

Peer Review Information

Journal: Nature Cell Biology

Manuscript Title: Polycomb repressive complexes 1 and 2 are each essential for maintenance of X inactivation in extraembryonic lineages

Corresponding author name(s): Dr Edith Heard

Editorial Notes:

Reviewer Comments & Decisions:

Decision Letter, initial version:
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Dear Professor Heard,

Your manuscript, "Polycomb complexes PRC1 and PRC2 are each essential for maintenance of X inactivation in extraembryonic lineages", has now been seen by 3 referees, who are experts in X inactivation and embryogenesis (referee 1); Polycomb complex and X inactivation (referee 2); and X inactivation (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. I should stress that the referees' concerns point to a premature dataset and these points would need to be addressed with experiments and data, and reconsideration of the study for this journal and re-engagement of referees would depend on strength of these revisions.

In particular, it would be essential to:

a) experimentally strengthen the claim on PRC1-independent recruitment of PRC2, as noted by:

Referee 1:

A quantification of H3K27me3 (% cells with H3K27me3 enrichment on the Xi) in the Ring1A/B deletion needs to be provided. The authors indicate that H3K27me3 is mostly unaffected by Ring1A/B deletion, but from the immunostaining images it seems like there may be some reduction in the number of cells

that have H3K27me3 enrichment compared to WT embryos. Thus, although H3K27me3 is able to accumulate on the Xi in the absence of H2AK119ub1, there might still be some degree of dependence on PRC1 for PRC2 recruitment. This should be quantified in embryonic and extraembryonic tissues and maybe at different developmental time points after deletion (even if the embryos are already appearing to be dying). One concern is that the interdependence of the two polycomb marks is not seen because they have distinct dynamics of loss.

Similarly, there is no quantification in Figure 2d for the H3K27me3 accumulation on the Xi in Δ Ring1A/B TGCs. Moreover, H2Aub in Figure 3b needs to be quantified.

Referee 2:

The major concern I have, which the authors also expressed in their discussion section, is that ~2 days of RING1A/B deletion may not be enough time to detect an effect on PRC2 accumulation. At E7.5, the authors only detected PRC1-mediated H2AK119ub loss in the extraembryonic tissues of 4 of 16 embryos profiled, whereas once RING1A/B have been deleted, 100% of the embryos should lack H2AK119ub. The fact that loss of H2AK119ub was observed in only 25% of the extraembryonic samples profiled raises some doubts about the conclusions that PRC2 is completely independent from PRC1 at E7.5 -- it is plausible that the E7.5 timepoint is just on the cusp of the timepoint at which PRC2 would be lost on the Xi due to loss of RING1A/B, although I agree with the authors that their TGC experiment lends confidence to the independence. Still, the data are not so strong that a proponent of the PRC1-dominant model could not argue these results away.

2) In E8.5 embryos that are Normal or Small, could the authors perform a similar quantitative study of H3K27me3 and H2AK119ub in WT vs RING1A/B DKO genotypes?

[AU: we have consulted with referee 1 regarding this particular experiment. This referee agrees that an examination at a later time point will be an essential experiment to perform.]

Referee 3:

1, The authors examined the role of PRC1 in H2AK119ub1 accumulation on the Xi in mouse embryos bearing a constitutively mutated Ring1A allele and a conditional inactivated Ring1B allele. In a total of 15 E7.5 embryo sections only 4 lacked H2AK119ub1. The authors explained this by saying the loss of this mark occurs progressively. But it is also possible that the Ring1B depletion is not equally effective in the 15 embryos. The authors should examine expression and protein level of Ring1B to exclude this possibility.

4, Related to above point 1, while the authors found de-repression of Atrx and Huw1e in three PRC1-null embryos without H2AK119ub, did they also examine gene expression in the other PRC1-null embryos that retain H2AK119ub accumulation? These observations could contribute to separate the functions of the PRC1 complex and of the H2AK119ub mark, as well as serve as a control. In the text the authors state "...were compared to one control female retaining H2AK119ub1 accumulation on the Xi". It would be helpful if the authors clarify if the PRC1-null embryos with H2AK119ub were included as controls.

b) show the developmental phenotypes for males and females of Ring1A/B or Ezh2 mutants to support that the embryonic lethality phenotype is due to a defect in XCI maintenance in extraembryonic tissue as opposed to regulation of genes outside of the X chromosome, as noted by Referee 1:

How certain can the authors be that the embryonic lethality phenotype in these polycomb deletions is due to a defect in XCI maintenance in extraembryonic tissue as opposed to regulation of genes outside

of the X chromosome. This question is particularly important for the Ring1A/B deletion where the authors do not show figures or tables with the developmental phenotypes for males and females. Similarly, such data are only discussed in the text for male/female Ezh2 KO mice without presentation of the numbers and assessment per time point. It would be important for the authors to provide the actual data if they want to make the point that there is a female-specific enhanced lethality for PRC1 and PRC2 depletion.

c) add more analyses such as RNA-seq of PRC2 mutants for a better comparison between PRC1 loss vs PRC2 loss as noted by referee 1:

Much more analysis is done with the PRC1 deletion than the PRC2 deletion, which makes the paper end in an incomplete fashion. For instance, there are no RNA-seq data, which would be nice for a comparison of which genes are de-repressed upon PRC1 vs PRC2 depletion. Similarly, the FISH's of diverse X-linked gene transcripts could be extended to the different extra-embryonic lineages and embryonic cells.

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

e) 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 www.nature.com/nature/authors/).
- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.
- provide the completed Editorial Policy Checklist (found here <https://www.nature.com/authors/policies/Policy.pdf>), and Reporting Summary (found

here <https://www.nature.com/authors/policies/ReportingSummary.pdf>). This is essential for reconsideration of the manuscript and these documents 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.

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

[Redacted]

*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We would like to receive a revised submission within six months. We would be happy to consider a revision even after this timeframe, however if the resubmission deadline is missed and the paper is eventually published, the submission date will be the date when the revised manuscript was received.

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,

Jie Wang

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Nature Cell Biology

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Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

In this manuscript, the authors assess the role of PRC1 and PRC2 in imprinted X-inactivation. Prior reports with an Xi-linked GFP reporter have implicated PRC2 in the maintenance of imprinted X-

inactivation, yet, it has remained unclear how the role of PRC2 extends to endogenous genes on the X-chromosome in vivo, what role PRC1 plays in imprinted XCI in vivo and the dependencies of these two polycomb complexes.

Using conditional mouse models for the Rnf2 and Ezh2 subunits of PRC1 and PRC2, the paper shows that depletion of PRC1 by Ring1A KO and Ring1B conditional KO ablates H2AK119ub1 marks and derepresses some genes in embryonic and extra-embryonic cells, without affecting H3K27me3 marks. Conversely, depletion of PRC2 by Ezh2 conditional KO ablates H3K27me3 marks and derepresses some genes in extra-embryonic cells, without affecting H2AK119ub1 marks. Thus, there is an extraembryonic tissue specific role of PRCs in XCI. Moreover, it appears that PRC1 and PRC2 seem to be recruited to the Xi via independent mechanisms, which is different from what happens in the cells of the epiblast as shown by published Xist B-repeat papers. Overall, this paper defines a critical role for PRC1 and PRC2 in imprinted X-inactivation and provides a link between PRC-dependence and CpG islands. Moreover, it is important because it explores processes in vivo. However, there are some inconsistencies and missing data in the figures and the following points should be addressed before publication.

Specific Points:

A quantification of H3K27me3 (% cells with H3K27me3 enrichment on the Xi) in the Ring1A/B deletion needs to be provided. The authors indicate that H3K27me3 is mostly unaffected by Ring1A/B deletion, but from the immunostaining images it seems like there may be some reduction in the number of cells that have H3K27me3 enrichment compared to WT embryos. Thus, although H3K27me3 is able to accumulate on the Xi in the absence of H2AK119ub1, there might still be some degree of dependence on PRC1 for PRC2 recruitment. This should be quantified in embryonic and extraembryonic tissues and maybe at different developmental time points after deletion (even if the embryos are already appearing to be dying). One concern is that the interdependence of the two polycomb marks is not seen because they have distinct dynamics of loss.

Similarly, there is no quantification in Figure 2d for the H3K27me3 accumulation on the Xi in Δ Ring1A/B TGCs. Moreover, H2Aub in Figure 3b needs to be quantified.

