Rosmaninho et al EMBO J, 2018). We also **think that the critisized activation and repressing levels in Fig. 4C and D look convincing and support our conclusion!** I have seen published really bad reporter assay data, and these do not belong to this group.

6) Antibody controls are missing for Figure 2A. A straightforward control would be to create KO clones with CRISPR, and there should be no signal. We used here validated commercial antibodies, and **will further show controls** (e.g. siRNA kd for the factors).

7) An additional control for Figure 2B would be to attempt the in situ proximity ligation assay between Zeb1 and a factor that it should not interact with (NLS-GFP may be one example), to confirm that the signal is truly showing specific interactions within the nucleus, and not just random interactions between two highly expressed nuclear factors .**Will be done**

8) For the CO-IP experiments, shouldn't the IP lane produce significantly more protein than the input, which is not the case with Zeb1. In fact, the amount of protein in the input lane is never comparable to the IPs. The efficiency of the IPs depends on the specific efficency of the antibody on the spcific factor. **IPs for ZEB1 are always less efficient (probably due to ist huge size of 230 kd) than for small factors like Jun or Fos.** The presence of pulldown in the IgG lanes raises concerns about specificity and whether these are real interactions or just artifacts. **We do not agree to this critics.** There is an extremely weak pulldown in the IgG lanes, by far weaker than in the ZEB1, Jun and FOSL1 lanes, clearly indicating specificity.

9) With the ChIP-seq and ATAC-seq data, the authors can make precise editing of target sites of interest with CRISPR to evaluate the consequences on gene expression of manipulating Zeb1 binding sites in different contexts. This would be more convincing than the artificial luciferase assays. **ZEB1 sites are missing at most activated genes. I think again, we should make this more clear, since the reviewer overlooked this key finding of our paper in several comments!** Moreover we refer to our answer to points 4 and 5 that CRISPR modifications at enhancers are out of range for this manuscript and a project in this direction just started.

10) While the authors perform some genetic perturbations of Zeb1 and the other factors, an experiment that would add more depth to these studies would be to extrinsically modulate these factors where possible. As the Hippo pathway can be regulated by extrinsic cues, the

transcriptional activation of Zeb1-YAP loci should be interrogated in culture conditions favoring differential Hippo activation. We have previously demonstrated that siRNA depletion of YAP inhibits expression of Zeb1/YAP common target genes (Lehmann et al. 2016). As suggested, **we will further address this point** including newly identified Zeb1/YAP/AP1 target genes, which do not overlap with the Zeb1/YAP target gene signature and will also use pharmacological inhibition (eg. Verteporfin for YAP and SP600125 for JNK/AP1).

11) In Figure 7 the authors attempt to link their findings in MB231 cells to human breast tumors. Unfortunately, to be convincing, the authors would need to demonstrate that similar mechanisms are at play within these breast cancers. Do the authors see that Zeb1 only tumors show a transcriptomic profile with elevated features linked to the Zeb1-only ChIP-seq sites? Do Zeb1+FOSL1 tumors show a transcriptomic profile with features linked to the Zeb1+API ChIP-seq sites? Again I fear that the reviewer did not fully understand the conclusion of our findings. There is no ZEB1 only and seperate ZEB1/YAP/AP1 type of breast cancer! But only a ZEB1 negative type (all subtypes besides Claudin-low) and a ZEB1 poisitive subtype (Claudin low), which in most cases also co-expresses AP1 and YAP. (Only) in the latter both mechanisms of ZEB1 (repression and activation) are active in parallel on different target gene sets (differentiation and tumor-promoting, resp.). Thus the suggested questions and investigations are invalid. Instead **we will take up his/her general suggestion and could correlate ZEB1 expression with downstream target genes (activated or repressed) in breast cancer (this means Claudin low vs. other subtypes).**

Other issues:
1. No supplemental figures and tables were included in this submission. **This is not true**, but might have influenced the general attitude of the reviewer to our manuscript. Moreover he/she probably did not see all necessary data. It might also explain, why he/she misunderstood many of our conclusions?

Referee #2:

In this study, the authors perform CHIP-seq analysis of Zeb1 on MDA-MB-231 cells to explore its binding sites in this cell line. The introduction sets up the rationale for the study: uncover an explanation for the seemingly controversial role of Zeb1 in cancer metastasis/invasive program. This is however rather misleading and confusing. The description of the literature surrounding the controversy of EMT transcription factors in distinct cancer types is inaccurate and superficial (and perhaps unnecessary given the limited scope of the present study on Zeb1). The authors present a confusing picture of the distinct functions attributed to EMT program. For example, deletion of certain transcription factors (not Zeb1) showed impact on therapy resistance but not metastatic dissemination (Zheng 2015). Deletion of Zeb1 in pancreatic cancer suppressed both primary tumor growth and metastasis (Krebs 2017). Zeb1 deletion in breast cancer mouse models in vivo has not been realized, and it is unclear what EMT phenotype will be affected (chemoresistance, metastasis, or both). A revision of the introduction may be needed to bring into better focus the study question and clarify the interpretation of the main conclusions. **We are sorry for generating confusion in the introduction**.We did not want to focus on the controversy around EMT, but simply wanted to point out that context-dependent differences in the function of EMT-transcription factors could be one reason. As mentioned above **we agree to this critics and can easily rewrite not only the introduction but also other manuscript parts** to set a different focus (less on breast cancer vs. glioblastoma, but more on characterising a subtype of breast cancer) The study presented does not place the data in the context of breast cancer EMT outcome: there is no clear indication as to what is the functional EMT outcome of the distinct Zeb1 transcription regulation shown here (similar to

reviewer 1 he did not fully understand the point, which **clearly shows that we must explain it better**: the distinct functional Zeb1 regulation does not separate breast tumor types, but act in parallel in one cell and characterize the most agressive subtype, Claudin-low), no indication as to what are the genes repressed/activated and their distinct roles in migration, stemness, metabolism, resistance to apoptosis, etc **(We agree and can easily deliver additional data to this critics)**. As detailed below, the design of the experiment is limited to a single cell line. This should be addressed experimentally. It is noted in the abstract, introduction, discussion, and concluding model figure, that a tissue specific feature has arose from their study, implicating distinct Zeb1 transcriptional partners in breast cancer vs. glioblastoma (published dataset, obtained using three glioma cell lines (Rosmaninho). **This is not true**! Rosmaninho only perfomed ZEB1 ChIP-Seq in one glioblastoma line (NCH421k), and not even used LEF1 ChIP Seq. (see also rebuttal to referee1, point 2). But we will take up this critics and verify specific results in other ZEB1 expressing br ca lines). This is presented as a main conclusion from the study, but in fact has hardly no representation in the manuscript. The authors should consider performing their own analysis on distinct cell types to strengthen this conclusion. **As already stated above, we will weaken the conclusion.**
Like for referee 1, we do not see an overall negative review till here, but an encouragement to address the points before publication.

1) A critical drawback from the presented study is the generalization made on data obtained from ChIP-seq data of a single breast cancer cell line. The cell line studied, MDA-MB-231, represents a metastatic, mesenchymal-like cell line, thus bringing into question the conclusions made with respect to the role of Zeb1 in inducing acquisition of EMT/mesenchymal features The conclusion is only made for ZEB1 expressing breast cancers, which are according to our data only the Claudin low subtype. Does the induction of EMT (by TGFb, for example) in breast cancer cell lines that does not normally express ZEB1 result in a switch from Zeb1-only (repressor) to Zeb-1/Yap/JUN (activator). I fear that also this reviewer did not fully understand our conclusion, we will make it more clear in the revised mansucript (maybe the word „switch“ generates this problem, we will omit it): a cell that does not express Zeb1 can also have no ZEB1 repressor function. As mentioned above, if ZEB1 is expressed it exerts both functions (repressive and activating) in the same cell in parallel. The use of a single cell line is (not?) sufficient to generalize findings to breast cancer. Please see our the statement to ZEB1 ChIP Seq in one cell line above (Ref 1, point 2). **We agree and 1. will validate findings in other breast cancer cell lines and 2. weaken a bit the overall conclusions.**

2) It is unclear what is shown in Supplementary Figure 1D. It is assumed that the 45.1% activated genes represent the transcripts downregulated in the Zeb1 knockdown cells compared to control cells (please clarify) **Can be clarified very easy**. Can a heat map and pathway analysis be performed on these published data to validate the EMT related signature of Zeb1 knockdown, thereby affirming whether the genes activated are pro-mesenchymal/stem cell/migratory genes while the genes repressed are epithelial genes? **OK, good point, will be done.**

3) Could the author ascertain whether the analysis shown in Figure 1D extend to published ChIP-seq data set of the AP-1 factor JUN. He/she misunderstood the Fig. 1D, we will give a better explanation of these often a bit cryptic ChIP-Seq data presentations: shown is an overlap between Jun and ZEB1 peak summits (defined as within +/- 200), which demonstrates that summits have very low distance) ? What fraction of gene promoters in the current analysis shown in Figure 1D overlap with analysis from correlation with JUN ChIP-seq data? Shown in Fig. 1C are all 12617 ZEB1 peaks, the JUN overlapping 4201 peaks are shown beteen the dotted lines in Fig. 1D.

