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Peer Review Information

Journal: Nature Cell Biology
Manuscript Title: Loss of PRC2 or KMT2D-COMPASS Unlocks Distinct EMT Trajectories that Contribute Differentially to Metastasis
Corresponding author name(s): Yun Zhang, Robert A. Weinberg

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Cell Biology submission NCB-Z45438
Message:

*Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Dr Weinberg,

Your manuscript, "Loss of PRC2 or KMT2D-COMPASS Unlocks Distinct EMT Trajectories that Contribute Differentially to Metastasis", has now been seen by 3 referees, who are experts in metastasis (referee 1); cancer epigenetics (referee 2); and EMT (referee 3). As you will see from their comments (attached below) they find this work of potential interest, but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, it would be essential to:

A) Validate the key conclusions as requested by reviewers;

Reviewer 1
"A major drawback of this study is that key results derive mostly from HLMER, an immortalized epithelial cell line. It is well established that cancer subtypes have different epigenetic signatures, therefore it is unclear whether PRC2 and KMT2D/COMPASS will play a similar role in cancer cells of different epigenomic landscape. Several key observations need to be confirmed using additional models recapitulating major subtypes of breast cancers. These key points include the existence of plastic clones and nonconvertible clones, the loss of PRC2 or KMT2D/COMPASS during the acquisition of EMP, the distinct EMT states upon PRC2 or KMT2D KO, and the quasi-mesenchymal state induced by PRC2 KO."

"There is a concern about the data generated by the KO cells derived from single-cell clones using a single sgRNA in this study. Considering the possibility of off-target in CRISPR-CAS9 system and the heterogeneous nature of parental cells, at least two to three additional KO clone using sgRNAs targeting a different region of EED or KMT2D needs to be tested side by side in some key experiments."

Reviewer 3
" To further validate the critical roles of PRC2 and KMT2D in restricting EMP, will exogenous expression of EZH2 or KMT2D in C2-mesenymal clone switch and lock cells into epithelial state?"

B) Strengthen the mechanistic dataset as raised by all reviewers;

Reviewer 1
"The study seems unidirectional (from Epithelial to Mesenchymal). The reciprocal process (i.e., MET) needs to be examined. It seems clear that the PRC2 complex is maintaining homeostasis in immortalized cells. Based on multiple studies suggesting the role of the PRC2 complex in promoting EMT and metastasis (PMID: 22187039, PMID: 26848980, PMID: 30353102), it remains uncertain whether EED depletion would lead to different outcome if the initial cells were mesenchymal (e.g. MDA-MB-231)? Moreover, the study suggests that blocking both PCR2 complex and KMT2D-COMPASS promotes EMT and tumor growth in lung. It would be highly informative to evaluate whether lung lesions maintain their EMP phenotype or revert to an epithelial state? Similarly, the fact that EED inhibition promotes EMT via Zeb1 may suggest that overexpressing EED and/or other PRC2 members may reverse the EMT phenotype in mesenchymal cells. However, this was not sufficiently addressed in the study."

"Knocking down EED may impact the non-enzymatic function of EZH2, which was previously shown to activate stemness related pathway. Therefore, to confirm that increased EMT and metastasis formation is a direct reflection of EED depletion, other components of the PRC2 complex must (including EZH2) must be depleted and evaluated similarly to the above. Using a pharmacological approach may not be sufficient as non-enzymatic functions may also be involved. A previous study suggest that cytoplasmic EZH2 following p38-mediated phosphorylation at T367 promotes breast cancer metastasis (PMID: 30022044)."

" Basically, the different mesenchymal states resulting from knock out of EED and KMT2D were well characterized in epithelial HMLER clones, but the underlying mechanism appears unclear. First, since ZEB1 and ZEB2 were upregulated in both EED and KMT2D knockout cells, the link between EMT-TFs to the distinct mesenchymal states is missing. Evaluating this may be key to comprehend the mechanistic differences underlying EED and KMT2D KO."

"Though the authors did a comprehensive comparison between the parental epithelial cells and mesenchymal fractions of sgEED or sgKMT2D cells, the comparison in the epithelial fractions of control cells and KO cells is still needed. Such analysis might provide clues on how the plastic clones are initiated and how they are directed to divergent EMT trajectories. This is also relevant to the single-cell trajectory map in Fig 3B. The epithelial subpopulation in EED KO cells locates in the transition zone between C1 control cells and the epithelial KMT2D KO cells. This data suggests that there is another layer of epigenetic or genetic barrier that prevents the epithelial EED KO cells from further evolving to the trajectory represented by KMT2D KO cells. This barrier can be further characterized."

"The connection between the EED or KMT2D KO cells and the convertible clones appears weak. The authors did some preliminary comparisons among the epithelial C2 fraction and EED or KMT2D KO cells in Fig 2 and extended fig 6. Such comparison may be misleading as both EED and KMT2D KO cells were a mixture of epithelial and mesenchymal cells whereas the C2 fraction was purified epithelial cells. It is difficult to understand if the observed differences on EMT markers or selected promoter occupancy were due to the different plasticity status or the different proportion of the mesenchymal population in the mixtures. Importantly, whether the EED or KMT2D KO in nonconvertible clones could truly recapitulate some of the preexisted, naturally occurred plastic clones in HMLER cells was still unclear. Therefore, an unbiased and systemic comparison using the epithelial fraction of genetic KO clones and plastic single-cell clones may clarify this point."

Reviewer 2
"The only weakness of the paper is the lack of mechanistic insights as to how/if the gene expression changes observed upon loss of PRC2 or KMT2D lead to the quasi-mesenchymal or highly mesenchymal states, respectively. Nor is it clear whether non-transcriptional, non-histone targets of these enzymes might also contribute to these different states. However, this paper clearly provides much fertile ground for future studies."

Reviewer 3
"In Extended Data Fig 6b, it is better to include the measurement of H3K4me2/3 status, because KMT2D is known to regulate H3K4me1/2/3. In addition, KMT2D-KO significantly increased H3K27me3, suggesting that PRC2 activity was increased in C1-sgKMT2D cells. This is somewhat against the conclusion of reduced EZH2/PRC2 association at its target gene promoters (including ZEB1/2) in the C1-

sgKMT2D cells (Figs2e-2f). Have ChIP-seq using H3K4me3 and H3K27me3 antibodies been performed on these clones and compared with the results on Figs 2e-2f?"

"For the C1 clone, the result on EED-KO (Fig 2d) is somewhat different from the result using two different PRC2 inhibitors (TAZ and EED226 in Fig 5e). C1 cells with EED-KO (C1-sgEED) have a large population in mesenchymal state, however, treatment with TAZ or EED226 did not lead a significant population of cells in mesenchymal state."

"On Extended Data Fig 4e, only a minor fraction of cells were in mesenchymal state after EED- or KMT2D-KO in lung cancer HCC827 cells. Does TGFβ increase EMT in these KO cells?"

C) All other referee concerns pertaining to strengthening existing data, providing controls, methodological details, clarifications and textual changes, as applicable should also be addressed.

D) Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and https://www.nature.com/nature/for-authors).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Reporting Summary (found here https://www.nature.com/documents/nr-reporting-summary.pdf). This is essential for reconsideration of the manuscript will be available to editors and referees in the event of peer review. For more information see http://www.nature.com/authors/policies/availability.html or contact me.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

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Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

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We would like to receive a revised submission within six months.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

Best wishes,
Zhe Wang

Zhe Wang, PhD
Senior Editor
Nature Cell Biology

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email: zhe.wang@nature.com

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
This manuscript elucidated the complexity of epigenomic barriers during epithelial to mesenchymal plasticity (EMP). Using molecular techniques including a CRISPR KO system, they identified two methyl transferase complexes (PRC2 and KMT2D/COMPASS) that play the role of “guardian” in epithelial cells. Loss of either potentiated TGFβ-induced EMT. Loss of PRC2 in epithelial cells leads to a quasi-mesenchymal state, which makes cells more capable of lung colonization. The authors also provided evidence supporting that the loss-of-function of PRC2 predicts poorer survival in breast cancer patients. Overall, the finding challenges current knowledge on the role of these complexes in cancer. Although the conclusion is intriguing, more validation is required to extrapolate to a general cancer context.
Major comments:
1- A major drawback of this study is that key results derive mostly from HLMER, an immortalized epithelial cell line. It is well established that cancer subtypes have different epigenetic signatures, therefore it is unclear whether PRC2 and KMT2D/COMPASS will play a similar role in cancer cells of

different epigenomic landscape. Several key observations need to be confirmed using additional models recapitulating major subtypes of breast cancers. These key points include the existence of plastic clones and nonconvertible clones, the loss of PRC2 or KMT2D/COMPASS during the acquisition of EMP, the distinct EMT states upon PRC2 or KMT2D KO, and the quasi-mesenchymal state induced by PRC2 KO.

2- There is a concern about the data generated by the KO cells derived from single-cell clones using a single sgRNA in this study. Considering the possibility of off-target in CRISPR-CAS9 system and the heterogeneous nature of parental cells, at least two to three additional KO clone using sgRNAs targeting a different region of EED or KMT2D needs to be tested side by side in some key experiments.

3- The study seems unidirectional (from Epithelial to Mesenchymal). The reciprocal process (i.e., MET) needs to be examined. It seems clear that the PRC2 complex is maintaining homeostasis in immortalized cells. Based on multiple studies suggesting the role of the PRC2 complex in promoting EMT and metastasis (PMID: 22187039, PMID: 26848980, PMID: 30353102), it remains uncertain whether EED depletion would lead to different outcome if the initial cells were mesenchymal (e.g. MDA-MB-231)? Moreover, the study suggests that blocking both PCR2 complex and KMT2D-COMPASS promotes EMT and tumor growth in lung. It would be highly informative to evaluate whether lung lesions maintain their EMP phenotype or revert to an epithelial state? Similarly, the fact that EED inhibition promotes EMT via Zeb1 may suggest that overexpressing EED and/or other PRC2 members may reverse the EMT phenotype in mesenchymal cells. However, this was not sufficiently addressed in the study.