How certain can the authors be that the embryonic lethality phenotype in these polycomb deletions is due to a defect in XCI maintenance in extraembryonic tissue as opposed to regulation of genes outside of the X chromosome. This question is particularly important for the Ring1A/B deletion where the authors do not show figures or tables with the developmental phenotypes for males and females. Similarly, such data are only discussed in the text for male/female Ezh2 KO mice without presentation of the numbers and assessment per time point. It would be important for the authors to provide the actual data if they want to make the point that there is a female-specific enhanced lethality for PRC1 and PRC2 depletion.

The text mentions that TGCs endoreplicate and therefore typically have more than 2 copies of genes. However, the graphs indicate only data for bi-allelic expression of X-linked genes, which therefore could be two active X-chromosomes? Should this rather be plotted as expression on the Xist-coated X, for both PRC1 and PRC2 deletion.

The p values in Fig 2A are only marginally significant. Along with this, the control counts in ED2A are high sometimes and similar to KO mice. Would it be possible to add more counts?

Much more analysis is done with the PRC1 deletion than the PRC2 deletion, which makes the paper end in an incomplete fashion. For instance, there are no RNA-seq data, which would be nice for a comparison of which genes are de-repressed upon PRC1 vs PRC2 depletion. Similarly, the FISH's of diverse X-linked gene transcripts could be extended to the different extra-embryonic lineages and embryonic cells.

How do the data compare to the published EED KO TSC data from the Kalantry lab?

Pcgf3/5 defines the PRC1 complex subtype while Ring1A/B is responsible for catalysis of all PRC1 complex subtypes. Do the authors have insights into which PRC1 complexes are present in extraembryonic cells and how much of the result is due to deletion of canonical versus noncanonical PRC1 complexes?

Reviewer #2:

Remarks to the Author:

Masue, Corbel and colleagues report a study of how conditional knockout of PRC1 or PRC2 affect maintenance of XCI early during mouse development (E6-7.5). This is important work because prior studies of PRC1 and PRC2 did not necessarily survey endogenous genes nor did they effectively examine interdependency of the two modifications during early XCI maintenance. The main findings are that PRC1 and PRC2 are each important for early maintenance of gene silencing and appear to act independently of each other, in that when one complex was conditionally deleted, it did not affect the visible accumulation of the other. For the most part, the data and the interpretations were clear. This is an important study for the field given the complex history of this topic.

The major concern I have, which the authors also expressed in their discussion section, is that ~2 days of RING1A/B deletion may not be enough time to detect an effect on PRC2 accumulation. At E7.5, the authors only detected PRC1-mediated H2AK119ub loss in the extraembryonic tissues of 4 of 16 embryos profiled, whereas once RING1A/B have been deleted, 100% of the embryos should lack H2AK119ub. The fact that loss of H2AK119ub was observed in only 25% of the extraembryonic samples profiled raises some doubts about the conclusions that PRC2 is completely independent from PRC1 at E7.5 -- it is plausible that the E7.5 timepoint is just on the cusp of the timepoint at which PRC2 would be lost on the Xi due to loss of RING1A/B, although I agree with the authors that their TGC experiment lends confidence to the independence. Still, the data are not so strong that a proponent of the PRC1-dominant model could not argue these results away.

I also found the language that described the critical H3K27me3 data in the results section to be unclear or tempered in a way that I did not understand -- specifically:

"H3K27me3 accumulation was also observed in embryos which still exhibited H2AK119ub1 accumulation on the Xi in some embryonic lineages (Extended Data Fig. 1a, b)."

Wouldn't this be expected? Is it not the opposite set of embryos that we should be focusing on -- the embryos that lost K119 but still retained H3K27me3? I apologize if I am missing something.

In terms of concrete requests:

- 1) Is it possible for the authors to quantify H3K27me3 and H2AK119ub more rigorously in the images which they have already captured? Perhaps in a subset of nuclei from the embryonic and extraembryonic E7.5 sections, what fraction of nuclei have H3K27me3 and H2AK119ub clouds in WT vs RING1A/B KO embryos?
- 2) In E8.5 embryos that are Normal or Small, could the authors perform a similar quantitative study of H3K27me3 and H2AK119ub in WT vs RING1A/B DKO genotypes?
- 3) The authors should also consider briefly mentioning Carolyn Brown's recent work on Xist-mediated recruitment of PRC1 and PRC2 during the maintenance of human XCI, which in my opinion support the same conclusions drawn by the authors in this study: that PRC1 and PRC2 are not necessarily recruited to the Xi in a dependent manner.

Reviewer #3:

Remarks to the Author:

The authors present new and interesting evidence of separate roles for the PRC complexes in maintenance of X-chromosome inactivation (XCI). The results are based on studies in mouse embryos with conditional deletions of critical components of the PRC1 or PRC2 complex. The paper presents experimental data based on RNA-FISH and immunostaining of histone modifications to follow alterations in epigenetic marks and allele-specific expression of X-linked genes in the engineered mice. With these observations, the authors conclude that PRC1 and PRC2 complexes independently contribute to the maintenance of XCI in extraembryonic lineages. This is a novel finding of potential interest. However, more evidence is needed to fully support this claim, especially since some aspects of these results contradict previous studies.

Major comments:

- 1, The authors examined the role of PRC1 in H2AK119ub1 accumulation on the Xi in mouse embryos bearing a constitutively mutated Ring1A allele and a conditional inactivated Ring1B allele. In a total of 15 E7.5 embryo sections only 4 lacked H2AK119ub1. The authors explained this by saying the loss of this mark occurs progressively. But it is also possible that the Ring1B depletion is not equally effective in the 15 embryos. The authors should examine expression and protein level of Ring1B to exclude this possibility.
- 2, The authors measured gene silencing on the inactive X in PRC1-null embryos by RNA FISH of only two X-linked genes Atrx and Huwe1. Considering the total number of X-linked genes, the authors should examine the expression of more than these two genes by RNA FISH if RNA-seq is not applicable. In addition, only 1 control embryo was used in scoring, which would not provide enough statistical power to adjust the variances between normal embryos.
- 3, The authors examined the role of PRC1 in XCI maintenance in E7.5 EPC and EPC-derived TGCs. How were the EPCs were selected? Do they have H2AK119ub1 accumulation? In the earlier context, the authors showed that EPCs from the three PRC1-null embryos without H2AK119ub1 already showed a substantial de-repression of Atrx and Huwe1 by RNA-FISH. Later in the manuscript, the authors

demonstrate de-repression by RNA-seq without mentioning H2AK119ub1. Does it mean the EPCs in PRC1-null embryos have impaired XCI regardless of H2AK119ub1 mark? The authors should provide clarification.

4, Related to above point 1, while the authors found de-repression of *Atrx* and *Huw1e* in three PRC1-null embryos without H2AK119ub, did they also examine gene expression in the other PRC1-null embryos that retain H2AK119ub accumulation? These observations could contribute to separate the functions of the PRC1 complex and of the H2AK119ub mark, as well as serve as a control. In the text the authors state "...were compared to one control female retaining H2AK119ub1 accumulation on the Xi". It would be helpful if the authors clarify if the PRC1-null embryos with H2AK119ub were included as controls.

Minor comments;

1, In the section "Role of PRC1 in XCI maintenance in E7.5 post-implantation embryos tissues" the authors state "To assess whether these female-specific abnormalities...". The authors should provide information about male embryos, including numbers examined, in order to demonstrate the abnormalities are female-specific.

2, The authors investigated the role of CpG islands (CGI) in PRC1-mediated gene silencing in XCI. They state that "We found that 95/116 (81.9%) informative X-linked genes have a promoter associated CGI, showing significantly higher expression from the Xi compared to the other informative X-linked genes that do not have CGIs (21/116; 18.1%) ($P = 1.32 \times 10^{-4}$) in *Ring1A/B* mutant EPCs (Extended Data Fig. 4d)". This sentence is misleading. In the Extended Data Fig. 4d, they are not comparing gene expression from the Xi between genes with and without CGI in the mutant mouse. Instead, they are comparing the allelic ratio diff (*Ring1A/B* – Control) in the two groups of genes.

3, A recent publication (Dixon-McDougall T, Brown CJ: Independent domains for recruitment of PRC1 and PRC2 by human XIST. *PLoS Genet* 2021, 17:e1009123), demonstrates the independence of the PRC1 and PRC2 pathways in human cells. The authors should cite and discuss this study.

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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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TITLE – should be no more than 100 characters including spaces, without punctuation and avoiding technical terms, abbreviations, and active verbs..

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AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

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

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see <https://www.nature.com/licenceforms/nrg/competing-interests.pdf>.

REFERENCES – are limited to a total of 70 for Articles, Resources, Technical Reports; and 40 for Letters. This includes references in the main text and Methods combined. References must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

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".