4) Can the author clarify the discrepancy in the p-values shown in Figure 1A and

Supplementary Table 4. **Can be easily done**: Fig. 1A corresponds to Suppl Table 1, based on ChIP Seq, Suppl. Table 4 is based on ATAC Seq. The rank listing shows AP-1 at number 10 in the top 25 enriched motifs, but is listed as rank 2 in Figure 1A and in the text. Please include an explanation of the curated table shown in Figure 1A. **OK, will be done.**

5) Consider providing a circle plot to show the Zeb1, YAP, AP-1 (JUN, FOSL1), and LEF/TCF peak intensity for all chromosomes to support the claim made regarding Zeb1 acting 'genome wide', and to aid in the visualization of Zeb1/AP-1 in breast cancer vs. Zeb1/LEF/TCF in glioblastoma **(Yes, we will present circle plot).**

6) Figure 2D: Can the authors confirm that modulation in Zeb1 protein content impact JUN pull down? In other words, would the suppression of Zeb1 result in loss of JUN pull down, or would high expression of Zeb1 (via induction of EMT) in breast cancer cells would enhance JUN pull down? Would this be observed in glioblastoma cells? If really wanted, we could show this in ZEB1 depleted clones. But does it really make sense? Without ZEB1, there is no ZEB1 IP. We did not claim that ZEB1 modification affects JUN or YAP DNA binding, but that DNA-binding of JUN and YAP is important for ZEB1 DNA-binding. **We will address this question of the reviewer by additional experiments.** Consider a pull down assay for ZEB1 in the MCF7 lines with E-box mutants. We are not sure, what is meant here? Probably a Zeb1 ChIP for WT and mut ANKRD1 luc construct, referring to Fig. 5C and D? **We will try this.**

7) Figure 3C-D: what are the genes (pathways at least) repressed vs. activated in the Zeb1 only vs. Zeb1/Yap/JUN promoter peaks? **We can easily include it in a revised version.** It is unclear whether the distinction between the targets for Zeb1 only and the targets of Zeb1/Yap/JUN are functionally relevant and associated with distinct EMT gene expression outcome. **We can also easily include it in a revised version (**GO term analyses, GSEA,, show differentiation vs. umor promoting, make again clear that this goes on in parallel in one cell!).

8) Please justify the rationale for transfecting 4x the amount of ZEB1 as YAP/Jun/FOSL1 in these assays in the methods. **We titrated to find the optimal ratio for target gene activation and reporter assays**, because ZEB1 overexpression was generally weaker than for the other factors.

9) Figure 4 attempts to make a connection between the Zeb1 only vs. Zeb1/Yap/JUN functional targets, but compares cells without induction of EMT, which is presented in the introduction as a pro-migratory and resistance/stem cell program. There is a disconnect between the data presented and the functional contribution of these observations (pro-metastatic features of EMT program controlled by Zeb1 in breast cancer cells). Is LLGL2 associated with breast cancer metastasis? What about ANKRD1 and DOCK9? Are those specifically associated with metastatic dissemination/migration/stemness. **Will be easily done** (explain known functions of shown genes and refer to further functional connections in new GO term and GSEA analyses). Are there any genes/candidates that would distinguish the distinct functional outcome of EMT of breast cancer cells and are differentially regulated by Zeb1 only vs. Zeb-1/Yap/JUN?. Again, as stated above, the reviewer did not fully understand, that the different modes of action take place in one cell**. We will explain this better.**

10) The data in Figure 4 suggest that Zeb1-only targets are transcriptionally repressed by Zeb1, whereas Zeb1/Yap/JUN enhance transcription on targets (Figure 1C). Can this be generalized and functionally linked to pro-metastatic EMT program? If not, is it rather associated with a resistance/pro-survival program? The experiment in Figure 4 should be interpreted in the context of inducing the invasive EMT program the authors introduce. **We agree, and all points can be easily addressed** (see above, by GO, GSEA, etc.).

11) It is unclear what the meaning of the data shown in Figure 7 is with respect to the main conclusions of this manuscript. The authors cannot distinguish between Zeb1 only vs. Zeb1/Yap/JUN regulated targets in Figure 7D (Again this is due to misunderstanding that the different modes of action occur in **one cell, there is no sepation in ZEB1 only and ZEB1/YAP/AP1 type!** Thus in Fig. 7D we analysed hormone receptor negative breast cancers, which fall into a ZEB1 high, poor prognosis type (likely claudin-low) and a ZEB1 low, good prognosis type (likely basal-type, according to Fig. 7B). **We definitely will explain this better**) and there is no clinical data associated with the data shown in Figure 7C. These data should be carefully interpreted. Here we only correlated expression, there were no clinical data available for this set. Clinical follow up is shown in 7D. **In a revised version we will include additional data for clinical relevance of Zeb/YAP/AP1 target gene sets**.

Referee #3:

Triple-negative breast cancer is an aggressive subgroup of breast cancer with poor prognosis due to a complex genomic landscape.
In the manuscript entitled "Genome-wide cooperation of the EMT-activator ZEB1 with YAP and AP-1 factors in breast cancer" Dr. Brabletz and colleagues describe a genome-wide interaction of ZEB1 (known as pivotal cancer progression stimulator in many tumor types). The authors already nicely published in 2016 the clinical relevance of ZEB1 in breast cancer, and here they investigated the role of ZEB1 on a genome-wide level in a cancer of epithelial origin, that is aggressive breast cancer. To do that they use the triple negative breast cancer cell line MDA-MB-231. They corroborate the observation that ZEB1 has different mode of action and show convincing evidence for the mode of action of EMT-TFs in cancer.

Nevertheless, considering the multifaceted activity di ZEB1 in epithelial and non-epithelial tumors, and the translational importance of these findings, the authors should explore more deeply the findings presented in Figure 7.

• It is worthy to investigate the expression and correlation level of ZEB1 and FOSL1 in in silico available data set (TGCA for instance) to validate the results in a bigger cohort. **We agree.** As suggested, we will try directly for ZEB1, but in most breast cancers subtypes ZEB1 is expressed only in stroma, and most TCGA data sets are not generated from microdissected tumors. Therefore the better alternative is probably to correlate clinical outcome with the expression of a specific Zeb1/YAP/AP1 target gene set, which we will do.
• Moreover, would be interesting to investigate the correlation of ZEB1 mRNA expression with the expression of YAP, FOSL1 and JUN in human primary culture. We will also try to use published data sets from patient derived tumor cell cultures.

## 1st Revision - Editorial Decision 11th Dec 2019

Dear Simone, dear Thomas,

Thank you for contacting me regarding our decision and for your patience with my response, which as mentioned got much delayed due to protracted consultation with the referees. We have now carefully assessed your rebuttal letter together with the study, and related literature and internally re-discussed the points raised in the reviewers' reports and your point-by-point response.

We appreciate your outline for a substantive experimental revision, and realise that you would - judging from the information provided in the rebuttal letter - be potentially able to address important issues raised by the referees.

We thus invite you to work towards a re-review and will be able to return a revised version to the referees for evaluation. Please note however that it would be essential to address the core concerns raised regarding generality and pathophysiological relevance of the findings as well as more detailed characterization of the downstream genes and pathways affected by ZEB1-only versus co-occupied sites in compelling manner and to the satisfaction of the referees. As you point out in your response, this might include complementary melanoma data. At the same time, we agree it should be reasonable to de-emphasize the comparison with glioblastoma and focus on the current ZEB1-AP1-YAP cooperation and its control.

We editorially concur that while per se well taken, the referees' arguments regarding CRISPR editing of the triple binding site are to be considered as out of scope for the current work.

Please note, that given the referees' critical view, we cannot at this stage guarantee that enthusiasm for EMBO Journal will be sufficiently high.