4- Knocking down EED may impact the non-enzymatic function of EZH2, which was previously shown to activate stemness related pathway. Therefore, to confirm that increased EMT and metastasis formation is a direct reflection of EED depletion, other components of the PRC2 complex must (including EZH2) must be depleted and evaluated similarly to the above. Using a pharmacological approach may not be sufficient as non-enzymatic functions may also be involved. A previous study suggest that cytoplasmic EZH2 following p38-mediated phosphorylation at T367 promotes breast cancer metastasis (PMID: 30022044).

5- Basically, the different mesenchymal states resulting from knock out of EED and KMT2D were well characterized in epithelial HMLER clones, but the underlying mechanism appears unclear. First, since ZEB1 and ZEB2 were upregulated in both EED and KMT2D knockout cells, the link between EMT-TFs to the distinct mesenchymal states is missing. Evaluating this may be key to comprehend the mechanistic differences underlying EED and KMT2D KO.

6- Though the authors did a comprehensive comparison between the parental epithelial cells and mesenchymal fractions of sgEED or sgKMT2D cells, the comparison in the epithelial fractions of control cells and KO cells is still needed. Such analysis might provide clues on how the plastic clones are initiated and how they are directed to divergent EMT trajectories. This is also relevant to the single-cell trajectory

map in Fig 3B. The epithelial subpopulation in EED KO cells locates in the transition zone between C1 control cells and the epithelial KMT2D KO cells. This data suggests that there is another layer of epigenetic or genetic barrier that prevents the epithelial EED KO cells from further evolving to the trajectory represented by KMT2D KO cells. This barrier can be further characterized.

7- The connection between the EED or KMT2D KO cells and the convertible clones appears weak. The authors did some preliminary comparisons among the epithelial C2 fraction and EED or KMT2D KO cells in Fig 2 and extended fig 6. Such comparison may be misleading as both EED and KMT2D KO cells were a mixture of epithelial and mesenchymal cells whereas the C2 fraction was purified epithelial cells. It is difficult to understand if the observed differences on EMT markers or selected promoter occupancy were due to the different plasticity status or the different proportion of the mesenchymal population in the mixtures. Importantly, whether the EED or KMT2D KO in nonconvertible clones could truly recapitulate some of the preexisted, naturally occurred plastic clones in HMLER cells was still unclear. Therefore, an unbiased and systemic comparison using the epithelial fraction of genetic KO clones and plastic single-cell clones may clarify this point.

8- The authors demonstrate that TGFβ is required for the mesenchymal transition of C1-sgEED and C1-sgKMT2D cells as SB-431542 (TGFB inhibitor) abrogates CD44hi conversion. However, in a previous study (PMID: 29029452), the histone demethylase KDM6A was reduced following TGFB-mediated E-to-M transition and re-expressed during the M-to-E transition. Additionally, the KDM6A was found to be lower in stem-like cells compared to non-stem cell counterparts. More experiments/discussions may be required to reconcile these findings.

Minor comments:
1- In clinical data analysis, the authors should examine if LOF mutations of PRC2 and KMT2D/COMPASS are enriched in specific breast cancer subtypes and/or perform the survival analysis in a specific subtype of cancer patients.

2- Spontaneous metastasis from the primary tumor should be examined to verify the metastatic potential of cells with different EMT states.

3- The authors have shown that the quasi-mesenchymal states induced by genetic KO or pharmaceutical inhibitors are not reversible by withdrawing inhibitors. It is necessary to test if re-expression of these genes could block the TGFβ induced EMT in the KO cells.

4- Extended Figure 1f: It is not clear how the co-culture was performed and how each cell was discriminated after sequencing. More specific information will help better understand the results.

5- In extended Figure 2, despite the lack of significant differences between c1 and c2-Epi in terms of TGF signaling, there appears to be a response following TGFβ treatment on both models. Could this suggest indirect effects of TGFβ?

Reviewer #2:
Remarks to the Author:
Zhang et al use elegant single cell approaches to identify factors that regulate epithelial-mesenchymal plasticity (EMP) in vitro. First, they demonstrate that single cell clones of HMLER epithelial cells exist in two subpopulations, which upon extended propagation either maintain their epithelial identity or exhibit extensive EMP. The authors then did a series of KO screens to identify factors required to suppress EMP in the stable epithelial clones. Remarkably, these screens indicated that loss of two histone methyltransferase complexes, PRC2 and KMT2D-COMPASS, triggered EMP. These two activities have opposite effects on gene expression. PRC2 mediates gene silencing through methylation of histone H3 K27, whereas KMT2D-Compass activates gene transcription by methylation of H3 K4. RNA-seq analyses, protein expression patterns, and functional assays all indicate that loss of PRC2 leads to a quasi-mesenchymal state that facilitates metastatic properties, whereas loss of KMT2D leads to a highly mesenchymal state and metastatic dormancy.

These findings provide important advances in our understanding of the regulation of EMP and how different EMP states contribute to metastatic potentials. As such, this paper will be of broad interest to both basic biologists and clinicians. These findings will/should have immediate impact on stratification of patients in clinical trials of PRC2 inhibitors as cancer therapies.

The only weakness of the paper is the lack of mechanistic insights as to how/if the gene expression changes observed upon loss of PRC2 or KMT2D lead to the quasi-mesenchymal or highly mesenchymal states, respectively. Nor is it clear whether non-transcriptional, non-histone targets of these enzymes might also contribute to these different states. However, this paper clearly provides much fertile ground for future studies.

One minor point: On page 8, the authors state that KMT2D-COMPASS implements H4K4me1 at enhancers, which is true, but it is also required for H3K4me3 in certain contexts (see Dhar et al, PMID 29861161). The authors should correct the wording of the sentence to reflect the broader functions of KMT2D.

Reviewer #3:
Remarks to the Author:
EMT is a developmental program that provides cells with the increased morphological and cellular plasticity required during embryonic development, tissue remodeling, wound healing and metastasis. Although this reversible and dynamic process, which is often propelled by microenvironmental signals, is regulated at the epigenetic level, many mechanistic details remain unclear. The major reason is that the induction of EMT (by TGFβ) in vitro is a highly inefficient process (Brown KA, Breast Cancer Res, 2004;6(3):R215-31), only a few cell lines, including NMuMG (mouse), MCF10A (human), and HMLE (human), are commonly used for studying EMT in vitro. This casts technical challenge to identify critical epigenetic regulators that either restrict or permit the reversible and dynamic EMT process operated during metastatic cascade and therapeutic treatments. In this study, Zhang and colleagues isolated a relative stable C1 clone that remained in epithelial state from parental HMLER cells. Interestingly, TGFβ failed to induce EMT in this C1 clone, suggesting that an intrinsic "memory" machinery is operated to "locks" these cells in epithelial state. Using unbiased CRISPR-Cas9 screening, the authors further found that two epigenetic complexes, PRC2 and KMT2D-Compass, played critical roles in restricting the EMT process. Intriguingly, EED-KO conferred C1 clone in a quasi-mesenchymal state and restored the cellular plasticity (EMP) required for lung metastasis in vivo. However, KMT2D-KO changed C1 clone all the way to mesenchymal state and these cells lost the ability to metastasize. These findings are very interesting and novel, as these indicate that distinct EMT trajectories or identities from E to M spectrum are regulated by different chromatin modifiers. Overall, this study represents a new advance in the EMT field and sheds new insights in the epigenetic regulation and cancer biology. Experiments were well designed and executed; robust quality data were presented and they were clear and supportive to the main conclusion. A few suggestions are recommended to further strengthen the conclusion and improve the visibility of this study.

(1) To further validate the critical roles of PRC2 and KMT2D in restricting EMP, will exogenous expression of EZH2 or KMT2D in C2-mesenymal clone switch and lock cells into epithelial state?

(2) As EMT is tightly associated with drug resistance, have C1, C2 (epithelial or mesenchymal state), C1-sgEED, or C1-sgKMT2D been tested for their sensitivities to chemotherapeutic agents? Such information can correlate with their EMT status and lung metastasis in vivo.

(3) In Extended Data Fig 6b, it is better to include the measurement of H3K4me2/3 status, because KMT2D is known to regulate H3K4me1/2/3. In addition, KMT2D-KO significantly increased H3K27me3, suggesting that PRC2 activity was increased in C1-sgKMT2D cells. This is somewhat against the conclusion of reduced EZH2/PRC2 association at its target gene promoters (including ZEB1/2) in the C1-

sgKMT2D cells (Figs2e-2f). Have ChIP-seq using H3K4me3 and H3K27me3 antibodies been performed on these clones and compared with the results on Figs 2e-2f?

(4) For the C1 clone, the result on EED-KO (Fig 2d) is somewhat different from the result using two different PRC2 inhibitors (TAZ and EED226 in Fig 5e). C1 cells with EED-KO (C1-sgEED) have a large population in mesenchymal state, however, treatment with TAZ or EED226 did not lead a significant population of cells in mesenchymal state.

(5) On Extended Data Fig 4e, only a minor fraction of cells were in mesenchymal state after EED- or KMT2D-KO in lung cancer HCC827 cells. Does TGFβ increase EMT in these KO cells?

(6) In the CUT&RUN methodology, which antibody was used? Is EZH2 or EED?

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READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

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ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

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METHODS – Nature Cell Biology publishes methods online. The methods section should be provided as a separate Word document, which will be copyedited and appended to the manuscript PDF, and incorporated within the HTML format of the paper.

Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words. The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled "Statistics and Reproducibility".

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• At a minimum, please include a statement confirming that all relevant data are available from the authors, and/or are included with the manuscript (e.g. as source data or supplementary information), listing which data are included (e.g. by figure panels and data types) and mentioning any restrictions on availability.

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All imaging data should be accompanied by scale bars, which should be defined in the legend. Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it should only be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

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We accept files from the following graphics packages in either PC or Macintosh format:

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- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc.). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the “unprocessed scans” Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial http://www.nature.com/ncb/authors/submit/index.html#suppinfo; http://www.nature.com/ncb/journal/v14/n3/index.html#ed). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos much be accompanied by a separate Word document including titles and legends.

GUIDELINES FOR EXPERIMENTAL AND STATISTICAL REPORTING

REPORTING REQUIREMENTS – We are trying to improve the quality of methods and statistics reporting in our papers. To that end, we are now asking authors to complete a reporting summary that collects information on experimental design and reagents. The Reporting Summary can be found here

https://www.nature.com/documents/nr-reporting-summary.pdf)If you would like to reference the guidance text as you complete the template, please access these flattened versions at http://www.nature.com/authors/policies/availability.html.

STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from n<3. For sample sizes of n<5 please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled “Statistics Source Data”, and mentioned in all relevant figure legends.

--------- Please don't hesitate to contact NCB@nature.com should you have queries about any of the above requirements ---------

Author Rebuttal to Initial comments

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

This manuscript elucidated the complexity of epigenomic barriers during epithelial to mesenchymal plasticity (EMP). Using molecular techniques including a CRISPR KO system, they identified two methyl transferase complexes (PRC2 and KMT2D/COMPASS) that play the role of "guardian" in epithelial cells. Loss of either potentiated TGFβ-induced EMT. Loss of PRC2 in epithelial cells leads to a quasi-mesenchymal state, which makes cells more capable of lung colonization. The authors also provided evidence supporting that the loss-of-function of PRC2 predicts poorer survival in breast cancer patients. Overall, the finding challenges current knowledge on the role of these complexes in cancer. Although the conclusion is intriguing, more validation is required to extrapolate to a general cancer context.

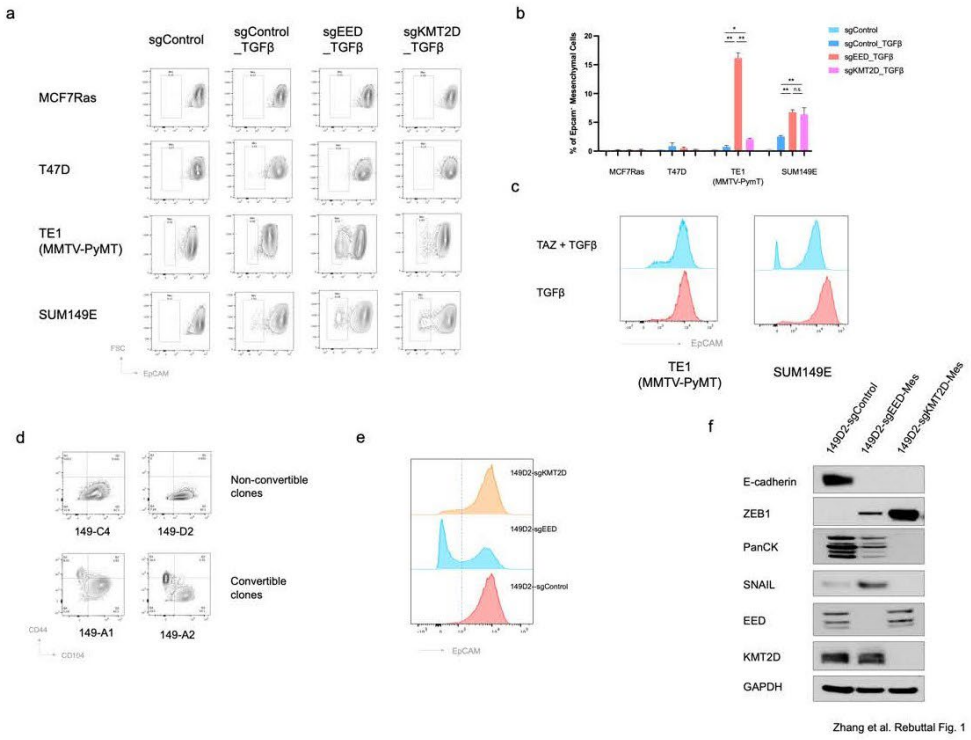
We appreciate the comprehensive and accurate summary of our study by the reviewer. We have addressed the questions raised by the reviewer shown below.

Major comments:

1- A major drawback of this study is that key results derive mostly from HLMER, an immortalized epithelial cell line. It is well established that cancer subtypes have different epigenetic signatures, therefore it is unclear whether PRC2 and KMT2D/COMPASS will play a similar role in cancer cells of different epigenomic landscape. Several key observations need to be confirmed using additional models recapitulating major subtypes of breast cancers. These key points include the existence of plastic clones and nonconvertible clones, the loss of PRC2 or KMT2D/COMPASS during the acquisition of EMP, the distinct EMT states upon PRC2 or KMT2D KO, and the quasi-mesenchymal state induced by PRC2 KO.

We thank the reviewer for this thoughtful suggestion. To explore the function of PRC2 and KMT2D-COMPASS complexes in distinct breast cancer subtypes, we have extended our analysis using additional cell line models representing these various subtypes. As shown in R-Fig.1a & b, we found that there were heterogeneous changes of EMP following loss of PRC2 or KMT2D-COMPASS, depending on the cell line being investigated. Thus, in MCF7Ras and T47D cell lines, which represent luminal subtypes of human breast cancer, we did not observe the induction of a mesenchymal cell population even in the presence of exogenous TGFβ. In contrast, we studied the behavior of two more epithelial mammary cell populations: (1) The TE1 cells, which represent a cell line developed from tumors of the MMTV-PyMT transgenic mammary tumor model showing an $EpCAM^{+}$ epithelial phenotype and (2) SUM149E cells, which represents a FACS-sorted subpopulation of human SUM149 breast cancer cells which exhibits an $EpCAM^{+}CD104^{+}CD44^{lo}$ epithelial phenotype. In each of these cell populations, we knocked out the genes encoding the EED and KMT2D individually and followed their responses. In both cases, we observed a gain of EMP (R-Fig. 1a & b) similar to the one that we described previously in the HMLER cells. In addition, in both

cell lines we observed the acquisition of EMP following loss of PRC2 function by treating the TE1 and SUM149E cells with Tazemetostat (TAZ), a pharmacological inhibitor of PRC2 (R-Fig. 1c). Hence, our initially described observations with HMLER cells were generalizable in at least two additional mammary carcinoma cell types. The MMTV-PyMT mammary tumor model, in which TE1 cells were derived, is commonly considered as a luminal-like model. While SUM149 represents triple-negative subtype of human breast cancer. Thus, the subtype of breast cancer is less likely to be the only determinant of these epigenetic complexes in regulating EMP. It remains to be determined whether additional factors modulate the ability of PRC2 and KMT2D-COMPASS in regulating EMP, which represents an open-ended question that is well beyond the scope of the current study.



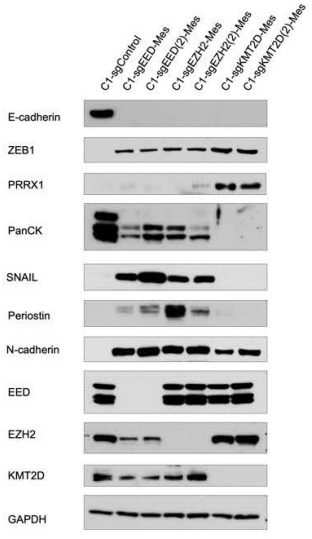
Zhang et al. Rebuttal Fig. 1

In addition, we uncovered other similarities between the previously reported HMLER cells and the SUM149 human breast cancer cells. As we reported previously, we observed two distinct types of epithelial cell populations, one type being convertible and thus prone to spontaneous generation upon propagation in culture of more mesenchymal cells, and the other type being stably established in the more epithelial state without giving evidence of any spontaneous generation of more mesenchymal cell types. Similar to the previously reported HMLER model, when we derived single-cell-derived clonal populations from SUM149E cells, we also found convertible clones such as A1 and A2, which could spontaneously convert into $CD44^{hi}$ mesenchymal states, and non-convertible clones such as C4 and D2, which stably persist in the $CD44^{lo}$ epithelial state when cultured in vitro (R- Fig. 1d).

In particular, knocking-out EED in the initially non-convertible SUM149-D2 cells elevated their EMP and generated a quasi-mesenchymal cell state. These cells, similar to what we previously reported with HMLER cells, moved from the epithelial state into a quasi-mesenchymal state characterized by retention of cytokeratin expression together with acquired expression of SNAIL. In contrast, On the other hand, knocking-out KMT2D in the cloned SUM149-D2 cells resulted in these previously epithelial cells migrating into a highly mesenchymal cell state (R-Fig. 1e & f). Hence, manipulation of cells derived from patient-derived human breast cancer model echoed and thus confirmed our previous observations in the HMLER system, including the pre-existence of epithelial cells that naturally exhibited distinct degrees of EMP, unlocking EMP upon knock-out of EED or KMT2D, and distinct mesenchymal states generated by loss of EED or KMT2D. These new results have been added into the revised manuscript (Extended Data Fig. 4f and 7g).

2- There is a concern about the data generated by the KO cells derived from single-cell clones using a single sgRNA in this study. Considering the possibility of off-target in CRISPR-CAS9 system and the heterogeneous nature of parental cells, at least two to three additional KO clone using sgRNAs targeting a different region of EED or KMT2D needs to be tested side by side in some key experiments.