All Nature Cell Biology manuscripts submitted on or after March 21 2016 must include a Data availability statement at the end of the Methods section. For Springer Nature policies on data availability see <http://www.nature.com/authors/policies/availability.html>; for more information on this particular policy see <http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf>. The Data availability statement should include:

- Accession codes for primary datasets (generated during the study under consideration and designated as "primary accessions") and secondary datasets (published datasets reanalysed during the study under consideration, designated as "referenced accessions"). For primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here <http://www.nature.com/authors/policies/availability.html#data>.
- Unique identifiers (accession codes, DOIs or other unique persistent identifier) and hyperlinks for datasets deposited in an approved repository, but for which data deposition is not mandated (see here for details <http://www.nature.com/sdata/data-policies/repositories>).
- 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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We recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. More details can found at www.nature.com/protocolexchange/about.

DISPLAY ITEMS – main display items are limited to 6-8 main figures and/or main tables for Articles, Resources, Technical Reports; and 5 main figures and/or main tables for Letters. For Supplementary Information see below.

FIGURES – Colour figure publication costs \$600 for the first, and \$300 for each subsequent colour figure. All panels of a multi-panel figure must be logically connected and arranged as they would appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

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.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

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

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- We accept PowerPoint (.PPT) files if they are fully editable. However, please refrain from adding PowerPoint graphical effects to objects, as this results in them outputting poor quality raster art. Text used for PowerPoint figures should be Helvetica (preferred) or Arial.
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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 must be accompanied by a separate Word document including titles and legends.

GUIDELINES FOR EXPERIMENTAL AND STATISTICAL REPORTING

REPORTING REQUIREMENTS – To improve the quality of methods and statistics reporting in our papers we have recently revised the reporting checklist we introduced in 2013. We are now asking all life sciences authors to complete two items: an Editorial Policy Checklist (found here <https://www.nature.com/authors/policies/Policy.pdf>) that verifies compliance with all required editorial policies and a reporting summary (found here <https://www.nature.com/authors/policies/ReportingSummary.pdf>) that collects information on experimental design and reagents. These documents are available to referees to aid the evaluation of the manuscript. Please note that these forms are dynamic 'smart pdfs' and must therefore be downloaded and completed in Adobe Reader. We will then flatten them for ease of use by the reviewers. 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

Responses to Reviewers

We would like to thank the reviewers for their very helpful and constructive input. We have tried to address all of their points below and in the text of the manuscript. In response to the comments, we have performed further experiments and included new data and figures. We believe these have considerably strengthened the manuscript and we are most grateful to the reviewers.

Below we provide a point-by-point rebuttal:

Reviewer #1:

Remarks to the Author:

In this manuscript, the authors assess the role of PRC1 and PRC2 in imprinted X-inactivation. Prior reports with an Xi-linked GFP reporter have implicated PRC2 in the maintenance of imprinted X-inactivation, yet, it has remained unclear how the role of PRC2 extends to endogenous genes on the X-chromosome in vivo, what role PRC1 plays in imprinted XCI in vivo and the dependencies of these two polycomb complexes.

Using conditional mouse models for the Rnf2 and Ezh2 subunits of PRC1 and PRC2, the paper shows that depletion of PRC1 by Ring1A KO and Ring1B conditional KO ablates H2Ak119ub1 marks and derepresses some genes in embryonic and extra-embryonic cells, without affecting H3K27me3 marks. Conversely, depletion of PRC2 by Ezh2 conditional KO ablates H3K27me3 marks and derepresses some genes in extra-embryonic cells, without affecting H2Ak119ub1 marks. Thus, there is an extraembryonic tissue specific role of PRCs in XCI. Moreover, it appears that PRC1 and PRC2 seem to be recruited to the Xi via independent mechanisms, which is different from what happens in the cells of the epiblast as shown by published Xist B-repeat papers. Overall, this paper defines a critical role for PRC1 and PRC2 in imprinted X-inactivation and provides a link between PRC-dependence and CpG islands. Moreover, it is important because it explores processes in vivo. However, there are some inconsistencies and missing data in the figures and the following points should be addressed before publication.

Specific Points:

A quantification of H3K27me3 (% cells with H3K27me3 enrichment on the Xi) in the Ring1A/B deletion needs to be provided. The authors indicate that H3K27me3 is mostly unaffected by Ring1A/B deletion, but from the immunostaining images it seems like there may be some reduction in the number of cells that have H3K27me3 enrichment compared to WT embryos. Thus, although H3K27me3 is able to accumulate on the Xi in the absence of H2AK119ub1, there might still be some degree of dependence on PRC1 for PRC2 recruitment. This should be quantified in embryonic

and extraembryonic tissues and maybe at different developmental time points after deletion (even if the embryos are already appearing to be dying). One concern is that the interdependence of the two polycomb marks is not seen because they have distinct dynamics of loss.

In the revised manuscript, we provide quantifications of all IF analyses shown in the Figures. These are now displayed in **Extended Data Fig. 1b-d** and described in the legend of original Figure 1 (**Fig. 1b**). Concerning H3K27me3 enrichment on the Xi, although the degree of H3K27me3 on the Xi was slightly diminished in some cells, quantification clearly showed that H3K27me3 was retained on the Xi in the majority of cells in Δ Ring1A/B female E7.5 embryonic and extraembryonic lineages (**Extended Data Fig. 1c**). We also examined E8.5 Δ Ring1A/B female embryos, and showed that H2AK119ub1 accumulation on the Xi is depleted in both embryonic and extraembryonic tissues. Here, however, H3K27me3 still accumulated on the Xi in extraembryonic tissues at comparable levels to the control females, supporting the idea that PRC2/H3K27me3 accumulation on the Xi is not reliant on PRC1/H2AK119ub1 in extraembryonic lineages, at least over several days of development (**Extended Data Fig. 2c**). On the other hand, we were not able to detect H3K27me3 accumulation on the Xi in embryonic tissues of E8.5 Δ Ring1A/B female embryos, suggesting the accumulation of PRC2/H3K27me3 on the Xi may depend on PRC1/H2AK119ub1 in embryonic tissues (**Extended Data Fig. 2c**). The reason for these differences between embryonic and extraembryonic tissues to PRC1 deletion in E8.5 embryos is not clear and could be due to lineage-specific differences PRC propagation mechanisms. Nevertheless, our results clearly show that H3K27me3 accumulation on the Xi is regulated independently of H2AK119ub1 in extraembryonic tissues at E7.5-8.5. This is discussed in the manuscript (**pages 16, 17, lines 491-517**).

Similarly, there is no quantification in Figure 2d for the H3K27me3 accumulation on the Xi in Δ Ring1A/B TGCs. Moreover, H2Aub in Figure 3b needs to be quantified.

We provided quantifications for this immunofluorescence data and present them in the Extended Data Figures: for **Fig. 2d** in **Extended Data Fig. 4b** and for **Fig. 3b** in **Extended Data Fig. 4c**.

How certain can the authors be that the embryonic lethality phenotype in these polycomb deletions is due to a defect in XCI maintenance in extraembryonic tissue as opposed to regulation of genes outside of the X chromosome. This question is particularly important for the Ring1A/B deletion where the authors do not show figures or tables with the developmental phenotypes for males and females. Similarly, such data are only discussed in the text for male/female Ezh2 KO mice without presentation of the numbers and assessment per time point. It would be important for the authors to provide the actual data if they want to make the point that there is a female-specific enhanced lethality for PRC1 and PRC2 depletion.

We thank the reviewer for raising this important point. In the revised manuscript, we provide a full set of E7.5 and 8.5 phenotype/genotype data including both males and females upon Ring1A/B deletion (**Fig. 1c, Extended Data Fig. 2a**). The new data clearly show that there is no female-specific embryonic lethality upon Ring1A/B KO at E7.5. However, by E8.5, female Δ Ring1A/B mutants showed more signs of embryonic death than male mutants. This female-biased embryonic lethality at E8.5 is not significantly represented probably due to the general important roles of PRC1 in development in both males and females (**Fig. 1c**). This is now discussed on **page 6 (lines 153-158)**. To examine the possible female-specific effect in more depth, we have also provided another dataset using a series of cryosection experiments with E8.5 embryos (**Extended Data Fig. 2a**). In these experiments, the appearance of Δ Ring1A/B female embryos are significantly more underrepresented than expected, indicating female Δ Ring1A/B mutants are dying at this stage. Taken together, all of these data support the idea that both female and male embryos are affected between E7.5 and E8.5 upon PRC1 deletion starting at E5.5, with more severity in female mutants albeit it subtle, and this effect may indeed be attributable to the role of PRC1 in the maintenance of X-linked genes on the Xi.