Please contact me if you have any questions related to this.

I am looking forward to your revised manuscript.

Kind regards,

Daniel

Daniel Klimmeck, PhD
Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-

specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

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

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

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Method) that follows the model below (see also http://emboj.embopress.org/authorguide#dataavailability). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

# Data availability

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

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

Our journal also 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 .

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (http://emboj.embopress.org/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
http://emboj.embopress.org/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 10th Mar 2020.

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## Reply to the editor's comments:

*We are grateful for the positive revision, the identification of priority points and your valuable suggestions, how to address some of the referees' queries. We improved the manuscript a lot and marked all changes using green writing.*
*Please see a point by point reply to your comments below and to the referee comments for more detailed explanations and for additional new experiments not listed in your priority list:*

Dear Simone, dear Thomas,

Thank you for contacting me regarding our decision and for your patience with my response, which as mentioned got much delayed due to protracted consultation with the referees. We have now carefully assessed your rebuttal letter together with the study, and related literature and internally re-discussed the points raised in the reviewers' reports and your point-by-point response.

We appreciate your outline for a substantive experimental revision, and realise that you would - judging from the information provided in the rebuttal letter - be potentially able to address important issues raised by the referees.

We thus invite you to work towards a re-review and will be able to return a revised version to the referees for evaluation. Please note however that it would be essential to address the core concerns raised regarding generality and pathophysiological relevance of the findings
*- As you will recognise, we addressed these important concerns, by including additional cell lines, characterizing tumor promoting functions of downstream targets and a stronger focus on expression patterns and clinical outcome in publicly available datasets.*
as well as more detailed characterization of the downstream genes and pathways affected by ZEB1-only versus co-occupied sites in compelling manner and to the satisfaction of the referees.
*- We now included a detailed characterisation of the ZEB1 downstream targets and pathways, comparing the ZEB1-only repressed targets with the ZEB1/YAP/AP-1(JUN) activated targets, showing that they have opposing functions and thus, that their opposing regulation synergistically leads to tumour progression and poor outcome.*
As you point out in your response, this might include complementary melanoma data.
*- We startet a cooperation with Jean-Christophe Marine's group from the University of Leuven (Belgium), who published a functional interaction of AP-1 and TEAD in invasive melanoma only with high Zeb1 expression (see reference: Verfaillie et al. Nat Commun 2015). In November we startet to generate and compare Zeb1, Ap-1 and Tead depending transcriptomes and ChIPSeq data in melanoma. However before finishing the data collection his lab was completely shut down due to the Coronavirus pandemic. We hope to restart the promising collaboration soon, but decided to leave this aspect out of the manuscript since it is not finished yet and we don't know who long this situation will last. We referenced this manuscript as supporting findings in melanoma by identifying AP-1/TEAD as main regulators of the invasive state that is marked by high ZEB1 expression.*
At the same time, we agree it should be reasonable to de-emphasize the comparison with glioblastoma and focus on the current ZEB1-AP1-YAP cooperation and its control.
*- We put the comparison to glioblastoma out of the focus throughout the whole manuscript and only discussed the different results in glioblastoma and breast cancer as well as potehtial explanations in a short discussion chapter. The manuscript is now strongly focussed on the results in breast cancer, with the main conclusion that our findings characterize the highly aggressive claudin-low subtype.*
We editorially concur that while per se well taken, the referees' arguments regarding CRISPR editing of the triple binding site are to be considered as out of scope for the current work.
*- Thank you for sharing our view. We recently got a three year DFG grant for in depth analyses of Zeb1 dependent enhancer identification and characterisation (in cancer), including in vivo analyses. So we definitely agree to this reviewers' comment, but want to do it carefully.*

Please note, that given the referees' critical view, we cannot at this stage guarantee that enthusiasm for EMBO Journal will be sufficiently high.

Please contact me if you have any questions related to this.

I am looking forward to your revised manuscript.

Kind regards,

Daniel

Daniel Klimmeck, PhD
Editor
The EMBO Journal

## Reply to Referee #1:

*Thank you for the positive evaluation of our manuscript. We are grateful for the critical and very helpful comments. Based on your suggestions we changed critical parts throughout the manuscript text, and particularly included clarifications and weakened conclusions. We apologise for causing confusion. The novel parts of the text are marked in green. Please see our point by point reply below your specific comments:*

Referee #1:

Cell state changes, and particularly those associated with features linked to cellular plasticity, such as EMT, are fundamentally important elements of tumor development, progression, and metastasis. Thus, studies that examine the molecular mechanisms that regulate such cell state changes are of high interest. In Feldker et al., the authors investigate the transcriptional control of Zeb1 in order to further elucidate how this transcription factor functions to regulate cellular state changes in breast cancer. The authors use ChIP-seq, ATAC-seq, and luciferase assays to identify target sites associated with Zeb1, to identify regulatory or co-occupied transcription factors associated with these sites, and to study the transcriptional dynamics active at these sites. The authors describe an interaction between Zeb1 and the AP1 and Hippo pathway factors that regulate the transcriptional activity of these molecules, providing interesting insight into how differential transcriptional behavior associated with Zeb1 may be regulated. The authors deduce from their data that Zeb1 acts as a repressor when bound alone to its target site, while it acts as an activator when bound in conjunction with AP1/HIPPO factors in breast cancer, or other factors in glioblastoma.

While the data provide some interesting insight into the function and regulation of Zeb1 and Zeb1-target genes, there are significant issues that need to be addressed before this manuscript would be suitable for publication:

1) Statistical analyses for the Venn diagrams (1C, 1E) are missing.
*We included statistical analyses for the Venn diagrams. See new Figs 1C/G and Fig EV1G.*

2) The authors do not detect LEF/TCF motifs within the ZEB1 peaks in the breast cancer cells, and draw attention to the fact that such motifs were found in glioblastoma cancer cells, leading them to suggest that ZEB1 interacts with YAP/AP1 in breast cancer and TCF/LEF in glioblastoma. These findings are significantly overstated.
*We agree with these critics about the comparison between breast cancer and glioma. We now took the comparison to glioblastoma out of the focus throughout the whole manuscript. We now included an additional Figure (new EV5A) showing that LEF1 is generally lower expressed in breast cancer cell lines than in glioblastoma lines, offering a possible explanation for a minor role in the breast cancer context and only discussed the different results in glioblastoma and breast cancer in a short discussion chapter. Overall the manuscript is now strongly focussed on the results in breast cancer, with the main conclusion that our findings characterize the highly aggressive claudin-low subtype.*
First, this is based on analysis of a single breast cancer cell line.
*We agree that it is always problematic to use only one cell line. However, it turned out that ChIP-seq for transcription factor Zeb1 was much more complicated than e.g. for Histone marks. Labs working with the factor Zeb1 know, how difficult it is to handle this factor. In particular good antibodies for ChIP are not available. We started Zeb1 ChIP-Seq with three Zeb1 expressing breast cancer cell lines and finally got reliable results (according to our cooperation partner at the MPI for Epigenetics in Freiburg) only for MDA-MB 231. Additionally, in other referenced papers accepted in high-ranked journals, ChIP-Seq was also only done for one cell line (e.g. Zanconato et al , Nat Cell Biol using MDA-MB231, and even Rosmaninho et al. (also published in EMBO J !) only used one glioblastoma line for ZEB1 ChIP-Seq. To evaluate that our findings are valid, we now repeated important following experiments with two additional basal type breast cancer cell lines, namely BT549 and*

*Hs578T. We now show the proximity ligation assay for all three cell ines (see Figs 2A and new EV2B). Importantly, we also verified the target gene regulation via Luciferase reporter assays in BT549 and Hs578T (new Fig EV4B) and in a completely different system, the MCF10A epithelial mammary cell line that nicely responds to TGFß treatment by upregulating ZEB1 and undergoing an EMT (new Figs EV4A and Fig 4A).*

Second, breast cancer cell lines are a model system that cannot directly be equated with breast cancer, and certainly this one cell line bears little resemblance to the diverse spectrum of diseases collectively called breast cancer.

*We apologize for having not been clear enough. We are of course aware that breast cancer is not breast cancer and tried to state this throughout the manuscript. We showed that our findings are valid for and characterize one of the most aggressive breast cancer types, the claudin-low subtype. We now stated this already in the abstract and tried to make it clearer throughout the whole manuscript.*

Third, there multiple other differences besides just a BC vs. a GBM cell line, such as culture conditions (suspension vs. adherent) and culture media that may account for differential activity of Wnt signaling and impact TCF/LEF and Zeb1 function. This aspect of the paper either needs significantly more experimental attention, or should be removed from the abstract and main results.