Following the reviewer's suggestion, we have designed additional guide RNAs against EED and KMT2D targeting different genomic segments of their corresponding genes. As shown in R-Fig. 2, these new sgRNAs including sg-EED(2) and sg-KMT2D(2) also increased EMP in initially non-convertible HMLER-C1 cells. Importantly, precisely recapitulating our previously reported observations, both sgRNAs targeting EED resulted in spontaneous migration toward a quasi-mesenchymal cell states as shown by retained expression of cytokeratin and acquired high expression levels of SNAIL. Once again, and replicating our previous observations, both sgRNAs targeting KMT2D enabled previously non-convertible epithelial cells to enter into a highly mesenchymal cell state (R-Fig. 2). These data have been added into the revised manuscript (Fig. 3g) and the text has been changed accordingly.



Zhang et al. Rebuttal Fig. 2

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3- The study seems unidirectional (from Epithelial to Mesenchymal). The reciprocal process (i.e., MET) needs to be examined. It seems clear that the PRC2 complex is maintaining homeostasis in immortalized cells. Based on multiple studies suggesting the role of the PRC2 complex in promoting EMT and metastasis (PMID: 22187039, PMID: 26848980, PMID: 30353102), it remains uncertain whether EED depletion would lead to different outcome if the initial cells were mesenchymal (e.g. MDA-MB-231)? Moreover, the study suggests that blocking both PCR2 complex and KMT2D-COMPASS promotes EMT and tumor growth in lung. It would be highly informative to evaluate whether lung lesions maintain their EMP phenotype or revert to an epithelial state? Similarly, the fact that EED inhibition promotes EMT via Zeb1 may suggest that overexpressing EED and/or other PRC2 members may reverse the EMT phenotype in mesenchymal cells. However, this was not sufficiently addressed in the study.

We agree with the reviewer that additional analysis is necessary to examine the effects of PRC2 complex in regulating EMP. In response to these suggestions, we have now generated new data to clarify these three issues:

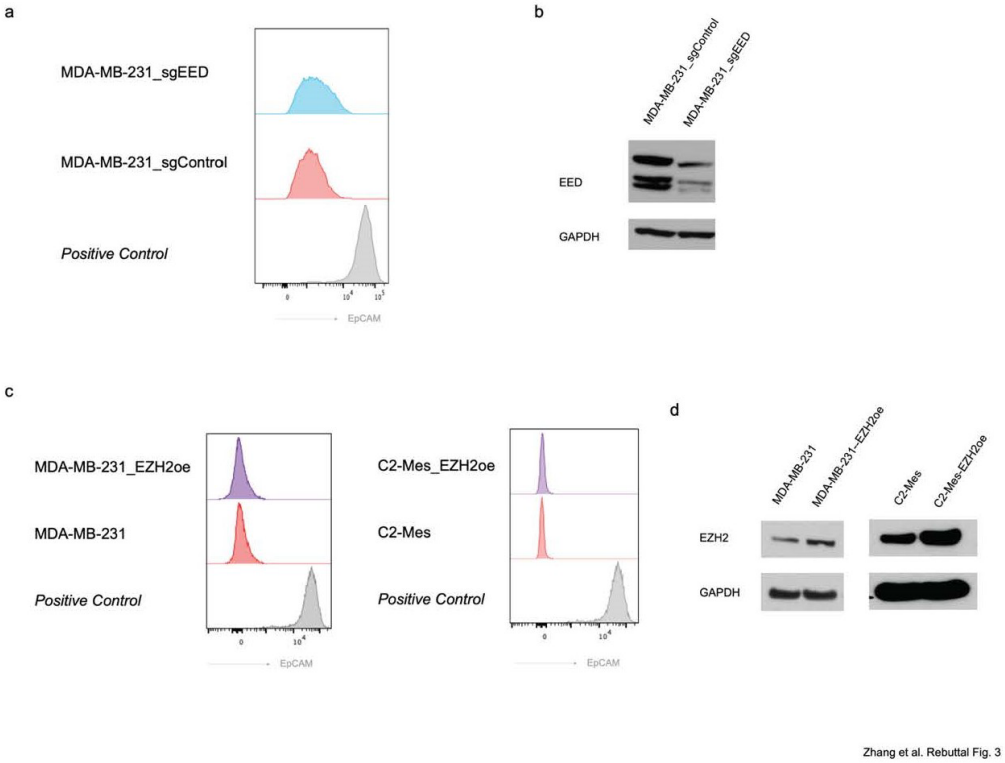
1) Knock-out EED in the initially mesenchymal MDA-MB-231 cells did not induce a mesenchymal-to-epithelial transition process (R-Fig. 3a & b); Hence EED action operates to maintain residence in an existing epithelial phenotypic state but does not seem to participate in maintain an existing more mesenchymal cell, as least in the human breast cancer cells examined by us.

2) The reviewer asked whether the lung lesions initiated by EED-KO quasi-mesenchymal cells maintained their mesenchymal phenotype or revert to an epithelial state. As shown in Fig. 4e & f, immunostaining of lung metastases initiated by C1-sgEED-Mes showed cells remaining in an E-cadherin negative, cytokeratin and periostin positive quasi-mesenchymal cell state. These data indicates that at least in this system, it is not necessary for these cells to convert back to an E-cadherin positive epithelial state to form lung metastases.

3) When EZH2, the catalytic subunit of PRC2 complex, was overexpressed in MDA-MB-231 or C2-Mes cells, we did not see a conversion of the mesenchymal cell state into an epithelial one (R-Fig. 3c & d). This is consistent with the previous observation that once HMLER-C1 cells entered the $CD44^{hi}Epcam^{-}$ mesenchymal state following treatment of PRC2 inhibitors, removal of PRC2 inhibitor failed to force the cells to trigger a mesenchymal-to-epithelial transition (Extended Data Fig. 10c & d).

When taken together, the observations showed that although loss of PRC2 in non-convertible epithelial HMLER cells elevated EMP, restoration of PRC2 function by removing PRC2 inhibitors or overexpression of EZH2, the catalytic subunit of PRC2 complex, was not sufficient to trigger an MET process in mesenchymal cells. As we have observed in this experiment and in other work not described here, the EMT and MET are not fully reversible processes and cells that have transited into a phenotypic state through a drug treatment or gene knock-out cannot always exit that

phenotypic state and return to their original phenotypic state simply by withdrawing the drug or re-expressing the gene. In other words, the EMT- and MET-induced cell state changes are not simply reversible for reasons that we and others do not fully understand. This phenomenon is likely caused by extensive transcriptional and epigenetic reprogramming that accompanies the process of EMT, such as the establishment of certain robust, self-reinforcing autocrine positive feedback loops, the details of which are out of the scope of our paper.



Zhang et al. Rebuttal Fig. 3

4- Knocking down EED may impact the non-enzymatic function of EZH2, which was previously shown to activate stemness related pathway. Therefore, to confirm that increased EMT and metastasis formation is a direct reflection of EED depletion, other components of the PRC2 complex must (including EZH2) must be depleted and evaluated similarly to the above. Using a pharmacological approach may not be sufficient as non-enzymatic functions may also be involved. A previous study suggest that cytoplasmic EZH2 following p38-mediated phosphorylation at T367 promotes breast cancer metastasis (PMID: 30022044).

We agree with the reviewer that additional analysis of EZH2, the catalytic subunit of PRC2, should be included to examine its function on regulating EMP. To address these concerns, we have now knocked out the gene encoding EZH2 in HMLER-C1 cells and found that it enabled these initially non-convertible epithelial cells to convert into mesenchymal cell state, which is similar to EED knock-out (R-Fig. 2). This result is also consistent with 1) our previously described CRISPR screening results, in which we found that multiple components of the PRC2 complex, namely EED, EZH2 and SUZ12, were functionally important in HMLER-C1 cells to maintain the stable epithelial

state; and 2) pharmacological inhibition of PRC2 in HMLER-C1, MCF10A, TE1 and SUM149E cells led to elevated EMP. In addition, by knocking-out EZH2 in HMLER-C1 cells, we have now shown that similar to loss of EED, loss of EZH2 resulted in a quasi-mesenchymal cell state, as revealed by its retained expression of cytokeratin and acquired high expression of SNAIL (R-Fig. 2). These results have been added into the revised manuscript (Fig. 3g). Collectively, our previous and new data provided strong evidence that the epigenetic regulatory complex PRC2 plays an essential role in maintaining cells in a stable epithelial state and loss of PRC2 generated a quasi-mesenchymal cell state.

We thank the reviewer to mention an earlier publication suggesting EZH2 phosphorylation promotes breast cancer metastasis (PMID: 30022044). An important clinical observation from this study is that cytoplasmic localization of EZH2, which is mediated by its T367 phosphorylation, is associated with breast cancer invasiveness and metastasis. This post-translational modification of EZH2 leads to reduced nuclear function (presumably as a component of PRC2) as shown by lower H3K27me3 levels. This clinical observation is indeed consistent with our data in which reduced PRC2 function was found to elevate EMP and generate a metastatic, quasi-mesenchymal cell state. In certain contexts, the cytoplasmic, phosphorylated EZH2 might further promote invasion and metastasis, as suggested by this report.

5- Basically, the different mesenchymal states resulting from knock out of EED and KMT2D were well characterized in epithelial HMLER clones, but the underlying mechanism appears unclear. First, since ZEB1 and ZEB2 were upregulated in both EED and KMT2D knockout cells, the link between EMT-TFs to the distinct mesenchymal states is missing. Evaluating this may be key to comprehend the mechanistic differences underlying EED and KMT2D KO.