In order to address whether PRC2 mutation results in a female-specific embryonic lethality, we examined conditional knockout mice of *Eed*, a core component of PRC2. Despite a robust derepression of X-linked genes from the Xi in E7.5 female Δ Eed embryos (new **Fig. 4**), we could not detect a sex-specific morphological phenotype in these embryos. However, both males and females were severely affected at this stage, suggesting the loss of PRC2 may have more severe general effects in both sexes, over and above its role in XCI. This is described in the text (**pages 12, 13, lines 375-377**).

The text mentions that TGCs endoreplicate and therefore typically have more than 2 copies of genes. However, the graphs indicate only data for bi-allelic expression of X-linked genes, which therefore could be two active X-chromosomes? Should this rather be plotted as expression on the Xist-coated X, for both PRC1 and PRC2 deletion.

When endoreduplication occurs, the newly synthesized chromatin of each chromosome typically remains clustered together in the nucleoplasm and RNA or DNA FISH probes thus detect clusters of multiple spots in the same region of the nucleus. The endoreduplicated active and inactive X chromosomes can thus be easily distinguished based on the Xist RNA signal. However, the reviewer rightly points out that the term “biallelic expression” can be misleading. In the revised manuscript, we replace it with “Expression on both Xa and Xi”.

The p values in Fig 2A are only marginally significant. Along with this, the control counts in ED2A are high sometimes and similar to KO mice. Would it be possible to add more counts?

We thank the reviewer but we do not think it is necessary to examine more control embryos. The reason for this is that we have extensively analysed control embryos for X-linked gene expression at these stages and in these tissues in our previous work (Corbel et al, 2013). Also we think that the one control sample used in this dataset gives a sufficient statistical power because we used a one-sample t-test rather than a two-sample t-test. The following site describes the method and we determined that a one-sample t-test would provide sufficient statistical power in this experiment.

<http://sciences.usca.edu/biology/zelmer/305/tee/>

Much more analysis is done with the PRC1 deletion than the PRC2 deletion, which makes the paper end in an incomplete fashion. For instance, there are no RNA-seq data, which would be nice for a comparison of which genes are derepressed upon PRC1 vs PRC2 depletion. Similarly, the FISH's of diverse X-linked gene transcripts could be extended to the different extra-embryonic lineages and embryonic cells.

As pointed out by the reviewer, our study had initially focused more on PRC1. In our revised manuscript, we have now performed an extensive allele-specific RNA-seq analysis using ΔEed PRC2-null E7.5 EPCs and compared the results with our $\Delta Ring1A/B$ PRC1-null RNA-seq data. These analyses are now provided in a new figure, **Fig. 4**. We show that a substantial number of X-linked genes are significantly derepressed from the Xi upon *Eed* deletion. Importantly, some of these *Eed*-dependent escapees are also significantly derepressed from the Xi in $\Delta Ring1A/B$ E7.5 EPCs, indicating that PRC1 and PRC2 independently maintain gene silencing of these X-linked genes. Furthermore, several other X-linked genes are derepressed only upon PRC2 deletion, and others only upon PRC1 deletion. This indicates that some X-linked genes are silenced by a single PRC and do not require the other PRC. We believe that this is an important and insightful result about the differential requirements of loci for PRC1 and PRC2 for maintenance of their repressed state and thank the reviewer for encouraging us to perform this more extensive analysis.

The reviewer also asked us to extend our RNA FISH analysis to other embryonic and extraembryonic lineages. Actually, multiple lineages were analysed in **Fig. 2a** and these were selected by their ease of distinction through simple DAPI staining. We think it will be difficult to distinguish further lineages such as allantois, amnion, definitive endoderm and parietal endoderm at this stage without any lineage-specific marker staining, as they often have unclear borders to or even intermingle with adjacent tissues. Therefore, we hope that the reviewer will agree that our current RNA FISH data presented in **Fig. 2a** already covers the major and most readily distinguishable lineages at E7.5.

[How do the data compare to the published EED KO TSC data from the Kalantry lab?](#)

Sundeep Kalantry's laboratory previously reported that several X-linked genes are derepressed from the Xi in *Eed* KO female TSCs (Maclary et al., Genome Biol., 2017). However their experimental system differed substantially from ours, in at least two

ways. First, although TSCs are primary cultured cells, derived from the trophectoderm, their presence, especially as undifferentiated TSCs represents only a very minor population in the growing mouse embryos that we used. Second, Maclary et al. used Adenovirus Cre expression to induce *Eed* KO and subcloned those infected TSCs, which could have produced a bias towards a limited population of TSCs and may have hindered the detection of a direct effect of PRC2 loss. In our study we have used TAM-induced conditional KO in developing embryos, in order to detect all of the acute and direct effects of Polycomb inactivation *in vivo*, under more physiological conditions.

We would also like to mention another recent study, that came out during the revision of our manuscript. Alex Meissner's group reported an analysis of Polycomb-dependent escapees on the Xi using zygotic KO PRC1 (*Rnf2/Ring1B*) and PRC2 (*Eed*) embryos looking at E6.5 extraembryonic ectoderm (ExE) by allele-specific RNA-seq (Andergassen et al., Dev. Cell, 2021). Their study indicated that all PRC1(*Rnf2*)-dependent escapees (N = 35) are included in the fraction of PRC2(*Eed*)-dependent escapees (n = 74), which is in contrast to our study where we identified several X-linked genes (21 out of 31) that are significantly derepressed by PRC1(*Ring1A/B*) mutation, but not by PRC2(*Eed*) mutation. As Andergassen et al. did not provide the list of X-linked genes expressed from the Xi in their mutant ExEs, we re-analysed their raw data and made a comparison with our study. This comparison revealed that X-linked genes derepressed in PRC1(*Rnf2*) or PRC2(*Eed*) inactivation in E6.5 ExEs do not have a significant overlap with the list of X-linked genes that were identified in our study which investigated Δ PRC1(Δ Ring1A/B) or Δ PRC2(Δ Eed) E7.5 EPCs (see **Supplementary Analysis** attached to this document). This difference could be attributable to the difference in the timing of KO induction (E3.5 or E5.5 in our study vs zygotic induction in the Andergassen study); or the timing of the analysis (E7.5 our study vs E6.5 theirs); or to the tissues analysed in the two studies (EPC vs ExE). Importantly, as they used a zygotic KO, their system cannot distinguish an effect on the different phases of XCI, in particular initiation and maintenance and it should be noted that PRC is actually responsible for the *Xist* imprint on the maternal X at early pre-implantation stages which means that the KO could have multiple direct and indirect effects on the status of the two X chromosomes prior to E6.5. Our study, on the other hand, focuses on the maintenance of XCI by inducing *Ring1A/B* KO at E5.5 or *Eed* KO at E3.5, when imprinted XCI is already established. We have included some discussion about the Andergassen et al. paper in the manuscript (**pages 15, 16, lines 473-490**) and also provide a detailed comparison between two studies as a **Supplementary Analysis** for the reviewers only, that we attach to this document.

Pcgf3/5 defines the PRC1 complex subtype while Ring1A/B is responsible for catalysis of all PRC1 complex subtypes. Do the authors have insights into which PRC1 complexes are present in extraembryonic cells and how much of the result is due to deletion of canonical versus noncanonical PRC1 complexes?

We thank the reviewer for this important question. As pointed out, RING1A/B proteins are present both in the canonical and non-canonical forms of PRC1 complexes and our study cannot distinguish which ones are acting, although both must be affected. To address the question of which non-canonical forms of PRC1 might be active in extraembryonic cells at the stage analysed, we calculated the expression levels of the *Pcgf* genes within our E7.5 EPC RNA-seq data used in this study. The results indicate that all *Pcgf* genes are present in E7.5 EPC with expression levels > 4 FPKM (*Pcgf1*; 5.5, *Pcgf2*; 5.9, *Pcgf4*; 39.7, *Pcgf4*; 4.9, *Pcgf5*; 36.0, *Pcgf6*; 22.8 [FPKM]). These data suggest the presence of both canonical (*Pcgf2/4*) and non-canonical (*Pcgf1/3/5/6*) PRC1 subcomplexes in extraembryonic lineage EPC. To address the importance of canonical and non-canonical PRC1 subcomplexes in the maintenance of XCI, it would be necessary to knockout each of the PRC1 subcomplexes under identical conditions, which would be beyond the scope of this study.