*As mentioned above, we agree that we had to de-emphasise the comparison of BC to GBM, what we did and also offered the generally low expression as a possible reason for the minor role of LEF1 in BC (new Fig EV5A). Of note, both cell types grow under adherent conditions and Rosmaninho et al. (EMBO J, 2011) showed that Wnt signaling was not involved in its function together with ZEB1*

3) The authors showed coincidence between JUN and ZEB1 peak summits and infer direct interaction between Zeb1 and Jun at these sites. However, this conclusion is not justified in the absence of a direct experiment to demonstrate this for the following reason. The authors show that 32% of the ZEB1 peaks do not have a high affinity ZEB1 motif and JUN ChIP-seq peaks only overlap with about 1/3 ZEB1 peaks.

*We do not understand this critical point here, but thank you to alert us that our description was not clear enough, which might cause confusion. The mentioned numbers (from Fig. 1A, 68% of Zeb1 peaks are at Zeb1 motifs and therefore 32 % not, and Fig. 1c one third of the Zeb1 peaks overlap with Jun peaks) fit almost perfectly (32% of peaks without Zeb1 motif, is equivalent to one third overlap with Jun peaks) and fully support the hypothesis that Zeb1 at these peaks binds indirectly through Jun/Fos to AP1 sites. We made this more clear in the manuscript now.*

Further, ZEB1 physically interracts with JUN and FOSL1. Therefore, it is necessary to show that within JUN & ZEB1 overlapping peaks, such peaks are enriched for both JUN and ZEB1 motifs and both proteins are actually interacting in those sites before claiming the cooperation between ZEB1 and JUN in chromatin binding.

*We again apologise for not explaining this result properly and peformed additional experiments to support a cooperation. We here show that overlapping peaks for Jun and Zeb1 do not enrich for the classical Zeb1 motif! Therefore, we postulated a likely indirect recruitment of Zeb1 to DNA in this context: Indeed, 32% of the ZEB1 peaks do not have a high affinity ZEB1 binding motif (Fig 1A). This nicely fits to our result, that DNA-binding in the ZEB1/YAP/JUN common peaks does not need this motif as is shown in Fig 5C and new Fig 5D. Therefore, it's not expected that common ZEB1/YAP/JUN peaks are enriched for the ZEB1 motif, but for AP-1- and TEAD-motifs, whereas it is the other way round in the ZEB1-only peaks (Fig 5A). We tried to confirm the cooperative binding of all three factors, which is not easy because a Re-ChIP experiment relies on crosslinked chromatin and would therefore give false positive results, even if the factors bind independantly. To circumvent this, we performed electromobility shift assays to demonstrate cooperative binding within native (not cross-linked) protein complexes. With this, we managed to show that binding of a specific competible high molecular weight complex to a TEAD motif is sterically inhibited by specific antibodies not only against TEAD (the DNA-binding partner of YAP1) himself, but also against both, ZEB1 and JUN, whereas the non-specific control antibodies have no influence*

*(new Fig 5D left). An indirect DNA binding of ZEB1 to TEAD sites (likely through interaction with AP-1 and YAP1) that do not contain the high affinity ZEB1 motif was further confirmed by the fact that recombinantly expressed ZEB1 binds the ZEB1 motif but does not bind the TEAD or the AP-1 motif (new Fig. 5D right).*

4) ZEB1 only peaks are enriched near promoters while ZEB1/YAP/AP1 peaks are enriched in the distal regions (presumably enhancers). However, when the author tried to associate these two different groups of ZEB1 peaks to either repression or activation, only the promoter proximal ZEB1/YAP/AP1 peaks (not the majority of these jointly bound peaks) were used. Thus, these data do not support the conclusion that "ZEB1 in cooperation with YAP and AP-1 factors seems to act mainly as a transcriptional activator."
*Here, we firstly refer to the ATAC-Seq data in Fig 6B that fully support our conclusion: Zeb1/Yap/AP1 peaks enrich in open chromatin regions (also mainly distal regions!), whereas Zeb1 only peaks are found mainly in closed chromatin. Furthermore, we performed experiments for both promoter and enhancer (Dock9) regions (Fig 4E). However, we completely agree, that thorough analysis of those peaks in the putative enhancer regions is of great interest. We currently carefully analyse these regions, also including identification of the respective genes (by defining enhancers with ChromHMM and annote them to genes with e.g. TAD boundary files, etc.) and further functional in vitro and in vivo validation (by CRISPR modification in chromatin context). This is a long project which just started with a new grant support and goes beyond inclusion in this manuscript. In this study, we focused on promoters because they can be clearly attributed to genes, whose transcripts we have shown to be also depending on Zeb1 expression. Importantly, we have got highly significant results even from the few promoter regions we could use (Fig 3D/E).*

5) Other studies to confirm this point involved the use of transient assays with co-transfected proteins and luciferase expression. It is well known that the DNA in such transient studies is not chromatinized in the same way as endogenous genes. This might explain the huge fold induction observed, but only the very modest effects on "repression." More convincing would have been direct analysis of transcripts produced from endogenous genes engineered by CRISPR to contain or lack YAP/AP1 sites and to analyze expression mediated by expression of endogenous proteins expressed at physiologic levels.
*We agree, that luciferase reporter assays are not completely mirroring the endogenous situation. However, they are still standard to measure the effects of transcription factors. Also DNA in transient studies is chromatinized, of course we agree, not fully in the same way. And we completely agree, that repression of an endogenous factor has milder effects than recombinant overexpression. This is expectable and sound. To further confirm the regulation of target genes, we now included direct analyses of transcripts firstly, after TGF$\beta$ mediated ZEB1 upregulation and secondly, after following siRNA transfection targeting ZEB1, YAP or the AP-1 factor FOSL1 (new Fig EV4A and new Fig 4A).*

6) Antibody controls are missing for Figure 2A. A straightforward control would be to create KO clones with CRISPR, and there should be no signal.
*We included antibody controls using specific siRNAs (new Supplementary Fig 1A). We certainly used two different si RNAs for each factor, but show here only one representative example.*

7) An additional control for Figure 2B would be to attempt the in situ proximity ligation assay between Zeb1 and a factor that it should not interact with (NLS-GFP may be one example), to confirm that the signal is truly showing specific interactions within the nucleus, and not just random interactions between two highly expressed nuclear factors .
*We thank the referee for indicating this weakness. We have now included the Hippo factor TAZ that is nuclearly expressed in our cell lines (new Fig EV2A) in the PLA assays. As shown in new Fig 2A and new Fig EV2B, we don't see an interaction between TAZ and ZEB1 in any of the three cell lines, confirming the specificity of the interaction between ZEB1 and*

*JUN and ZEB1 and FOSL1. (As a side effect this result points out to differences between TAZ and its closely related factor YAP, which however is not the topic of this manuscript).*

8) For the CO-IP experiments, shouldn't the IP lane produce significantly more protein than the input, which is not the case with Zeb1.
*The amount of protein in the input lane is not comparable to the IPs. The efficiency of the IPs depends on the specific efficency of the antibody for the specific factor. IPs for ZEB1 are unfortunately in general less efficient (probably due to ist huge size of 230 kd) than for small factors like Jun or Fos.*
The presence of pulldown in the IgG lanes raises concerns about specificity and whether these are real interactions or just artifacts.
*Indeed, we were quite happy to achieve these co-precipitations, and are convinced that they are no artefacts. There is only an very weak pulldown in the IgG lanes, by far weaker than in the ZEB1, Jun and FOSL1 lanes, clearly indicating specificity. Further, they do not stand alone, but are supported by the PLA-assays, the electromobility shift assays, the ChIP-seq data and all our functional analyses.*

9) With the ChIP-seq and ATAC-seq data, the authors can make precise editing of target sites of interest with CRISPR to evaluate the consequences on gene expression of manipulating Zeb1 binding sites in different contexts. This would be more convincing than the artificial luciferase assays.
*We again apologise for causing confusion. ZEB1 motifs are missing in most of the activating peaks. In those regions, ZEB1 likely binds via its co-factors YAP and AP-1 (see Fig 5). In addition, I refer to the above reply to point 5, hoping to convinced the reviewer to a certain level. Of course, CRISPR modifications of enhancer regions and their in depth in vitro and in vivo characterisation are planned for our ongoing project and will be included in a future manuscript.*