The EMT-TFs ZEB1 and ZEB2 were both expressed in the mesenchymal cells generated by EED or KMT2D knock-out. However, ZEB1 expression was much higher in the KMT2D-KO highly mesenchymal state at mRNA level (Fig. 3f). In addition, we did observe some differential expression patterns of other EMT-TFs, including SNAIL and PRRX1. Thus, the quasi-mesenchymal cell state generated by EED-KO showed higher level of SNAIL while the KMT2D-KO highly mesenchymal cell state expressed higher level of PRRX1. This observation is similar to a recent report in which these two EMT-TFs were found to distinguish two distinct mesenchymal states (PMID: 31712603). Importantly, in this report the SNAIL-high quasi-mesenchymal state was found to associate with poor prognosis in breast cancer patients, which is consistent with our observation that EED-KO quasi-mesenchymal state (SNAIL-high) showed highly metastatic abilities. Therefore, the quasi-mesenchymal and highly mesenchymal cell states generated by EED-KO and KMT2D-KO respectively do show different expression patterns of certain EMT-TFs.

We agree with the reviewer that more studies are needed to understand the exact mechanisms of how loss of these epigenetic regulatory complexes determine the two distinct EMT trajectories. We plan to address these questions in a follow-up study.

6- Though the authors did a comprehensive comparison between the parental epithelial cells and mesenchymal fractions of sgEED or sgKMT2D cells, the comparison in the epithelial fractions of

control cells and KO cells is still needed. Such analysis might provide clues on how the plastic clones are initiated and how they are directed to divergent EMT trajectories. This is also relevant to the single-cell trajectory map in Fig 3B. The epithelial subpopulation in EED KO cells locates in the transition zone between C1 control cells and the epithelial KMT2D KO cells. This data suggests that there is another layer of epigenetic or genetic barrier that prevents the epithelial EED KO cells from further evolving to the trajectory represented by KMT2D KO cells. This barrier can be further characterized.

We appreciate the reviewer's raising of this important question about the epithelial fractions in EED-KO and KMT2D-KO cells. In HMLER-C1 cells, we did notice that the cells underwent complete loss of EED or KMT2D will take some time to complete the EMT process that lead to either the quasi-mesenchymal or fully mesenchymal phenotypic state. This is reflected in the single-cell trajectory map we generated in which a proportion of EED-KO or KMT2D-KO cells remains largely in the epithelial state. We think this is likely due to a process in EED-KO and KMT2D-KO cells whereby they gradually lose H3K27me3 marks and change additional epigenetic/transcriptional programs to enable the EMT process. The EED-KO and KMT2D-KO epithelial states are not stable - If we culture these cells long enough, all the EED-KO and KMT2D-KO cells will eventually enter their respective mesenchymal states and stably reside in such states in culture.

The reviewer asked us to further characterize the epithelial fractions of control cells and KO cells. Indeed, the CUT&RUN experiments were performed using the epithelial fraction of sgControl, sgEED and sgKMT2D cells (Fig. 2 e-g and Extended Data Fig. 6), in order to explore how gene knock-out of either EED or KMT2D elevated EMP. From these experiments we found that in C1-sgControl cells, PRC2 bind to promoter regions of ZEB1/2 genes and this binding is lost in both sgEED and sgKMT2D conditions. A more detailed description of this procedure has now been added to the figure legends and the Methods section.

7- The connection between the EED or KMT2D KO cells and the convertible clones appears weak. The authors did some preliminary comparisons among the epithelial C2 fraction and EED or KMT2D KO cells in Fig 2 and extended fig 6. Such comparison may be misleading as both EED and KMT2D KO cells were a mixture of epithelial and mesenchymal cells whereas the C2 fraction was purified epithelial cells. It is difficult to understand if the observed differences on EMT markers or selected promoter occupancy were due to the different plasticity status or the different proportion of the mesenchymal population in the mixtures. Importantly, whether the EED or KMT2D KO in nonconvertible clones could truly recapitulate some of the preexisted, naturally occurred plastic clones in HMLER cells was still unclear. Therefore, an unbiased and systemic comparison using the epithelial fraction of genetic KO clones and plastic single-cell clones may clarify this point.

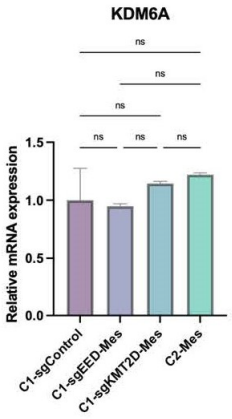
As mentioned above, the CUT&RUN experiment was performed using epithelial C1-sgControl cells and the epithelial fraction of C1-sgEED, C1-sgKMT2D and C2 cells. Thus, none of the samples used in this experiment was a mixture of epithelial and mesenchymal cells. As a result, the possible different epigenetic configuration of mesenchymal cells compared with epithelial cells should not contaminate the result from this experiment.

From this experiment, we found that the loss of PRC2 binding to the promoter region of *ZEB1/2* is a common feature for all the phenotypically plastic epithelial cells, including C1-sgEED (Epi), C1-sgKMT2D (Epi) and naturally plastic C2-Epi cells, suggesting this is a major mechanism constraining EMP in this system.

8- The authors demonstrate that TGFβ is required for the mesenchymal transition of C1-sgEED and C1-sgKMT2D cells as SB-431542 (TGFB inhibitor) abrogates CD44hi conversion. However, in a previous study (PMID: 29029452), the histone demethylase KDM6A was reduced following TGFB-mediated E-to-M transition and re-expressed during the M-to-E transition. Additionally, the KDM6A was found to be lower in stem-like cells compared to non-stem cell counterparts. More experiments/discussions may be required to reconcile these findings.

We thank the reviewer for raising this interesting question about KDM6A in regulating EMP. KDM6A (also known as UTX) encodes one of human histone H3K27 demethylases, which could potentially counteract PRC2 function, though KDM6A is also known to play functional roles independent of its H3K27 demethylase activity (PMID: 21095589, 22949634, 23028370).

In our study, using genetic and pharmacological methods, we provided strong evidence showing that loss of PRC2 elevated EMP. As mentioned above, we also found that once the cells entered a mesenchymal state, manipulating PRC2 function does not lead to significant change of their epithelial-mesenchymal status. Thus, we think the observation that KDM6A is lower in mesenchymal state following TGFβ-induced EMT in the previous study does not necessarily contradict our findings. In addition, in our system, we did not notice a significant change of KDM6A expression among the epithelial C1-sgControl, C1-EED-KO quasi-mesenchymal, C1-KMT2D-KO highly mesenchymal and C2-Mes cells (R-Fig. 4). It will be interesting in the future to explore the function of KDM6A in regulating EMP and cancer stemness in both normal and PRC2-null conditions, though we feel that such research is out of the scope of the current study.



Zhang et al. Rebuttal Fig. 4

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Minor comments:

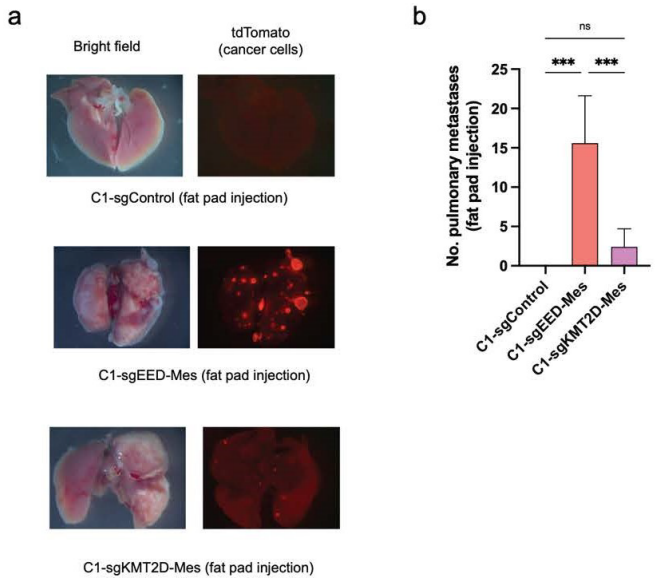
1- In clinical data analysis, the authors should examine if LOF mutations of PRC2 and

KMT2D/COMPASS are enriched in specific breast cancer subtypes and/or perform the survival analysis in a specific subtype of cancer patients.

We thank the reviewer for the suggestion. However, given the limited number of patients with LOF mutations of PRC2 or KMT2D-COMPASS components in the TCGA database, it is statistically challenging/impossible to perform survival analysis of such mutations in different subtypes in a statistically significant way. Nevertheless, as shown in Fig. 5d, using a gene signature for EED-KO quasi-mesenchymal cell state, we did find that in ER-negative patient cohort, the association between this signature and poor prognosis is more readily apparent.

2- Spontaneous metastasis from the primary tumor should be examined to verify the metastatic potential of cells with different EMT states.

We agree with the reviewer that spontaneous metastasis from the primary tumors should be examined to test the abilities of cells with PRC2 or KMT2D-COMPASS loss to complete the whole invasion-metastasis cascade. To do so, C1-sgControl, C1-sgEED-Mes and C1-sgKMT2D-Mes cells were implanted into orthotopic sites, i.e., the mammary fat pad of NSG mice, and lung metastases were measured 8 weeks later. Echoing the results from the tail-vein injection assay, we found that EED-KO quasi-mesenchymal cells displayed the strongest ability in forming lung metastases (R-Fig. 5). These new findings have been added to the revised manuscript (Extended Data Fig. 8e & f).



Zhang et al. Rebuttal Fig. 5

3- The authors have shown that the quasi-mesenchymal states induced by genetic KO or pharmaceutical inhibitors are not reversible by withdrawing inhibitors. It is necessary to test if re-

expression of these genes could block the TGFβ induced EMT in the KO cells.