Reviewer #2:

Remarks to the Author:

Masui, Corbel and colleagues report a study of how conditional knockout of PRC1 or PRC2 affect maintenance of XCI early during mouse development (E6-7.5). This is important work because prior studies of PRC1 and PRC2 did not necessarily survey endogenous genes nor did they effectively examine interdependency of the two modifications during early XCI maintenance. The main findings are that PRC1 and PRC2 are each important for early maintenance of gene silencing and appear to act independently of each other, in that when one complex was conditionally deleted, it did not affect the visible accumulation of the other. For the most part, the data and the interpretations were clear. This is an important study for the field given the complex history of this topic.

The major concern I have, which the authors also expressed in their discussion section, is that ~2 days of RING1A/B deletion may not be enough time to detect an effect on PRC2 accumulation. At E7.5, the authors only detected PRC1-mediated H2AK119ub loss in the extraembryonic tissues of 4 of 16 embryos profiled, whereas once RING1A/B have been deleted, 100% of the embryos should lack H2AK119ub. The fact that loss of H2AK119ub was observed in only 25% of the extraembryonic samples profiled raises some doubts about the conclusions that PRC2 is completely independent from PRC1 at E7.5 -- it is plausible that the E7.5 timepoint is just on the cusp of the timepoint at which PRC2 would be lost on the Xi due to loss of RING1A/B, although I agree with the authors that their TGC experiment lends confidence to the independence. Still, the data are not so strong that a proponent of the PRC1-dominant model could not argue these results away.

We thank the reviewer for their supportive comments and for raising this pertinent point about timing. We agree about the potential issue of sufficient time elapsed post KO when looking at E7.5 Δ Ring1A/B embryos. In the revised manuscript, we therefore

performed new experiments looking one day later (which was as late as we could look before death finally affects the mutant embryos) and have now added this new data of E8.5 Δ Ring1A/B embryos. Details are described below in point 2.

I also found the language that described the critical H3K27me3 data in the results section to be unclear or tempered in a way that I did not understand -- specifically:

"H3K27me3 accumulation was also observed in embryos which still exhibited H2AK119ub1 accumulation on the Xi in some embryonic lineages (Extended Data Fig. 1a, b)."

Wouldn't this be expected? Is it not the opposite set of embryos that we should be focusing on -- the embryos that lost K119 but still retained H3K27me3? I apologize if I am missing something.

We apologize for this lack of clarity. What we meant to say was that H3K27me3 accumulates on the Xi, irrespective of the H2AK119ub1 accumulation state. We agree with the reviewer and this sentence has now been removed from the revised manuscript.

In terms of concrete requests:

1) Is it possible for the authors to quantify H3K27me3 and H2AK119ub more rigorously in the images which they have already captured? Perhaps in a subset of nuclei from the embryonic and extraembryonic E7.5 sections, what fraction of nuclei have H3K27me3 and H2AK119ub clouds in WT vs RING1A/B KO embryos?

In the revised manuscript, we provide quantifications of H3K27me3 and H2AK119ub1 for all images used in this study by classifying each cell into three categories, i.e. strong accumulation, weak accumulation and no accumulation: nuclei were identified upon DAPI staining and IF staining for each mark was recorded as cloud of different intensity, which corresponds to the Xi. These quantifications are now presented in the **Extended Data Fig. 1, 4** and indicated in the legends of the original Figures.

2) In E8.5 embryos that are Normal or Small, could the authors perform a similar quantitative study of H3K27me3 and H2AK119ub in WT vs RING1A/B DKO genotypes?

As briefly mentioned above, we performed a new analysis of Xist/H2AK119ub1/H3K27me3 staining with E8.5 female control and Δ Ring1A/B embryos, which is now presented in **Extended Data Fig. 2**. We examined three Δ Ring1A/B and two control female embryos. In these female E8.5 mutants, we could not detect the accumulation of H2AK119ub1 on the Xi in both embryonic and extraembryonic lineages, indicating that H2AK119ub1 is completely removed from the Xi at this stage upon deletion of

Ring1A/B at E5.5. In this situation, we saw a comparable level of H3K27me3 accumulation to control embryos on the Xi in extraembryonic lineages in these three mutants, supporting the idea that PRC2/H3K27me3 accumulation is maintained on the Xi independently of PRC1/H2AK119ub1. On the other hand, we could not detect H3K27me3 accumulation on the Xi in embryonic lineages of these three Δ Ring1A/B E8.5 females, suggesting the accumulation of PRC2/H3K27me3 on the Xi may depend on PRC1/H2AK119ub1 in embryonic tissues at these stages. The reason for these differential responses between extraembryonic and embryonic tissues to PRC1 deletion is currently unknown. However, our results clearly show that H3K27me3 accumulation on the Xi is regulated independently of H2AK119ub1 in extraembryonic tissues at E7.5-8.5. We discuss this in the manuscript (**page 17, lines 513-517**).

3) The authors should also consider briefly mentioning Carolyn Brown's recent work on Xist-mediated recruitment of PRC1 and PRC2 during the maintenance of human XCI, which in my opinion support the same conclusions drawn by the authors in this study: that PRC1 and PRC2 are not necessarily recruited to the Xi in a dependent manner.

As pointed out by the reviewer, Carolyn J. Brown's laboratory recently reported that different domains of Human XIST are responsible for the recruitment of PRC1 and PRC2 in human HT1080 fibrosarcoma cell line (Dixon-McDougall and Brown, PLoS Genet., 2021). We now cite this paper in the revised manuscript (**page 3, lines 61-63**). Indeed this study illustrates the potential differences that there may be in PRC recruitment by human XIST RNA, compared to the mouse or to different cellular contexts. In the case of murine Xist, although the conserved BC repeats of mouse Xist RNA are important for recruitment to the Xi of both PRC1 and PRC2 (Pintacuda et al., Mol. Cell, 2017, Bousard et al., EMBO Rep., 2019) at least in undifferentiated murine ES cells, it is currently not clear how mouse Xist RNA recruits PRC1 and PRC2 to the Xi in differentiated tissues, such as in the post-implantation embryos used in this study. We cannot rule out the possibility that mouse Xist RNA changes its mode of PRC action in post-implantation embryos and uses a similar mechanism to human XIST to recruit PRC1 and PRC2 via different domains of Xist.

Reviewer #3:

Remarks to the Author:

The authors present new and interesting evidence of separate roles for the PRC complexes in maintenance of X-chromosome inactivation (XCI). The results are based on studies in mouse embryos with conditional deletions of critical components of the PRC1 or PRC2 complex. The paper presents experimental data based on RNA-FISH and immunostaining of histone modifications to follow alterations in epigenetic marks and allele-specific expression of X-linked genes in the engineered mice. With these observations, the authors conclude that PRC1 and PRC2 complexes independently contribute to the maintenance of XCI in extraembryonic lineages. This is a novel

finding of potential interest. However, more evidence is needed to fully support this claim, especially since some aspects of these results contradict previous studies.

We thank the reviewer for their supportive comments. We have provided more experimental evidence to strengthen our conclusions. Furthermore, we hope that our study will actually help to reconcile some of the discussions surrounding Polycomb function in XCI, as we clearly show that both of these complexes are needed, independently of each other, to maintain the inactive state of the Xi in extraembryonic tissues. Our study also contributes to showing just how different the requirements for PRC1 and PRC2 are at different stages of development and in different lineages, and that generalisations cannot be made about their functions in the process of XCI.

Major comments:

1, The authors examined the role of PRC1 in H2AK119ub1 accumulation on the Xi in mouse embryos bearing a constitutively mutated *Ring1A* allele and a conditional inactivated *Ring1B* allele. In a total of 15 E7.5 embryo sections only 4 lacked H2AK119ub1. The authors explained this by saying the loss of this mark occurs progressively. But it is also possible that the *Ring1B* depletion is not equally effective in the 15 embryos. The authors should examine expression and protein level of *Ring1B* to exclude this possibility.

We thank the reviewer for raising this point. Our attempts to quantitate RING1B protein levels by IF/RNA FISH analyses were unfortunately unsuccessful, probably because the anti-RING1B antibody used in these experiments is not compatible with IF/RNA FISH. Therefore, we instead quantitated *Ring1B* mRNA levels in E7.5 Δ *Ring1A/B* EPCs used for the allele-specific RNA-seq (**Extended Data Fig. 5c**). The data indicates that *Ring1B* was deleted in three Δ *Ring1A/B* female embryos (100%, 100% and 95.8% deletions, respectively), suggesting that the RING1B protein was also likely to be absent.