10) While the authors perform some genetic perturbations of Zeb1 and the other factors, an experiment that would add more depth to these studies would be to extrinsically modulate these factors where possible. As the Hippo pathway can be regulated by extrinsic cues, the transcriptional activation of Zeb1-YAP loci should be interrogated in culture conditions favoring differential Hippo activation.
*We agree with the referee, that extrinsic modulation of the factors would add depth to our study and performed additional experiments. Since we have previously demonstrated that siRNA depletion of YAP inhibits expression of Zeb1/YAP common target genes (Lehmann et al. 2016),we tried to pharmacologically manipulate this pathway. Addition of the available YAP inhibitor Verteporfin in commonly used concentrations unfortunately killed our cells. We also faced the problem that extrinsic AP-1 activation via TPA (12-O-tetradecanoylphorbol-13-acetate)-treatment led to massive overgrowth of the cells impairing comparability to control-treated cells. However we got supportive results when we extrinsically activated EMT via TGF$\beta$ in MCF10A breast epithelial cells, that endogenously express YAP, JUN and FOSL1 (new Supplementary Fig 1D) and induce ZEB1 in response to TGF$\beta$ (new Fig EV4A). As expected, TGFb induced ZEB1 expression also activated expression of ZEB1/YAP/AP-1 target genes but repressed the ZEB1-only target CDH1 (new Fig EV4A). Transient siRNA mediated knockdown of ZEB1 and to a lesser extent also FOSL1 and YAP, inhibited TGF$\beta$ mediated activation (new Fig 4A).*

11) In Figure 7 the authors attempt to link their findings in MB231 cells to human breast tumors. Unfortunately, to be convincing, the authors would need to demonstrate that similar mechanisms are at play within these breast cancers. Do the authors see that Zeb1 only tumors show a transcriptomic profile with elevated features linked to the Zeb1-only ChIP-seq sites? Do Zeb1+FOSL1 tumors show a transcriptomic profile with features linked to the Zeb1+API ChIP-seq sites?
*Thank you for this comment, which alerted us that we likely have failed in explaining our findings properly but instead caused some confusion: What we show and claim here is that*

*ZEB1 represses and activates in parallel in the same cell / tumour! There is no ZEB1 only and seperate ZEB1/YAP/AP1 type of breast cancer! But only a ZEB1 negative type (all subtypes besides claudin-low) and a ZEB1 positive subtype (claudin-low), which in most cases also co-expresses AP1 and YAP. In the latter, both mechanisms of ZEB1 (repression and activation) are active in parallel on different target gene sets (differentiation and tumor-promoting, respectively.) We now deepened the analyses shown in previous Fig 7 and included activated, as well as repressed target genes in the heatmap of the RNA expression data of all CCLE breast cancer cell lines. This nicely shows the opposing expression patterns of the two target gene subsets (new Fig7B) within the same cell line, particularly of the claudin-low type, but also in the opposite direction in the LuminalA,B and Her2 subtype lacking Zeb1 expression! Further, we now included additional data confirming the clinical relevance of ZEB1/YAP/AP-1 common activated target genes comprising the correlated expression in breast cancer cases and the prognostic significance of an activated target gene set (new Fig 8 C-F).*

Other issues:

1. No supplemental figures and tables were included in this submission.

*We very much regret that you didn't receive the suplementary Figures with our first manuscript! We have no explanation for this, since the other two referees obviously had access.*

## Reply to Referee #2:

*Thank you for the positive evaluation of our manuscript. We are grateful for the critical and very helpful comments. We apologise for causing confusion. Based on your suggestions we changed critical parts throughout the manuscript text, and particularly included clarifications and weakened conclusions. The novel parts of the text are marked in green. Please see our point by point reply below your specific comments:*

Referee #2:

In this study, the authors perform CHIP-seq analysis of Zeb1 on MDA-MB-231 cells to explore its binding sites in this cell line. The introduction sets up the rationale for the study: uncover an explanation for the seemingly controversial role of Zeb1 in cancer metastasis/invasive program. This is however rather misleading and confusing. The description of the literature surrounding the controversy of EMT transcription factors in distinct cancer types is inaccurate and superficial (and perhaps unnecessary given the limited scope of the present study on Zeb1). The authors present a confusing picture of the distinct functions attributed to EMT program. For example, deletion of certain transcription factors (not Zeb1) showed impact on therapy resistance but not metastatic dissemination (Zheng 2015). Deletion of Zeb1 in pancreatic cancer suppressed both primary tumor growth and metastasis (Krebs 2017). Zeb1 deletion in breast cancer mouse models in vivo has not been realized, and it is unclear what EMT phenotype will be affected (chemoresistance, metastasis, or both). A revision of the introduction may be needed to bring into better focus the study question and clarify the interpretation of the main conclusions.
*We are sorry for generating confusion in the introduction. We agree to the critics and completely rewrote the abstract and the introduction to clarify. We did not want to write a manuscript about the controversy around EMT-transcription factors and apologize that the discussion about different roles of Zeb1 in glioblastoma and breast cancer gave this impression. We now clearly changed the focus from the glioblastoma comparison to the biological and pathophysiological role of ZEB1 function in a subtype of breast cancer where it acts in parallel as a transcriptional repressor and as an activator.*
The study presented does not place the data in the context of breast cancer EMT outcome: there is no clear indication as to what is the functional EMT outcome of the distinct Zeb1 transcription regulation shown here, no indication as to what are the genes repressed/activated and their distinct roles in migration, stemness, metabolism, resistance to apoptosis, etc
*The manuscript is now strongly focussed on the results in breast cancer, with the main conclusion that our findings characterize the highly aggressive claudin-low subtype. We now better explain that there is no distinct outcome, but that both ZEB1 functions (repressor and activator) synergize within the same cancer cell in driving tumour progression by either inhibiting tumor-suppressing or activating tumor-promoting factors. To this end we also included GO analyses of the repressed (ZEB1-only) and the activated (ZEB1/YAP/AP-1) target genes as shown in new Fig 3F and scanned the literature for cancer related functions (Table EV3). Additionally, we included target genes in various analyses (new Figs 4A, EV4A, 7B, 8C, D, E, F).*
As detailed below, the design of the experiment is limited to a single cell line. This should be addressed experimentally. It is noted in the abstract, introduction, discussion, and concluding model figure, that a tissue specific feature has arose from their study, implicating distinct Zeb1 transcriptional partners in breast cancer vs. glioblastoma (published dataset, obtained using three glioma cell lines (Rosmaninho).
*We agree that it is always problematic to use only one cell line. However, it turned out that ChIP-seq for transcription factor Zeb1 was much more complicated than e.g. for Histone marks. Labs working with the factor Zeb1 know, how difficult it is to handle this factor. In particular good antibodies for ChIP are not available. We started Zeb1 ChIP-Seq with three Zeb1 expressing breast cancer cell lines and finally got reliable results (according to our cooperation partner at the MPI for Epigenetics in Freiburg) only for MDA-MB 231.*

*Additionally, in other referenced papers accepted in high-ranked journals, ChIP-Seq was also only done for one cell line (e.g. Zanconato et al , Nat Cell Biol using MDA-MB231, and even Rosmaninho et al. (also published in EMBO J !) only used one glioblastoma line for ZEB1 ChIP-Seq. To evaluate that our findings are valid, we now repeated important following experiments with two additional basal type breast cancer cell lines, namely BT549 and Hs578T. We now show the proximity ligation assay for all three cell ines (see Figs 2A and new EV2B). Importantly, we also verified the target gene regulation via Luciferase reporter assays in BT549 and Hs578T (new Fig EV4B) and in a completely different system, the MCF10A epithelial mammary cell line that nicely responds to TGFß treatment by upregulating ZEB1 and undergoing an EMT (new Figs EV4A and Fig 4A).*

This is presented as a main conclusion from the study, but in fact has hardly no representation in the manuscript. The authors should consider performing their own analysis on distinct cell types to strengthen this conclusion.

*As mentioned above, we agree with the critics and weakened this point (different interaction partners of Zeb1 in glioblastoma vs. breast cancer cells) throughout the whole manuscript.*

1) A critical drawback from the presented study is the generalization made on data obtained from ChIP-seq data of a single breast cancer cell line. The cell line studied, MDA-MB-231, represents a metastatic, mesenchymal-like cell line, thus bringing into question the conclusions made with respect to the role of Zeb1 in inducing acquisition of EMT/mesenchymal features.