As mentioned above, using multiple different methods, including genetic knock-out of different members of PRC2 complex and treatment using PRC2 pharmacological inhibitors, we have convincingly shown that loss of PRC2 elevated EMP. We also found that once the cells enter the quasi-mesenchymal state, re-expression of catalytic subunit of PRC2 complex, EZH2, is insufficient to induce an MET process, suggesting the regulation of EMP by PRC2 complex relies on the initial cell state. This result is consistent with our observation that once the cells entered the quasi-mesenchymal state following PRC2 inhibitor treatment, withdrawing these pharmacologic inhibitors could not induce an MET program, i.e., as described above, the process is not reversible for reasons that at present remain mechanistically unclear.

4- Extended Figure 1f: It is not clear how the co-culture was performed and how each cell was discriminated after sequencing. More specific information will help better understand the results.

In the experiment shown in Extend Figure 1f, C1, C2 and parental HMLER cells were barcoded before co-culture and all the cells were sequenced simultaneously. In the process of data analysis, C1, C2 and parental HMLER cells were annotated by the expression of specific RNA-barcode. We have now added more specific information in the figure legends in the revised manuscript.

5- In extended Figure 2, despite the lack of significant differences between c1 and c2-Epi in terms of TGF signaling, there appears to be a response following TGFβ treatment on both models. Could this suggest indirect effects of TGFβ?

As shown in Extended Figure 2, we found that both the non-convertible C1 cells and the convertible C2-Epi cells expressed and secreted TGFβ. In addition, they both exhibited autocrine TGFβ signaling activity as measured by phospho-Smad2 levels. Moreover, as a well-established biological function of TGFβ signaling pathway, TGF-β-induced cytostatic effects were observed in both cell types. However, a TGF-β-induced EMT program could only be efficiently activated in convertible C2-Epi cells and not in the non-convertible C1 cells. This indicates that certain mechanisms operating in C1 cells downstream of TGF-β signaling pathway exist to prevent efficient EMT activation. Based on our study, it is likely that PRC2 binding to the ZEB1/2 promoter plays an important role in limiting TGF-β-induced EMT in these cells.

Reviewer #2:

Remarks to the Author:

Zhang et al use elegant single cell approaches to identify factors that regulate epithelial-mesenchymal plasticity (EMP) in vitro. First, they demonstrate that single cell clones of HMLER epithelial cells exist in two subpopulations, which upon extended propagation either maintain their epithelial identity or exhibit extensive EMP. The authors then did a series of KO screens to identify factors required to suppress EMP in the stable epithelial clones. Remarkably, these screens indicated that loss of two histone methyltransferase complexes, PRC2 and KMT2D-COMPASS, triggered EMP. These two activities have opposite effects on gene expression. PRC2 mediates gene silencing through methylation of histone H3 K27, whereas KMT2D-Compass activates gene transcription by methylation of H3 K4. RNA-seq analyses, protein expression patterns, and functional assays all indicate that loss of PRC2 leads to a quasi-mesenchymal state that facilitates metastatic properties, whereas loss of KMT2D leads to a highly mesenchymal state and metastatic dormancy.

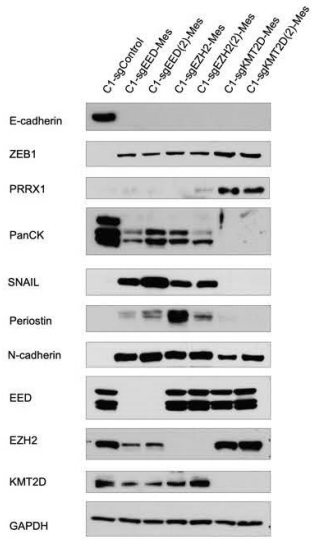
These findings provide important advances in our understanding of the regulation of EMP and how different EMP states contribute to metastatic potentials. As such, this paper will be of broad interest to both basic biologists and clinicians. These findings will/should have immediate impact on stratification of patients in clinical trials of PRC2 inhibitors as cancer therapies.

We appreciate the reviewer's comprehensive and accurate summary of our study.

The only weakness of the paper is the lack of mechanistic insights as to how/if the gene expression changes observed upon loss of PRC2 or KMT2D lead to the quasi-mesenchymal or highly mesenchymal states, respectively. Nor is it clear whether non-transcriptional, non-histone targets of these enzymes might also contribute to these different states. However, this paper clearly provides much fertile ground for future studies.

We agree with the reviewer that more mechanistic insights about how loss of PRC2 or KMT2D lead to the two distinct mesenchymal cell states would be important to explore further. In response to the reviewer's remarks, we now have made some progress in better understanding this question and describe these more recent observations in the revised manuscript. This includes:

1) Using multiple alternative guide RNAs to knock-out EED and the catalytic subunit of PRC2 complex, EZH2, we found that loss of either of these PRC2 components was able to induce HMLER-C1 cells to enter a quasi-mesenchymal cell state (R-Fig. 2). Although some of the PRC2 components have been reported to play PRC2-independent functions, these data, together with our previously described CRISPR screening results, in which we found that multiple components of the PRC2 complex, namely EED, EZH2 and SUZ12, were functionally important in HMLER-C1 cells for maintaining the stable epithelial state, suggest these proteins regulate EMP in a PRC2-dependent manner.

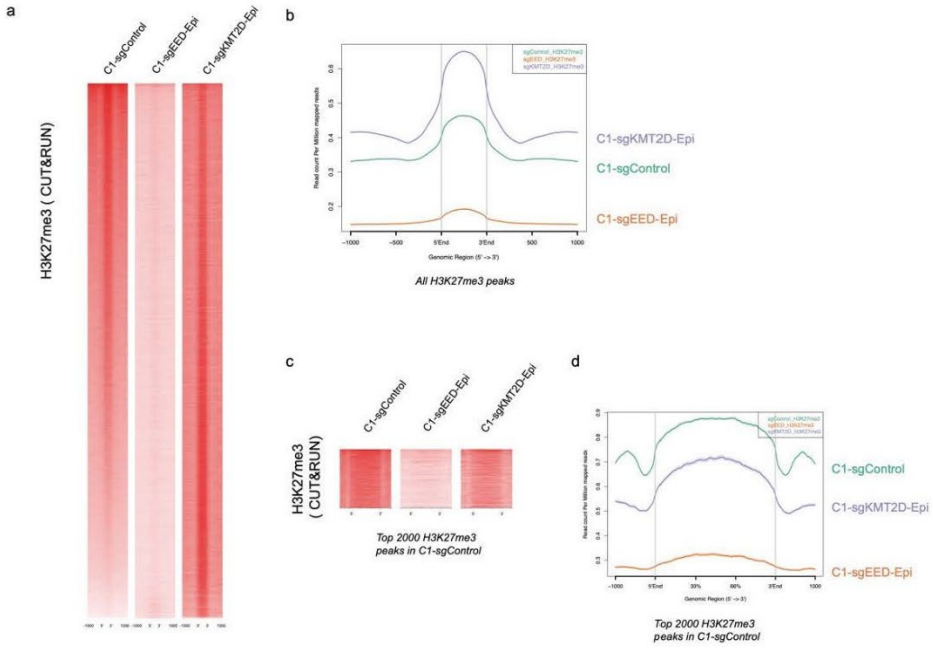


Zhang et al. Rebuttal Fig. 2

2) Trying to improve our understanding how EED-KO and KMT2D-KO generated two distinct mesenchymal cell states as the reviewer mentioned, we performed CUT&RUN experiment to examine the H3K27me3 status after loss of PRC2 or KMT2D. We found loss of PRC2 (EED-KO) almost completely remove this histone modification. Interestingly, however, KMT2D-KO leads to a global change of H3K27me3 mark distribution (R-Fig. 6) – many previously present H3K27me3-positive regions in epithelial HMELR-C1 cells showed lower signal and other regions gained H3K27me3 marks. This explains our previous observation that KMT2D-KO leads to an increase of total H3K27me3 level (Extended Data Fig. 6b). These results have now been added to the revised manuscript (Extended Data Fig. 6g-j). We think the changes of H3K27me3 marks and their associated facultative heterochromatin status might play important roles in determining these two distinct EMT subprograms. We plan to further study the mechanistic details of how KMT2D-KO leads to the global change of H3K27me3 mark distribution and how these two EMT trajectories are orchestrated by loss of PRC2 or KMT2D-COMPASS in a follow-up study.

One minor point: On page 8, the authors state that KMT2D-COMPASS implements H4K4me1 at enhancers, which is true, but it is also required for H3K4me3 in certain contexts (see Dhar et al, PMID 29861161). The authors should correct the wording of the sentence to reflect the broader functions of KMT2D.

We thank the reviewer for the advice. This sentence in the manuscript has been modified and the reference mentioned by the reviewer has been cited accordingly.



Zhang et al. Rebuttal Fig. 6

Reviewer #3:
Remarks to the Author:

EMT is a developmental program that provides cells with the increased morphological and cellular plasticity required during embryonic development, tissue remodeling, wound healing and metastasis. Although this reversible and dynamic process, which is often propelled by microenvironmental signals, is regulated at the epigenetic level, many mechanistic details remain unclear. The major reason is that the induction of EMT (by TGFβ) in vitro is a highly inefficient process (Brown KA, Breast Cancer Res, 2004;6(3):R215-31), only a few cell lines, including NMuMG (mouse), MCF10A (human), and HMLE (human), are commonly used for studying EMT in vitro. This casts technical challenge to identify critical epigenetic regulators that either restrict or permit the reversible and dynamic EMT process operated during metastatic cascade and therapeutic treatments. In this study, Zhang and colleagues isolated a relative stable C1 clone that remained in epithelial state from parental HMLER cells.