On this topic of the E7.5 embryo sections lacking H2AK119ub1, during the revision process, we noticed an error in **Extended Data Fig.1a** that presents the number of female Δ *Ring1A/B* embryos analysed in this study. OR1-1 at the top row actually lacks H2AK119ub1 in both embryonic and extraembryonic lineages (**Fig. 1b**, upper part), but had been presented as H2AK119ub1-positive (+) in extraembryonic lineages. This error has been corrected in the revised manuscript and now presented as H2AK119ub1-negative (-) in extraembryonic lineages. This correction has also changed the total number of female Δ *Ring1A/B* embryos lacking H2AK119ub1 accumulation on the Xi in all embryonic and extraembryonic lineages: the number is five out of fifteen (rather than four out of fifteen). We apologize for this mistake.

2, The authors measured gene silencing on the inactive X in PRC1-null embryos by RNA FISH of only two X-linked genes *Atrx* and *Huwe1*. Considering the total number

of X-linked genes, the authors should examine the expression of more than these two genes by RNA FISH if RNA-seq is not applicable. In addition, only 1 control embryo was used in scoring, which would not provide enough statistical power to adjust the variances between normal embryos.

We agree with the reviewer's comment that more statistical power could have been obtained if multiple control samples had been used. Since we knew from our previous studies using exactly the same approach at the same stage in female WT embryos (Corbel et al., Development, 2013) that embryonic lineages have XCI while extraembryonic lineages show derepression of *Atrx* and *Huwe1* on the Xi, we used only 1 control embryo in the comparative study by RNA FISH in the context of the PRC1 mutant. In addition, we used a one-sample t-test rather than a two-sample t-test. The following site describes the method and we determined that a one-sample t-test would provide sufficient statistical power in this experiment.

<http://sciences.usca.edu/biology/zelmer/305/tee/>

As shown in **Fig. 2c**, most of lineages, except the extraembryonic EPC and Chorion (Ch), showed no reactivation from the Xi, i.e. strict XCI, in control. Because of this strict silencing of *Atrx* and *Huwe1* in control females and the usage of one-sample t-test mentioned above, we believe that the scoring with one control can still give enough statistical power for this analysis. We also performed allele-specific RNA-seq using EPCs, one of the extraembryonic tissues that showed more expression of both *Atrx* and *Huwe1* in the RNA FISH experiments upon *Ring1A/B* deletion, to detect the spectrum of X-linked gene derepression in a chromosome-wide manner (**Fig. 2e**). We believe that the results of this allele-specific RNA-seq cover the reviewer's concern.

3, The authors examined the role of PRC1 in XCI maintenance in E7.5 EPC and EPC-derived TGCs. How were the EPCs were selected? Do they have H2AK119ub1 accumulation? In the earlier context, the authors showed that EPCs from the three PRC1-null embryos without H2AK119ub1 already showed a substantial de-repression of *Atrx* and *Huwe1* by RNA-FISH. Later in the manuscript, the authors demonstrate de-repression by RNA-seq without mentioning H2AK119ub1. Does it mean the EPCs in PRC1-null embryos have impaired XCI regardless of H2AK119ub1 mark? The authors should provide clarification.

In all experiments using E7.5 EPCs for RNA-seq and TGC derivation, we could not pre-emptively test by IF-RNA FISH on the sample prior to establishing the EPCs. Instead we selected the EPCs after confirming the degree of *Ring1B* mRNA depletion using the other part of the embryo, containing epiblast and extraembryonic ectoderm, to test by RT-qPCR. In the case of EPC-derived TGCs (**Fig. 2b-d**), EPCs (derived from embryos where *Ring1A/B* was deleted at E5.5) were cultured for 3 to 4 days to derive TGCs and we could not detect H2AK119ub1 accumulation in these EPC-derived TGCs (**Fig. 2d**). In the case of Δ *Ring1A/B* and Δ *Eed* allele-specific RNA-seq data presented in **Fig. 2e, 4a**. In the majority of EPCs with CreERT2, we confirmed that *Ring1B* (or *Eed*) mRNA were almost totally depleted (> 95%, **Extended Data Fig.**

2c, d). However, as we could not assess the level of H2AK119ub1 accumulation on the Xi in these embryos at the time of EPC derivation, we cannot formally rule out the possibility that these selected EPCs still initially retained some level of H2AK119ub1 on the Xi. We therefore added one sentence to the revised manuscript to mention this possibility (**page 9, lines 257-260**). Perhaps consistent with this point raised by the reviewer, our allele-specific RNA-seq with Δ Ring1A/B E7.5 EPCs show varying degrees of X-linked gene derepression from the Xi (**Extended Data Fig. 6**), which may reflect the level of remaining H2AK119ub1 in the EPCs used for RNA-seq experiment.

4, Related to above point 1, while the authors found de-repression of *Atrx* and *Huwe1* in three PRC1-null embryos without H2AK119ub, did they also examine gene expression in the other PRC1-null embryos that retain H2AK119ub accumulation? These observations could contribute to separate the functions of the PRC1 complex and of the H2AK119ub mark, as well as serve as a control. In the text the authors state "...were compared to one control female retaining H2AK119ub1 accumulation on the Xi". It would be helpful if the authors clarify if the PRC1-null embryos with H2AK119ub were included as controls.

In the revised manuscript, we provide new RNA FISH data using two E7.5 Δ Ring1A/B female embryos which retain a substantial amount of H2AK119ub1 on the Xi despite their *Ring1B* deleted genotype. These two embryos, Δ 4 (OR1-4) and Δ 5 (OR1-9) are listed in **Extended Data Fig. 1a**, and displayed a lesser degree of derepression for *Atrx* and *Huwe1* from the Xi (shown in **Extended Data Fig. 3c**). We believe that these data support the notion that the degree of H2AK119ub1 depletion on the Xi correlates with the degree of X-linked gene derepression on the Xi.

Minor comments;

1, In the section "Role of PRC1 in XCI maintenance in E7.5 post-implantation embryos tissues" the authors state "To access whether these female-specific abnormalities...". The authors should provide information about male embryos, including numbers examined, in order to demonstrate the abnormalities are female-specific.

In the revised manuscript, we provide a full set of data including males and females at E7.5 and E8.5 (**Fig. 1c** and **Extended Data Fig. 2a**).

2, The authors investigated the role of CpG islands (CGI) in PRC1-mediated gene silencing in XCI. They state that "We found that 95/116 (81.9%) informative X-linked genes have a promoter associated CGI, showing significantly higher expression from the Xi compared to the other informative X-linked genes that do not have CGIs (21/116; 18.1%) ($P = 1.32 \times 10^{-4}$) in Ring1A/B mutant EPCs (Extended Data Fig. 4d)". This sentence is misleading. In the Extended Data Fig. 4d, they are not comparing gene expression from the Xi between genes with and without CGI in the

mutant mouse. Instead, they are comparing the allelic ratio diff (Ring1A/B – Control) in the two groups of genes.

We agree with the reviewer and thank them for this valuable comment. In the revised manuscript, we replaced this chart (**Extended Data Fig. 4d** in the original submission) with new charts together with ΔEed allele-specific RNA-seq (**Extended Data Fig. 5e, f**). In these charts, we added two parameters, *P* value and fold change of allelic ratio, instead of the original allelic ratio difference, to identify significant escapees upon *Ring1A/B* or *Eed* knockout (*P* values ≤ 0.05 , fold change of allelic ratio ≥ 2.0). In these analyses, we assessed the correlation between a presence of promoter-associated CGI and degree of derepression upon *Ring1A/B* or *Eed* deletion in each informative X-linked gene and revealed that X-linked genes with a promoter-associated CGI are significantly derepressed in $\Delta Ring1A/B$ mutant EPCs compared to X-linked genes without a promoter-associated CGI. In contrast, this CGI-directed derepression was not seen in ΔEed mutants where X-linked genes with and without a promoter-associated CGI are both similarly derepressed (**Extended Data Fig. 5f**). We discuss these important findings further in the text of the revised manuscript (**page 15, lines 466–472**) and believe that this new data should help readers to compare the two datasets ($\Delta Ring1A/B$ and ΔEed). We also provide details of all the informative X-linked genes analysed in RNA-seq experiments in **Supplementary table 2**, which should also help readers to understand our conclusions.

3, A recent publication (Dixon-McDougall T, Brown CJ: Independent domains for recruitment of PRC1 and PRC2 by human XIST. *PLoS Genet* 2021, 17:e1009123), demonstrates the independence of the PRC1 and PRC2 pathways in human cells. The authors should cite and discuss this study.

We thank the reviewer for this comment. As mentioned in our response to Reviewer 2 in point 3, we have now included reference to this important study, even though the comparisons between species and in different cell types are not easy to make, the Brown study nevertheless highlights the potentially important variations in Xist RNA-Polycomb recruitment mechanisms.