*We now included two additional metastatic, mesenchymal like cell lines (BT549 and Hs578T, see new Figs EV2B, EV4B) for validating crucial experimental results in MDA-MB-231, since ZEB1 is only expressed in those lines, all belonging to the poor prognosis, Claudin-low subtype of breast cancer. Importantly, all our conclusions relate to this subtype. In summary results in these two lines confirmed all our data (see also our answer above). Additionally, we already had reporter assays in the luminal type cell line MCF7 where overexpression of the factors also led to activation of activated target gene promoter/enhancer reporters. Plus, we now show the upregulation of target genes after TGF$\beta$ induced upregulation of ZEB1/EMT in MCF10A breast epithelial cells, that endogenously express YAP, JUN and FOSL1 (new Supplementary Fig 1D) and induce ZEB1 in response to TGF$\beta$ (new Fig EV4A). As expected, TGF$\beta$ induced ZEB1 expression also activated expression of ZEB1/YAP/AP-1 target genes but repressed the ZEB1-only target CDH1 (new Fig EV4A). Transient siRNA mediated knockdown of ZEB1 and to a lesser extent also FOSL1 and YAP, inhibited TGF$\beta$ mediated activation (new Fig 4A).)*

Does the induction of EMT (by TGFb, for example) in breast cancer cell lines that does not normally express ZEB1 result in a switch from Zeb1-only (repressor) to Zeb-1/Yap/JUN (activator).

*We are again sorry for causing confusion. We unfortunately used the word switch, but mean that ZEB1 acts in parallel as a repressor and activator in one cell!. We now tried to better explain in the revised manuscript.*

The use of a single cell line is sufficient to generalize findings to breast cancer.

*As stated above, we agree and validated main findinings in additional cell lines .*

2) It is unclear what is shown in Supplementary Figure 1D. It is assumed that the 45.1% activated genes represent the transcripts downregulated in the Zeb1 knockdown cells compared to control cells (please clarify).

*Supplementary Fig 1D is now Fig EV1D. We now tried to better explain in the Figure legend, that the genes that we designated as "activated" by ZEB1 are those that are highly expressed in the high ZEB1 expressing cell line MDA-MB-231, but were downregulated after ZEB1-knockdown in these cells.*

Can a heat map and pathway analysis be performed on these published data to validate the EMT related signature of Zeb1 knockdown, thereby affirming whether the genes activated are pro-mesenchymal/stem cell/migratory genes while the genes repressed are epithelial genes?

*That's a valid point. We verified this by including GO term analyses of the repressed (ZEB1-only) and the activated (ZEB1/YAP/AP-1) target genes as shown in new Fig 3F and scanned the literature for cancer related functions of the activated target genes (Table EV3). Additionally, we included target genes in various analyses (new Figs 4A, EV4A, 7B, 8C, D, E, F).*

3) Could the author ascertain whether the analysis shown in Figure 1D extend to published ChIP-seq data set of the AP-1 factor JUN?
*Yes, shown is an overlap between Jun and ZEB1 peak summits (defined as within +/- 200), which demonstrates that summits have very low distance). We better explain this in the Figure legend.*
What fraction of gene promoters in the current analysis shown in Figure 1D overlap with analysis from correlation with JUN ChIP-seq data?
*Shown in Fig. 1C are all 12617 ZEB1 peaks, the JUN overlapping 4201 peaks are shown beteen the dotted lines in Fig. 1D.*

4) Can the author clarify the discrepancy in the p-values shown in Figure 1A and Supplementary Table 4.
*We apologise confusion. Fig. 1A corresponds to Supplementary Table 1, based on ChIP-Seq, Supplementary Table 4 (new number Supplementary Table 6) is based on ATAC Seq, shown in Fig 6.*
The rank listing shows AP-1 at number 10 in the top 25 enriched motifs, but is listed as rank 2 in Figure 1A and in the text. Please include an explanation of the curated table shown in Figure 1A.
*We now included a longer explanation in the legend of Supplementary table 1 and all other known motif tables: 3+4 and 6+7.*

5) Consider providing a circle plot to show the Zeb1, YAP, AP-1 (JUN, FOSL1), and LEF/TCF peak intensity for all chromosomes to support the claim made regarding Zeb1 acting 'genome wide', and to aid in the visualization of Zeb1/AP-1 in breast cancer vs. Zeb1/LEF/TCF in glioblastoma
*We thank for this suggestion and added circle plots that show the distribution of all ZEB1, all YAP and all JUN ChIP-seq peaks (new Fig 1F) and the distribution of ZEB1-only vs. ZEB1/YAP/JUN common peaks (new Fig 3B) throughout the genome of MDA-MB-231 cells. We here did not include glioblastoma, since we withdraw the comparison from our main focus.*

6) Figure 2D: Can the authors confirm that modulation in Zeb1 protein content impact JUN pull down? In other words, would the suppression of Zeb1 result in loss of JUN pull down, or would high expression of Zeb1 (via induction of EMT) in breast cancer cells would enhance JUN pull down? Would this be observed in glioblastoma cells?
*We believe that this question arose because of us not properly explaining that ZEB1 repression and activation occurs in the same cell. It is not possible to get a pull down for ZEB1 in ZEB1-negative cells, accordingly JUN could not be co-precipitated. We now show that all three factors cooperate in DNA-binding in native electromobility shift assays (EMSA) in MDA-MB-231 cells (new Fig 5D).*

Consider a pull down assay for ZEB1 in the MCF7 lines with E-box mutants.
*Unfortunately, the MCF7 do not express ZEB1. We refer to the above mentioned EMSAs showing cooperative binding to a TEAD motif, not needing Z-Boxes, while recombinantly expressed DNA binding domain of ZEB1 alone interacts solely with the double Z-Box but not with the TEAD nor the AP-1 motif (new Fig 5D).*

7) Figure 3C-D: what are the genes (pathways at least) repressed vs. activated in the Zeb1 only vs. Zeb1/Yap/JUN promoter peaks? It is unclear whether the distinction between the

targets for Zeb1 only and the targets of Zeb1/Yap/JUN are functionally relevant and associated with distinct EMT gene expression outcome.
*We are thankful for this valid request. We now included pathway analyses that revealed ZEB1-only repressed genes to be involved in processes like cell adhesion whereas ZEB1/YAP/JUN common activated genes contribute to processes favouring tumour progression, like cell migration (new Fig 3F) and also invasion, metastasis and therapy resistance that is shown in a new Table EV3 for the top 23 activated genes with common peaks in their promoter regions. Thus, both ZEB1 functions support tumour progression. Importantly, both functions are carried out in the same cell. Thus, there is no distinct EMT gene expression outcome, but both repressed and activated genes drive the cell into a tumor-promoting state.*

8) Please justify the rationale for transfecting 4x the amount of ZEB1 as YAP/Jun/FOSL1 in these assays in the methods.
*We titrated to find the optimal ratio for target gene activation and reporter assays, because ZEB1 overexpression was generally weaker than for the other factors. Further, the ZEB1 expression plasmid is a lot larger than the other factors, because the ZEB1 open reading frame extends about 3300bp.*

9) Figure 4 attempts to make a connection between the Zeb1 only vs. Zeb1/Yap/JUN functional targets, but compares cells without induction of EMT, which is presented in the introduction as a pro-migratory and resistance/stem cell program. There is a disconnect between the data presented and the functional contribution of these observations (pro-metastatic features of EMT program controlled by Zeb1 in breast cancer cells). Is LLGL2 associated with breast cancer metastasis? What about ANKRD1 and DOCK9? Are those specifically associated with metastatic dissemination/migration/stemness. Are there any genes/candidates that would distinguish the distinct functional outcome of EMT of breast cancer cells and are differentially regulated by Zeb1 only vs. Zeb-1/Yap/JUN?.
*We are really sorry for causing this confusion. We hope that our above replies, especially to point 7, already clarified a lot. Specifically to the functions of the above mentioned factors: LLGL2 is an epithelial polarity gene suppressed by ZEB1 to induce EMT. Thus it is downregulated in breast and other cancers and its loss is associated with increased metastasis. ANKRD1 was found to induce therapy resistance and high ANKRD1 levels correlated with poor outcome in e.g. ovarian cancer (Scurr et al., Clin. Cancer Res. 2008). The DOCK protein family are nucleotide exchange factors involved in cell migration (Gadea et al., Eur. J. Cell. Biol. 2014). DOCK9 is suggested as a biomarker to predict prostate cancer progression (Alkhateeb et al., Cancer Inform. 2019) and is related to the tumorigenesis in thyroid carcinoma (Zhang et al., Med. Sci: Monit. 2019). The function of regulated target genes is now summarized in table EV3. In summary, there is no ZEB1-dependent function without EMT. Both, the repressing and the activating functions occur in the same cell and synergize towards tumour progression.*