Interestingly, TGFβ failed to induce EMT in this C1 clone, suggesting that an intrinsic "memory" machinery is operated to "locks" these cells in epithelial state. Using unbiased CRISPR-Cas9 screening, the authors further found that two epigenetic complexes, PRC2 and KMT2D-Compass, played critical roles in restricting the EMT process. Intriguingly, EED-KO conferred C1 clone in a quasi-mesenchymal state and restored the cellular plasticity (EMP) required for lung metastasis in vivo. However, KMT2D-KO changed C1 clone all the way to mesenchymal state and these cells lost the ability to metastasize. These findings are very interesting and novel, as these indicate that distinct EMT trajectories or identities from E to M spectrum are regulated by different chromatin modifiers. Overall, this study represents a new advance in the EMT field and sheds new insights in the epigenetic regulation and cancer biology. Experiments were well designed and executed; robust quality data were presented and they were clear and supportive to the main conclusion. A few suggestions are recommended to further strengthen the conclusion and improve the visibility of this study.

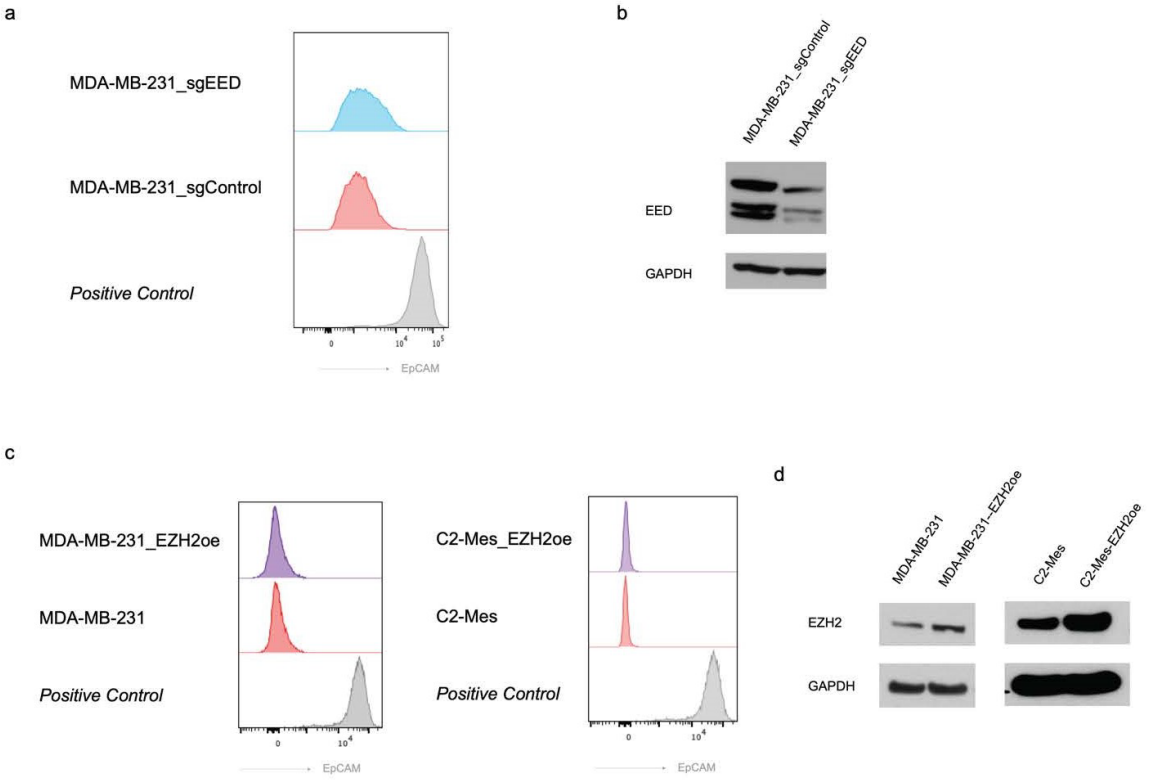
We thank the reviewer for the comprehensive and accurate summary of our study. We also would like to thank the reviewer for a number of insightful suggestions. We have addressed the reviewer's questions below.

(1) To further validate the critical roles of PRC2 and KMT2D in restricting EMP, will exogenous expression of EZH2 or KMT2D in C2-mesenymal clone switch and lock cells into epithelial state?

Following this suggestion, we overexpressed EZH2, the catalytic subunit of PRC2 complex, in both MDA-MB-231 and C2-Mes cells. We did not see a conversion of the mesenchymal cell state into an epithelial one (R-Fig. 3c & d). This is consistent with the previous observation that once HMLER-C1 cells have entered the $CD44^{hi}Epcam^{-}$ mesenchymal state following treatment of PRC2 inhibitors, removal of the inhibitor failed to force the cells to reverse the process by triggering a mesenchymal-to-epithelial transition (Extended Data Fig. 10c & d).

1

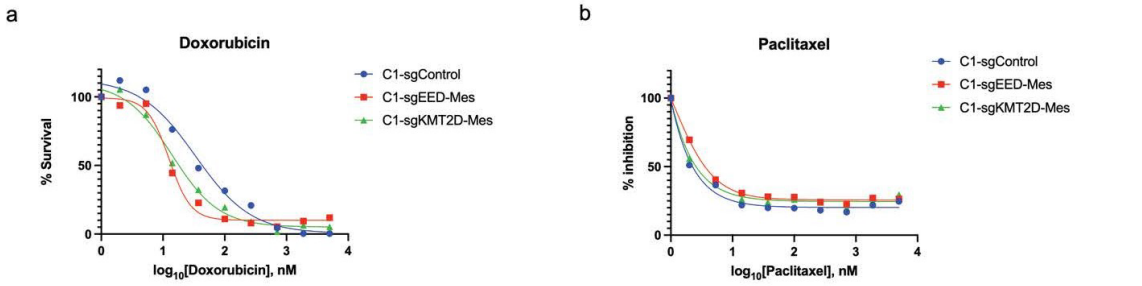
Thus, as stated in response to other reviewers' remarks above, entrance into certain phenotypic state following inhibition or overexpression of various regulators is often not reversible for reasons that are mechanistically unclear at present. This phenomenon might be caused by extensive transcriptional and epigenetic reprogramming that accompanies the process of EMT, such as the establishment of certain robust, self-reinforcing autocrine positive feedback loops.



Zhang et al. Rebuttal Fig. 3

(2) As EMT is tightly associated with drug resistance, have C1, C2 (epithelial or mesenchymal state), C1-sgEED, or C1-sgKMT2D been tested for their sensitivities to chemotherapeutic agents? Such information can correlate with their EMT status and lung metastasis in vivo.

In response to this question, we tested the sensitivity of HMLER-C1 cells as well as the sensitivities of C1-sgEED-Mes and C1-sgKMT2D-Mes cells to Doxorubucin and Paclitaxel, two chemo-therapeutic drugs that are commonly used to treat breast cancer patients. We found the differences of sensitivity to these drugs were minimal among C1-sgControl, C1-sgEED-Mes and C1-sgKMT2D-Mes cells (R-Fig. 7). This result might be influenced by certain factors when we performed the experiment, such as seeding density, the time of drug treatment etc. We think it will be interesting in the future to systematically screen drugs in a follow-up study to identify specific drugs that preferentially target these phenotypic states arrayed along the E-to-M spectrum. 32

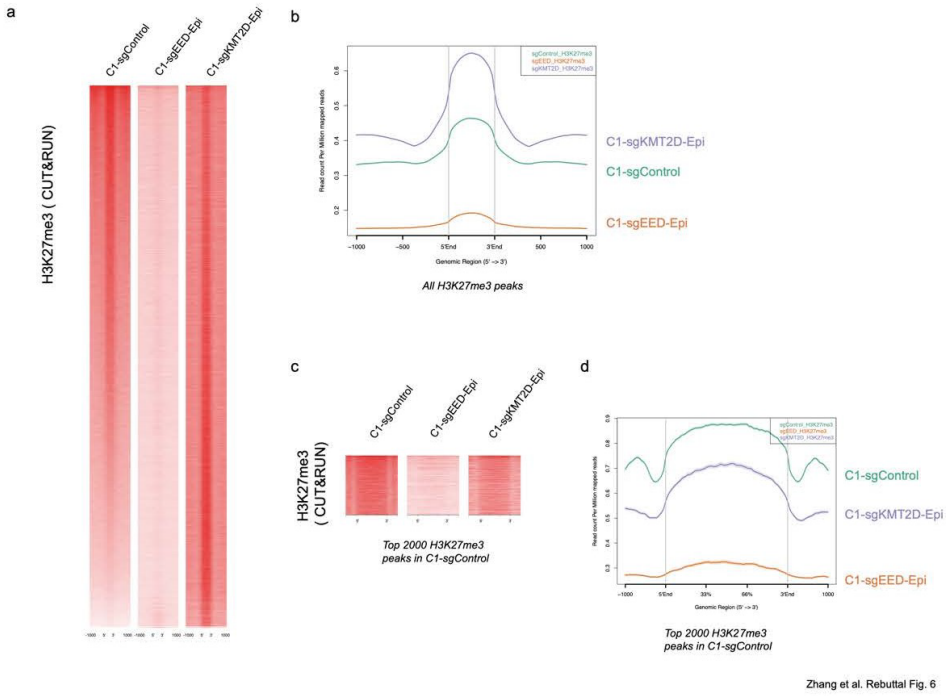


Zhang et al. Rebuttal Fig. 7

(3) In Extended Data Fig 6b, it is better to include the measurement of H3K4me2/3 status, because KMT2D is known to regulate H3K4me1/2/3. In addition, KMT2D-KO significantly increased H3K27me3, suggesting that PRC2 activity was increased in C1-sgKMT2D cells. This is somewhat against the conclusion of reduced EZH2/PRC2 association at its target gene promoters (including ZEB1/2) in the C1-sgKMT2D cells (Figs2e-2f). Have ChIP-seq using H3K4me3 and H3K27me3 antibodies been performed on these clones and compared with the results on Figs 2e-2f?