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Andergassen, D., Smith, Z. D., Kretzmer, H., Rinn, J. L. & Meissner, A. Diverse epigenetic mechanisms maintain parental imprints within the embryonic and extraembryonic lineages. *Dev. Cell* **56**, 2995-3005 (2021)

Bousard, A. et al., The role of Xist-mediated Polycomb recruitment in the initiation of X-chromosome inactivation. *EMBO Rep.* **20**, e48019 (2019)

Corbel, C., Diabangouaya, P., Gendrel, A. V., Chow, J. C. & Heard, E. Unusual chromatin status and organization of the inactive X chromosome in murine trophoblast giant cells. *Development* **140**, 861-872 (2013)

Dixon-McDougall, T. & Brown C. J. Independent domains for recruitment of PRC1 and PRC2 by human XIST. *PLoS Genet.* **17**, e1009123 (2021)

Maclary E. et al., PRC2 represses transcribed genes on the imprinted inactive X chromosome in mice. *Genome Biol.* **18**, 82 (2017)

Pintacuda, G. et al. hnRNPK Recruits PCGF3/5-PRC1 to the Xist RNA B-repeat to establish Polycomb-mediated chromosomal silencing. *Mol. Cell* **68**, 955-969 (2017)

Decision Letter, first revision:

Our ref: NCB-H45454A

20th July 2022

Dear Edith,

Thank you for submitting your revised manuscript "Polycomb complexes PRC1 and PRC2 are each essential for maintenance of X inactivation in extraembryonic lineages" (NCB-H45454A). As you know, per our previous interaction, 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. We expect the final version of the manuscript to be addressing all remaining comments provided by reviewer #1, as discussed per our previous interaction.

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.

Best wishes,
Stelios

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Reviewer #1 (Remarks to the Author):

In this manuscript, the authors define a critical role for both PRC1 and PRC2 in the maintenance of the

silencing of the Xi in extraembryonic lineages in vivo. The authors added some data and explanations during the revision, to strengthen their arguments about the independent role of PRC1 and PRC2 in the maintenance of XCI in extraembryonic lineages. However, I am still concerned about the timing of these experiments, which might have led to an apparent independence of these two PRC pathways.

Specifically, if H2Aub is beginning to be lost at around day 7.5 in 5 embryos (of 15), depletion of H3K27me3 might take another few days (cell cycles) even if it depended upon H2Aub. Is the cell division rate similar in the embryonic and extraembryonic compartment of Ring1a/b embryos? This could explain why the extraembryonic tissue does not yet show the loss of H3K27me3? It also seems one of the Ring1a/b KO d8.5 embryos had a weaker H3K27me3 staining – are the counts for this embryo given anywhere? Along this idea, the TGC data (expansion in vitro) indicate that the mutant TGCs are arrested and not dividing, which might make the loss of H3K27me3 difficult to obtain (and there is a change from 100% in control to 85% in Ring1a/b mutant TGCs).

Conversely, the early knockout phenotype of the Ezh2 deletion does not allow for an extended maintenance of the embryos. Consequently, the loss of H2Aub might not be observed just simply because the cells do not divide anymore and there is little time to achieve the depletion? It remains unclear in the manuscript if Ezh2 knockout TGCs are affected in their growth (similar to the Ring1a/b KO analysis).

Moreover, the use of the EED KO for the RNA-seq analysis of gene silencing on the Xi requires the transition to day 3.5 for the deletion and the lack of extensive overlap with the Ring1a/b KO might be due to the fact that different genes get regulated at different developmental stages. This idea is confirmed by the differences obtained in comparison to the recent Meissner study in which an even different timing for the deletions was utilized. For the EED KO it remains unclear how H2Aub and Xist behave (the Kalantry lab has shown before that the loss of EED in TSCs leads to the loss of Xist coating of the Xi in these cells).

Thus, overall, the paper is important as it further describes the role of PRC1 and PRC2 in the extraembryonic tissue. I am not completely convinced that there is no dependence of the two pathways. However, the data are what they are and maybe this caveat should be explained a bit more in the discussion.

Minor points:

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3. In Figures S1 and S2 – it would be great to clarify from how many replicate embryos the counts (quantifications) for H2Aub and H3K27me3 were obtained.
4. It seems the numbers for the X-linked gene results in Figure S3a are very different between the mutant and control, often higher in the mutant?
5. The discussion seems long.

Reviewer #2 (Remarks to the Author):

I would like to thank the authors for addressing my concerns. The persistence of H3K27me3 in PRC1-null embryos at E7.5 and again at E8.5, and again in TGCs, altogether was in my opinion compelling evidence that PRC2 can be recruited independently of PRC1 in the extra embryonic lineages. Considering also the allelic RNA-seq of EPC-derived tissue showing variably desilenced genes along the Xi in both PRC1 and PRC2 null embryos and a strong link between PRC1-dependence and CGIs, I feel like this work will be of broad interest and importance to the field.

Reviewer #3 (Remarks to the Author):

The authors have fully responded to previous reviews by providing new data and rewriting portions of this interesting paper. This has made the paper stronger.

Decision Letter, final checks:

Our ref: NCB-H45454A

2nd August 2022

Dear Dr. Heard,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Cell Biology manuscript, "Polycomb complexes PRC1 and PRC2 are each essential for maintenance of X inactivation in extraembryonic lineages" (NCB-H45454A). 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. Please also check and comment on any additional marked-up edits we have proposed within the text. 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.

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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 "Polycomb complexes PRC1 and PRC2 are each essential for maintenance of X inactivation in extraembryonic lineages". For those reviewers who give their assent, we will be

publishing their names alongside the published article.

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Best regards,

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Reviewer #1:

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In this manuscript, the authors define a critical role for both PRC1 and PRC2 in the maintenance of the silencing of the Xi in extraembryonic lineages in vivo. The authors added some data and explanations during the revision, to strengthen their arguments about the independent role of PRC1 and PRC2 in the maintenance of XCI in extraembryonic lineages. However, I am still concerned about the timing of these experiments, which might have led to an apparent independence of these two PRC pathways.

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Minor points:

1. It is difficult to see the Xist signal in the embryos in Figure S2. Is there less Xist coating in Ring1/b mutant embryos (in cells of the embryo proper) at d8.5? The same is true for the Xist signal in Figure 3 since there is no dapi stain – does Ezh2 loss change the number of Xist-positive cells?
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3. In Figures S1 and S2 – it would be great to clarify from how many replicate embryos the counts (quantifications) For H2Aub and H3K27me3 were obtained.
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5. The discussion seems long.

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Reviewer #3:

Remarks to the Author:

The authors have fully responded to previous reviews by providing new data and rewriting portions of this interesting paper. This has made the paper stronger.

Author Rebuttal, First Revision:

Referee 1

In this manuscript, the authors define a critical role for both PRC1 and PRC2 in the maintenance of the silencing of the Xi in extraembryonic lineages in vivo. The authors added some data and explanations during the revision, to strengthen their arguments about the independent role of PRC1 and PRC2 in the maintenance of XCI in extraembryonic lineages. However, I am still concerned about the timing of these experiments, which might have led to an apparent independence of these two PRC pathways.

Specifically, if H2Aub is beginning to be lost at around day 7.5 in 5 embryos (of 15), depletion of H3K27me3 might take another few days (cell cycles) even if it depended upon H2Aub. Is the cell division rate similar in the embryonic and extraembryonic compartment of Ring1a/b embryos? This could explain why the extraembryonic tissue does not yet show the loss of H3K27me3? It also seems one of the Ring1a/b KO d8.5 embryos had a weaker H3K27me3 staining – are the counts for this embryo given anywhere? Along this idea, the TGC data (expansion in vitro) indicate that the mutant TGCs are arrested and not dividing, which might make the loss of H3K27me3 difficult to obtain (and there is a change from 100% in control to 85% in Ring1a/b mutant TGCs).

We thank the referee for raising this point. We think that embryonic cells seem to have shorter cell cycle than extraembryonic cells at E7.5–8.5 in both control and Δ Ring1A/B female embryos although no systematic analyses have been made for this in this study. We agree with the referee that different cell cycle rates (i.e. chromatin replication rate) may explain the differential H3K27me3 accumulation/retention on the Xi in the two lineages at E7.5–8.5. Indeed, shorter cell cycles (more chromatin replication) could assist faster removal of H3K27me3 accumulation on the Xi in embryonic lineages and longer cell cycle (less chromatin replication) could allow extraembryonic cells, including Δ Ring1A/B EPC-derived TGCs, retain H3K27me3 accumulation on the Xi for longer time. We now emphasise this point in the Discussion (lines 523–533). Concerning Δ Ring1A/B E8.5 embryos, one of the three embryos showed a weaker enrichment of H3K27me3 on the Xi in extraembryonic tissues (#RAB8-40) where 42.4% of extraembryonic cells were H3K27me3-positive on the Xi. The other two clones (#RAB8-41 and 57) showed 85.2% and 95.5% of H3K27me3-positive cells in extraembryonic tissues, respectively. In the revised manuscript, we indicate these values in Extended Data Fig. 2b.