10) The data in Figure 4 suggest that Zeb1-only targets are transcriptionally repressed by Zeb1, whereas Zeb1/Yap/JUN enhance transcription on targets (Figure 1C). Can this be generalized and functionally linked to pro-metastatic EMT program? If not, is it rather associated with a resistance/pro-survival program? The experiment in Figure 4 should be interpreted in the context of inducing the invasive EMT program the authors introduce.
*Yes, as stated above the repressed as well as the activated targets contribute to tumour progression covering the classical EMT- as well as resistance- and survival-programmes. We now tried to better explain our results and especially Fig 4C (new Fig 4D) and also included ZEB1 knockdown in two additional mesenchymal type breast cancer lines to strengthen the result. Additionally, we now show the regulation of target genes after TGF$\beta$ induced upregulation of ZEB1/EMT in MCF10A breast epithelial cells, that endogenously express YAP, JUN and FOSL1 (new Supplementary Fig 1E) and induce ZEB1 in response to TGF$\beta$ (new Fig EV4A). As expected, TGF$\beta$ induced ZEB1 expression also activated expression of ZEB1/YAP/AP-1 target genes but repressed the ZEB1-only target CDH1 (new Fig EV4A).*

*Transient siRNA mediated knockdown of ZEB1 and to a lesser extent also FOSL1 and YAP, inhibited TGFb mediated activation (new Fig 4A). Additionally we charcterized the functions of repressed and activated target genes with a GO term analysis (new Fig 3F) and specify known functions (in the tumour context) of the top activated target genes in new Table EV3.*

11) It is unclear what the meaning of the data shown in Figure 7 is with respect to the main conclusions of this manuscript. The authors cannot distinguish between Zeb1 only vs. Zeb1/Yap/JUN regulated targets in Figure 7D and there is no clinical data associated with the data shown in Figure 7C. These data should be carefully interpreted.
*Sorry again for the sometimes confusing description in the first manuscript. We hope that we now managed to clarify that we do not distinguish between tumors with ZEB1-only repressed vs. tumors with ZEB/YAP/AP-1 activated target genes. Instead, we found a mechanism, which allows ZEB1 to both activate (tumor promoting) and repress (tumor suppressive, differentiation) target genes in the same tumor cell, which characterizes one of the most aggressive subtypes of breast cancer, claudin-low. Thus in previous Fig. 7D (now new Fig. 8A) we analysed hormone receptor negative breast cancers, which fall into a Zeb1 high, poor prognosis type (likely claudin-low) and a Zeb1 low, good prognosis type (likely basal-type). We now deepened the analyses shown in previous Fig 7 and included activated, as well as repressed target genes in the heatmap of the RNA expression data of all CCLE breast cancer cell lines. This nicely shows the opposing expression patterns of the two target gene subsets (new Fig7B) within the same cell line, particularly of the claudin-low type, but also in the opposite direction in the LuminalA,B and Her2 subtype lacking Zeb1 expression! Further, we now included additional data confirming the clinical relevance of ZEB1/YAP/AP-1 common activated target genes comprising the correlated expression in breast cancer cases and the prognostic significance of an activated target gene set (new Fig 8 C-F).*

## Reply to Referee #3:

*Thank you for the very positive evaluation of our manuscript. We are grateful for the critical and very helpful comments regarding the translational importance. We tried to extend our analyses where possible. The novel parts of the text are marked in green. Please see our point by point reply below your specific comments:*

Referee #3:

Triple-negative breast cancer is an aggressive subgroup of breast cancer with poor prognosis due to a complex genomic landscape.
In the manuscript entitled "Genome-wide cooperation of the EMT-activator ZEB1 with YAP and AP-1 factors in breast cancer" Dr. Brabletz and colleagues describe a genome-wide interaction of ZEB1 (known as pivotal cancer progression stimulator in many tumor types). The authors already nicely published in 2016 the clinical relevance of ZEB1 in breast cancer, and here they investigated the role of ZEB1 on a genome-wide level in a cancer of epithelial origin, that is aggressive breast cancer. To do that they use the triple negative breast cancer cell line MDA-MB-231. They corroborate the observation that ZEB1 has different mode of action and show convincing evidence for the mode of action of EMT-TFs in cancer.

Nevertheless, considering the multifaceted activity di ZEB1 in epithelial and non-epithelial tumors, and the translational importance of these findings, the authors should explore more deeply the findings presented in Figure 7.

• It is worthy to investigate the expression and correlation level of ZEB1 and FOSL1 in in silico available data set (TGCA for instance) to validate the results in a bigger cohort.
• Moreover, would be interesting to investigate the correlation of ZEB1 mRNA expression with the expression of YAP, FOSL1 and JUN in human primary culture.
*We absolutely agree that it would be interesting to look at correlation of ZEB1 and FOSL1 at the tumour level in addition to our analysis in cell lines. However, as we now also pointed out in the manuscript, most published tumour data sets like TCGA harbour the problem that they are based on none micro-dissected tumour material. Thus, gene expression of the tumour stroma can overlay the signal from the cancer cells. This is especially relevant for ZEB1 which is known to be highly expressed in the tumour stroma (Fig 8B, asteriks). We thus decided to look at correlation of ZEB1 and FOSL1 in immunohistological sections (Fig 8B) where we can differentiat between cancer and stromal cells. When we look at cancer cell line level, we see a very good correlation of ZEB1 and FOSL1 (Fig 7A). Here we also see a strong correlation of ZEB1 with common ZEB1/YAP/AP-1 activated target genes (Fig 7B, Supplementary Table 9). In addition, we also detected correlation of ZEB1 with common ZEB1/YAP/AP-1 activated targets in a published tumour dataset (Fig. 8D, Supplementary Table 10). We then evaluated the clinical relevance, by selecting a core subset of these targets and evaluatin its effect on survival. For two different tumour sets, stratifiing tumours according to high or low expression of the set clearly showed that high expression selects for worse survival (Fig 8E and F).*
*We agree that showing a correlation also in primary culture would be very helpful. Unfortunately, we did not succeed in finding published data of such cultures.*

## 2nd Revision - Editorial Decision 5th Jun 2020

Dear Simone, dear Thomas,

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your amended study was sent back to the three referees for re-evaluation. My apologies for getting back to you with delay. Please note that while referee #1 was at this time not able to re-assess your study, we have editorially re-evaluated your response to his-her concerns and found them reasonable. We did receive comments from the other two referees, which I enclose below. As you will see these referee finds that their concerns have been sufficiently addressed and they are now broadly in favour of publication, appreciating your diverse amendments.

Thus, in light of all information at hand, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues, which need to be adjusted at re-submission.

Please consider the remaining point of referee #2 regarding use of and emphasis on the term 'EMT' by introducing caveats where appropriate or revising the discussion We agree the main focus of the current work is ZEB1's role and mode of action rather then proof for EMT-dependent versus independent functional downstream consequences.

We in addition need you to consider a number of minor points related to formatting and data representation, which are listed below. Further, I will share additional changes and comments from our production team during the next days to be addressed.

Please contact me at any time if you need any help or have further questions.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel

Daniel Klimmeck PhD
Editor
The EMBO Journal.

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Formatting changes required for the revised version of the manuscript:

>> EV figures and tables are uploaded in one document. Please upload EV figures as separate figure files, and add their legends to the main manuscript, following main figure legends. Table EV3 should be renamed Table EV1 and uploaded as an editable file, with the legend included.

>> The "Supplementary Information" file contains 1 suppl. figure, 13 suppl. tables and source data. Please remove the source data and upload it as separate files, one file per figure. Rename the "Suppl. Infor." file "Appendix"; the suppl. fig. and suppl. tables should be renamed "Appendix Figure S1" and "Appendix Table S1" etc., and a ToC should be added to the file.

>> Remove Database links in the Data availability section.

>> Please avoid redundancy with your 2016 study, and alter phrasing in the introduction and results parts accordingly.

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please see also our instructions to authors
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Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 3rd Sep 2020.