We thank the reviewer for this insightful suggestion. In response, we have updated the Extended Data Fig.6b to include additional histone markers including H3K4me1/2/3. We observed that KMT2D-KO induced a lower level of global H3K4me1 and H3K4me3, though such change was not as obvious as EED-KO on H3K27me3 mark.

In order to better understand the observation that H3K27me3 global level is higher in KMT2D-KO cells, we performed CUT&RUN experiment to profile H3K27me3 genome-wide locations. As shown in R-Fig. 6, we found that compared with C1-sgControl cells, EED-KO almost completely eliminated this histone modification, which is consistent with the western result shown in Extended Data Fig. 6b. Interestingly, however, KMT2D-KO led to a global change of H3K27me3 marks – many previously present H3K27me3-positive regions in epithelial HMELR-C1 cells showed lower signals and other regions gained H3K27me3 marks. This explains why the global level of H3K27me3 is even higher in KMT2D-KO cells. These results have now been added to the revised manuscript (Extended Data Fig. 6g-j). To summarize, we conclude that although KMT2D-KO increased H3K27me3 global level, it led to a shift of H3K27me3 distribution, which presumably plays important roles in elevating EMP and determining the specific EMT subprogram. We plan to further study the mechanistic details how KMT2D-KO leads to the global change of H3K27me3 mark distribution and how these two EMT trajectories are orchestrated by loss of PRC2 or KMT2D-COMPASS in a follow-up study.



(4) For the C1 clone, the result on EED-KO (Fig 2d) is somewhat different from the result using two different PRC2 inhibitors (TAZ and EED226 in Fig 5e). C1 cells with EED-KO (C1-sgEED) have a large population in mesenchymal state, however, treatment with TAZ or EED226 did not lead a significant population of cells in mesenchymal state.

It is true that EED-KO showed a stronger elevation of EMP in HMLER-C1 cells. We think this is likely caused by the fact that genetic knock-out lead to a complete loss of function of PRC2, while treatment with PRC2 inhibitors may only partially block PRC2 function. Nevertheless, treatment with the TAZ or EED226 pharmacologic inhibitors alone (particularly TAZ) was sufficient to induce EMP albeit with slower kinetics.

(5) On Extended Data Fig 4e, only a minor fraction of cells were in mesenchymal state after EED- or KMT2D-KO in lung cancer HCC827 cells. Does TGFβ increase EMT in these KO cells?

In Extend Data Fig. 4e, the results were obtained using HCC827-sgEED and HCC827-sgKMT2D at the population level without subsequent single-cell cloning (cells were transduced with pLentiCRIPSRv2 containing designed guide RNA, then selected using puromycin but without single-cell cloning), which means that there might be certain cells in this potentially heterogeneous cell population without complete gene knock-out. This would seem to explain why only a minor fraction of cells was transited into mesenchymal state. Nevertheless, this result showed that in HCC827 cells, PRC2 and KMT2D-COMAPSS played a functional role in regulating EMP. In addition, we have

now shown the behavior of additional cell lines that rely on these two complexes to regulate EMP, including SUM149 and TE1 (a cell line derived from mouse MMTV-PyMT model) cells (R-Fig. 1).

(6) In the CUT&RUN methodology, which antibody was used? Is EZH2 or EED?

In the CUT&RUN experiment, we used EZH2 antibody (Cell Signaling, #5246) to map PRC2 binding sites. As shown in R-Fig. 2, in EED-KO cells, EZH2 level was reduced but still present.

Decision Letter, first revision:

Subject: Your manuscript, NCB-Z45438A
Message:

Our ref: NCB-Z45438A

22nd December 2021

Dear Dr. Weinberg,

Thank you for submitting your revised manuscript "Loss of PRC2 or KMT2D-COMPASS Unlocks Distinct EMT Trajectories that Contribute Differentially to Metastasis" (NCB-Z45438A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Cell Biology Please do not hesitate to contact me if you have any questions.

Sincerely,

Zhe Wang, PhD
Senior Editor
Nature Cell Biology

Tel: +44 (0) 207 843 4924
email: zhe.wang@nature.com

Reviewer #1 (Remarks to the Author):

The authors have provided more evidence to support their claim. It has become evident that the process driven by the PRC2 -mediated EMP is mostly unidirectional and might be restricted to specific models [as endocrine responsive cells such as T47D an MCF7 did not respond]. That said, the notion of plasticity still remains a minor concern for me as cells do not revert back to their initial state when PCR2 complex is rescued via overexpression of EED or EZH2 or retrieval of EZH2 inhibitor. Also, the increase in H3K27me3 following KDM2D downregulation, although redistributed, leads to more question that are not addressed in the manuscript. These remaining concerns may be addressed by appropriate wording and discussions.

Reviewer #2 (Remarks to the Author):

This revised paper by Zhang et al from the Weinberg group is even stronger now, with the addition of approaches and data to provide more mechanistic insights. The authors have thoughtfully addressed the concerns of all of the reviewers. As noted before, this work advances our understanding of EMP regulation and contributions to metastatic potential. The paper will be of broad interest to cancer biologists, chromatin researchers, and developmental biologists.

Reviewer #3 (Remarks to the Author):

The authors have thoroughly revised the manuscript based on the comments in previous cycle by adding new experimental data. The evidence provided further strengthen the major conclusion. The current

version is significantly improved and will be a general interest to the fields of EMT, epigenetic and metastasis. No further comments for this outstanding study.

Decision letter, final requests:

Subject: NCB: Your manuscript, NCB-Z45438A
Message: Our ref: NCB-Z45438A

10th January 2022

Dear Dr. Weinberg,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Cell Biology manuscript, "Loss of PRC2 or KMT2D-COMPASS Unlocks Distinct EMT Trajectories that Contribute Differentially to Metastasis" (NCB-Z45438A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within one week). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Cell Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Loss of PRC2 or KMT2D-COMPASS Unlocks Distinct EMT Trajectories that Contribute Differentially to Metastasis". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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If you have any further questions, please feel free to contact me.

Best regards,

Ziqian Li
Editorial Assistant
Nature Cell Biology

On behalf of

Zhe Wang, PhD
Senior Editor
Nature Cell Biology

Tel: +44 (0) 207 843 4924
email: zhe.wang@nature.com

Reviewer #1:
Remarks to the Author:

The authors have provided more evidence to support their claim. It has become evident that the process driven by the PRC2 -mediated EMP is mostly unidirectional and might be restricted to specific models [as endocrine responsive cells such as T47D an MCF7 did not respond]. That said, the notion of plasticity still remains a minor concern for me as cells do not revert back to their initial state when PCR2 complex is rescued via overexpression of EED or EZH2 or retrieval of EZH2 inhibitor. Also, the increase in H3K27me3 following KDM2D downregulation, although redistributed, leads to more question that are not addressed in the manuscript. These remaining concerns may be addressed by appropriate wording and discussions.

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Remarks to the Author:
This revised paper by Zhang et al from the Weinberg group is even stronger now, with the addition of approaches and data to provide more mechanistic insights. The authors have thoughtfully addressed the concerns of all of the reviewers. As noted before, this work advances our understanding of EMP regulation and contributions to metastatic potential. The paper will be of broad interest to cancer biologists, chromatin researchers, and developmental biologists.

Reviewer #3:
Remarks to the Author:
The authors have thoroughly revised the manuscript based on the comments in previous cycle by adding new experimental data. The evidence provided further strengthen the major conclusion. The current version is significantly improved and will be a general interest to the fields of EMT, epigenetic and metastasis. No further comments for this outstanding study.

Author Rebuttal, first revision:

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appropriate wording and discussions.

We agree with the reviewer that it remains to be seen precisely how loss of PRC2 and KMT2D specifies these two distinct mesenchymal cell states, including a deeper understanding of how the H3K27me3 genomic distribution was regulated by KMT2D-KO, which subsets of genes are essential in distinguishingthe two EMT subprograms, and why restoration of PRC2 function in the quasi-mesenchymal cells is insufficient to trigger an MET process. We have started a follow-up project and hopefully these questionscan be fully addressed.

Following the reviewer' suggestion, in the revised manuscript we have now added more discussion aboutthese issues in the second paragraph of the discussion section.

Reviewer #2:
Remarks to the Author:
This revised paper by Zhang et al from the Weinberg group is even stronger now, with the addition of approaches and data to provide more mechanistic insights. The authors have thoughtfully addressed theconcerns of all of the reviewers. As noted before, this work advances our understanding of EMP regulation and contributions to metastatic potential. The paper will be of broad interest to cancer biologists, chromatin researchers, and developmental biologists.

We appreciate the comments by the reviewer.

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The authors have thoroughly revised the manuscript based on the comments in previous cycle by addingnew experimental data. The evidence provided further strengthen the major conclusion. The current version is significantly improved and will be a general interest to the fields of EMT, epigenetic and metastasis. No further comments for this outstanding study.

We appreciate the comments by the reviewer.

Final Decision Letter:

Subject: Decision on Nature Cell Biology submission NCB-Z45438B
Message: Dear Dr Weinberg,

I am pleased to inform you that your manuscript, "Genome-wide CRISPR screen identifies PRC2 and KMT2D-COMPASS as regulators of distinct EMT trajectories that contribute differentially to metastasis", has now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

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Please feel free to contact us if you have any questions.

With kind regards,

Zhe Wang, PhD
Senior Editor
Nature Cell Biology

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