Conversely, the early knockout phenotype of the *Ezh2* deletion does not allow for an extended maintenance of the embryos. Consequently, the loss of H2Aub might not be observed just simply because the cells do not divide anymore and there is little time to achieve the depletion? It remains unclear in the manuscript if *Ezh2* knockout TGCs are affected in their growth (similar to the *Ring1a/b* KO analysis).

In a recent study by the Klose lab, they showed that H2AK119ub1 can be quickly removed from chromatin within 2 hours upon an acute degradation of RING1A/B proteins (Dobrinic et al., Nat Struct Mol Biol, 2021). This suggests that H2AK119ub1 can be quickly turned over without cell division. Because of this quick turnover property of H2AK119ub1, we think that our $\Delta Ezh2$ embryo experiments can still assess whether the accumulation of PRC1/H2AK119ub1 on the Xi depends on PRC2 or not. Furthermore, we did not observe any growth retardation or death phenotype in female $\Delta Ezh2$ TGCs compared to control TGCs. This point is now described in the manuscript (lines 351-352). As these $\Delta Ezh2$ female TGCs still retained H2AK119ub1 accumulation on the Xi, we concluded that the accumulation of PRC1/H2AK119ub1 on the Xi is independent of PRC2/H3K27me3, in this context.

Moreover, the use of the *EED* KO for the RNA-seq analysis of gene silencing on the Xi requires the transition to day 3.5 for the deletion and the lack of extensive overlap with the *Ring1a/b* KO might be due to the fact that different genes get regulated at different developmental stages. This idea is confirmed by the differences obtained in comparison to the recent Meissner study in which an even different timing for the deletions was utilized. For the *EED* KO it remains unclear how H2Aub and Xist behave (the Kalantry lab has shown before that the loss of *EED* in TSCs leads to the loss of Xist coating of the Xi in these cells).

We understand the referee's concern that the lack of extensive overlap of the *Eed* KO with the *Ring1A/B* KO RNA-seq data might be due to the fact that different X-linked genes would be affected at different developmental stages. However, given that imprinted XCI is already well established by E3.5 (Borensztein et al., Nat Struct Mol Biol, 2017), we know that the effects seen after the *Eed* knockout at E3.5 and the *Ring1A/B* knockout at E5.5, can only concern the maintenance of imprinted XCI and not its initiation. On the other hand, the timing of depletion used in the Meissner study is much earlier, as they used zygotic knockouts for both *Eed* and *Ring1B*. In this system, both initiation and maintenance of imprinted XCI could be impacted. Therefore, their study addresses the contributions of PRC1 and PRC2 to much earlier phases of imprinted XCI.

We have not examined H2AK119ub1 accumulation and Xist coating on the Xi at E7.5 upon *Eed* knockout at E3.5. We analysed the expression level of Xist RNA in EPCs of these ΔEed embryos by RT-qPCR and RNA-seq and found that Xist was expressed at similar levels to the control EPCs, suggesting that Xist coating was maintained in the short-term absence of *EED*. The report in the Kalantry paper (Maclay et al., Genome Biol, 2017) that ΔEed TSCs display a loss of Xist coating on the Xi could be due to the fact that they examined different cell types (in our study, we looked at whole EPCs from embryos vs their study which

looked only at cultured TSCs). The mode of knockout induction could also be the reason why differences are found as a conditional acute knockout was used in our study, compared to a constitutive long-term knockout in their study.

Thus, overall, the paper is important as it further describes the role of PRC1 and PRC2 in the extraembryonic tissue. I am not completely convinced that there is no dependence of the two pathways. However, the data are what they are and maybe this caveat should be explained a bit more in the discussion.

We thank the referee for these valuable comments. We have added some further text to our discussion to mention the caveats (lines 523-533). Although we cannot completely rule out the interdependency between the two PRCs in the *in vivo* setting we have looked at, our data strongly support the idea that the accumulation of both PRC1 and PRC2 are independently maintained on the Xi in post-implantation extraembryonic tissues and have independent but overlapping effects on the maintenance of imprinted XCI.

Minor points:

1. It is difficult to see the Xist signal in the embryos in Figure S2. Is there less Xist coating in Ring1/b mutant embryos (in cells of the embryo proper) at d8.5? The same is true for the Xist signal in Figure 3 since there is no dapi stain – does Ezh2 loss change the number of Xist-positive cells?

We have improved the contrast of Xist signal in Extended Data Fig. 2c. We observed that the number of Xist RNA-positive cells in Δ Ring1A/B E8.5 females (86.2%, n=65) was comparable to E8.5 control embryos (79.2%, n=77) in extraembryonic cells. In embryonic cells, however, the number of Xist-positive cells in Δ Ring1A/B E8.5 females (64.8%, n=54) was slightly lower than in the control (76.5%, n=68). In Δ Ezh2 E6.5 female embryos presented in Fig. 3b, we observed slightly more Xist-positive cells (80.4%, n=92) than in the control female embryos (58.3%, n=60). Therefore, although some of these experiments revealed lower percentages of Xist-positive cells, we think this can be attributed to the slight variations in Xist detection in immuno-RNA FISH experiments. Indeed, even control samples often show a variability of Xist RNA staining.

2. Is the proportion of Xist positive cells different across genotypes?

We do not think so but due to the varying detection of Xist RNA accumulation as mentioned above, it would be difficult to assess the effects of Polycomb mutations on Xist positive cells. Nevertheless, there

was no dramatic effect as we observed that Xist is expressed in >60% of cells in both Δ Ring1A/B and Δ Ezh2 mutant female post-implantation embryos.

3. In Figures S1 and S2 – it would be great to clarify from how many replicate embryos the counts (quantifications) For H2Aub and H3K27me3 were obtained.

In Extended Data Fig. 1, we show the quantifications of two Δ Ring1A/B E7.5 embryos (OR1-1 and OR3-2) for H3K27me3 and one Δ Ring1A/B E7.5 embryo (OR1-1) for H2AK119ub1, which displays loss of H2AK119ub1 on the Xi. In Extended Data Fig. 2, we quantify one Δ Ring1A/B E8.5 embryo (RAB8-57) for both H3K27me3 and H2AK119ub1.

4. It seems the numbers for the X-linked gene results in Figure S3a are very different between the mutant and control, often higher in the mutant?

This is because we only counted the cells that can be clearly identified as a certain embryonic or extraembryonic lineage. If we had any doubt for the lineage identity, we excluded those cells from further analysis. This is why the number of analyzed cells are different in each embryo section.

5. The discussion seems long.

We have shortened the discussion.

Referee 2

I would like to thank the authors for addressing my concerns. The persistence of H3K27me3 in PRC1-null embryos at E7.5 and again at E8.5, and again in TGCs, altogether was in my opinion compelling evidence that PRC2 can be recruited independently of PRC1 in the extra embryonic lineages. Considering also the allelic RNA-seq of EPC-derived tissue showing variably desilenced genes along the Xi in both PRC1 and PRC2 null embryos and a strong link between PRC1-dependence and CGIs, I feel like this work will be of broad interest and importance to the field.

We thank the referee for their encouraging comments. We believe that this study will be an important step for unveiling the complex functions of Polycomb complexes in XCI in extraembryonic tissues.

Referee 3

The authors have fully responded to previous reviews by providing new data and rewriting portions of this interesting paper. This has made the paper stronger.

We thank the referees for their valuable comments in the previous round, which substantially improved the manuscript.

References in the rebuttal

Borensztein, M. et al. Xist-dependent imprinted X inactivation and the early developmental consequences of its failure. *Nat. Struct. Mol. Biol.* **24**, 226-233 (2017)

Dorbrinić, P., Szczurek, A. T. & Klose, R. J. PRC1 drives Polycomb-mediated gene repression by controlling transcription initiation and burst frequency. *Nat. Struct. Mol. Biol.* **28**, 811-824 (2021)

Macray, E. et al. PRC2 represses transcribed genes on the imprinted inactive X chromosome in mice. *Genome Biol.* **18**, 82 (2017)

Final Decision Letter:

Dear Edith,

I am pleased to inform you that your manuscript, "Polycomb repressive complexes 1 and 2 are each essential for maintenance of X inactivation in extraembryonic lineages", has now been accepted for publication in Nature Cell Biology. Congratulations to you and your team!

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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With kind regards,
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