Link Not Available

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Referee #2:

The revised manuscript is clearer and more focussed, and the major concerns have been addressed. The focus of the work has shifted away from Zeb1 and EMT in breast cancer progression, and re-focussed on molecular subtypes of breast cancer with associated clinical features. The title and abstract however still suggest the work is about EMT. For example, Zeb1 is

more than just an EMT-activator as the title suggests. The authors conclude that their finding 'link three critical tumor-promoting pathways: EMT, AP-1, and Hippo', but in reality the study (convincingly) supports a link between Zeb1, AP-1 and Hippo. Should EMT be concluded upon, the data must then consistently show the downregulation of epithelial gene targets of Zeb1 in Figures 2, 4, 6, 8. The use of the terminology 'EMT' without the supporting data needs attention, and if missed, consider presenting it more clearly to establish that connection firmly.

Referee #3:

The authors made a substantive experimental revision and replied exhaustively to all the referees' comments

## Reply to the editor's comments:

*We are grateful for principle acceptance of our manuscript and now addressed all the remaining concerns as well as the formal requirements.*
*Please see a point by point reply to your comments below and to the comments of referee 2 for more detailed explanations.*

Dear Simone, dear Thomas,

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your amended study was sent back to the three referees for re-evaluation. My apologies for getting back to you with delay. Please note that while referee #1 was at this time not able to re-assess your study, we have editorially re-evaluated your response to his-her concerns and found them reasonable. We did receive comments from the other two referees, which I enclose below. As you will see these referee finds that their concerns have been sufficiently addressed and they are now broadly in favour of publication, appreciating your diverse amendments.
Thus, in light of all information at hand, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues, which need to be adjusted at re-submission.
Please consider the remaining point of referee #2 regarding use of and emphasis on the term 'EMT' by introducing caveats where appropriate or revising the discussion We agree the main focus of the current work is ZEB1's role and mode of action rather then proof for EMT-dependent versus independent functional downstream consequences.
*- We fully agree on the main focus of our work and changed some phrases mainly in the introduction and discussion section concerning the role of EMT-vs. non-EMT functions of ZEB1 target genes. In general such ZEB1-associated functions (e.g. survival, stemness, therapy resistance) are long known, and together with "classical" EMT-functions (e.g. loss of epithelial identidy, gain of motility) also detected in our current manuscript. We did not want to exclude or focus on any of the ZEB1-functions in the manuscript and made this more clear mainly in the introduction. We also included changes in the title, abstract and discussion, where appropriate. Moreover we sticked to the nomenclature recently proposed by the EMT-Society (ZEB1 as one of the five 'core' EMT transcription factors, and the definition of "classical" EMT vs. non-EMT associated functions exerted by EMT-TFs) and referenced the relevant publication (Yang et al. 2020). See also rebuttal to reviewer 2.*

We in addition need you to consider a number of minor points related to formatting and data representation, which are listed below. Further, I will share additional changes and comments from our production team during the next days to be addressed.
*- We addressed these points.*

Please contact me at any time if you need any help or have further questions.
Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.
Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel

Daniel Klimmeck PhD
Editor
The EMBO Journal.

**********

Formatting changes required for the revised version of the manuscript:

>> EV figures and tables are uploaded in one document. Please upload EV figures as separate figure files, and add their legends to the main manuscript, following main figure legends. Table EV3 should be renamed Table EV1 and uploaded as an editable file, with the legend included.

>> The "Supplementary Information" file contains 1 suppl. figure, 13 suppl. tables and source data. Please remove the source data and upload it as separate files, one file per figure. Rename the "Suppl. Infor." file "Appendix"; the suppl. fig. and suppl. tables should be renamed "Appendix Figure S1" and "Appendix Table S1" etc., and a ToC should be added to the file.
*- We addressed these points.*

>> Remove Database links in the Data availability section.
*- We deleted it.*

>> Please avoid redundancy with your 2016 study, and alter phrasing in the introduction and results parts accordingly.
*- We rephrased some parts in the manuscript to avoid unneccessary redundancy, where possible. At some points the 2016 study had to be mentioned as basis of the new study.*

## Reply to Referee #2:

*Thank you for the positive evaluation of our manuscript. We are grateful for the critical and very helpful comments. Please see our point by point reply below your specific comments:*

Referee #2:

The revised manuscript is clearer and more focussed, and the major concerns have been addressed. The focus of the work has shifted away from Zeb1 and EMT in breast cancer progression, and re-focussed on molecular subtypes of breast cancer with associated clinical features.
The title and abstract however still suggest the work is about EMT. For example, Zeb1 is more than just an EMT-activator as the title suggests.
*- We fully agree that the work is not (mainly) about EMT and rephrased some part to make this more clear. In general non-classical EMT -associated functions of ZEB1 (e.g. survival, stemness, therapy resistance) are long known, and together with "classical" EMT-functions (e.g. loss of epithelial identity, gain of motility) are also detected in our current manuscript. We did not exclude or focus on any of the ZEB1-functions in the manuscript and made this more clear mainly in the introduction section. We also included changes in the title, abstract and discussion, where appropriate. Thereby we sticked to the nomenclature recently proposed by the EMT-Society (ZEB1 is named as as one of the 'core' EMT transcription factors independently of its different downstream functions, and the definition of "classical" EMT vs. non-EMT associated functions exerted by EMT-TFs) and referenced the relevant publication (Yang et al. 2020). Consequently we changed "EMT-activator ZEB1" to "EMT-transcription factor ZEB1" already in the title and made clear that we write about both EMT and non-EMT functions of ZEB1 in the manuscript (main changes in the introduction section).*
The authors conclude that their finding 'link three critical tumor-promoting pathways: EMT, AP-1, and Hippo', but in reality the study (convincingly) supports a link between Zeb1, AP-1 and Hippo.
*- We again fully agree and changed this part (abstract, and discussion section) now with a focus on the transcription factors.*
Should EMT be concluded upon, the data must then consistently show the downregulation of epithelial gene targets of Zeb1 in Figures 2, 4, 6, 8. The use of the terminology 'EMT' without the supporting data needs attention, and if missed, consider presenting it more clearly to establish that connection firmly.
*- We did not want to conclude on EMT nor to exclude EMT, but, as mentioned above, show that ZEB1 exerts its effects on both 'classical' EMT- and non-EMT-associated downstream functions. This was made more clear in the text. We respectfully disagree to the concern, that we have not demonstrated EMT-effects: Also in this manuscript, we have demonstrated that ZEB1 acts on 'classical' EMT processes (see Fig. 3F: GO terms cell-cell junction, cell migration) and is associated with control of prototype epithelial target genes like the cell-cell adhesion factors E-cadherin (CDH1) (see Fig. 6C, Fig. 7B,EV1B, EV6B, Suppl. Table 9 ), Claudins 3 and 7 (see Fig. 7B, Suppl. Table 9), as well as the epithelial polarity factor LLGL2 (see Fig. 4C, 7B, EV4B, Suppl. Table 9).*

## Reply to Referee #3:

*Thank you for the positive evaluation of our manuscript.*

Referee #3:

The authors made a substantive experimental revision and replied exhaustively to all the referees' comments

## 3rd Revision - Editorial Decision 18th Jun 2020

Dear Simone, dear Thomas,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper.

Also in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
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On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work.
Please see the following link for a representative example:
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Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

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

Kind regards,

Daniel

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|---|
| Corresponding Author Name: Thomas Brabletz, Simone Brabletz |
| Journal Submitted to: The EMBO Journal |
| Manuscript Number: Manuscript EMBOJ-2019-103209R-Q |

**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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

## B- Statistics and general methods

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

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| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | See "Statistical analysis" section of manuscript. |
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| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | NA |
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| 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. | Yes, for each PLA we analysed 25 randomly chosen microscopic visual fields for analysis. |
| 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? | See "Statistical analysis" section of manuscript. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | See "Statistical analysis" section of manuscript. |
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| Is the variance similar between the groups that are being statistically compared? | See "Statistical analysis" section of manuscript. |

## C- Reagents

| | |
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| 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). | For all antibodies used, the respective companies and catalog numbers are provided in the 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. | All cell lines were purchased from American Type Culture Collection (ATCC). We always use low passage numbers and go back to frozen early passages latest after three months of culture. We do routine checks (for mycoplasma) on cell lines after thawing. |

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

## D- Animal Models

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| 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 |
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## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | Samples were retrieved from local archives and usage was approved by the Ethics Committee of the University of Erlangen-Nuremberg |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | Informed consent was obtained from all subjects and experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. |
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## F- Data Accessibility

| | |
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| 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'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | All genomic data sets generated in this study have been submitted to the Array Express database, as clearly stated in the "Data Availability" section which contains the required accession codes. |
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| 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 |
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| 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 |