SmcHD1 underlies the formation of H3K9me3 blocks on the inactive X chromosome in mice

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Referee reports from Review Commons

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

There are major issues affecting the conclusions.

Reviewer #1 (Significance (Required)):

The manuscript by Ichihara and colleagues follows up on the role of SmcHD1 in the X chromosome inactivation process. Previous studies have shown that SmcHD1 is recruited to the inactive X chromosome via hnRNP-K and PRC recruitment by Xist and that this intriguing protein is needed for the silencing of some genes on the X chromosome. It was previously shown a relatively large fraction of genes on the inactive X chromosome is not silenced in SmcHD1 mutant embryos and in MEFs derived from these mutant embryos. There are also studies that describe X-inactivation defects in differentiating embryonic stem cells during the initiation of XCI. Yet, the question of whether most genes are first properly repressed early in development and later reactivate has not been directly addressed before it seems. To address this question, the authors derive EpiSCs, which capture a primed pluripotent state present in early post-implantation embryos, and address how gene silencing is altered by SmcHD1 mutations. They find that relatively few genes are not undergoing X-inactivation in EpiSCs, and that the number of affected genes is much larger in MEFs. These results suggest that most genes become reactivated later in development.

The authors then pursue the question of whether the histone marks H3K27me3 and H3K9me3 change in EpiSCs carrying the SmcHD1 mutation. They look at this by immunostaining and Chip-seq. The most obvious and interesting result is that H3K9me3 appears to be lost in mutant cells. A possible link between SmcHD1 was previously applied by the association of SmcHD1 with HP1 and localization over H3K9me3 domains on the human inactive X chromosome, but whether ScmHD1 controls H3K9me3 levels on the X chromosome has not been shown before. Thus, this is potentially very interesting if it is properly done and if the causal relationship is revealed. Moreover, it remains unclear if this is a X chromosome specific result or affects the entire genome. It is implied that it only controls H3K9me3 on the Xi, raising the question of why. There are many other gene clusters that are known to be controlled by SmcHD1 - are those affected?

Overall, this manuscript is potentially interesting, but the ideas would need to be developed further and the analysis be altered. It is difficult to suggest more experiments and ask proper questions as most of the data are only qualitatively described. Therefore, it is unclear what is actually happening. Thus, most of the arguments are made based on a qualitative assessment of the data and it is very difficult to follow the conclusions or see the arguments. In addition, in many cases the authors could have explored questions much deeper.

Thus, at this point, I do not find the findings compelling. Even in Figures 1/2, the authors do not dig in a little more to better understand why some genes on the Xi apparently reactivate later in development in the absence of SmcHD1. For instance, are the active genes on the Xi that differ between EpiSCs and MEFs differentially expressed? There also have been prior papers that look at the effect of the SmcHD1 KO on the initiation of X-inactivation and the authors could have looked there as well for comparison. Lastly, there are no causal relationships between H3K27me3, H3K9me3, and silencing shown in this manuscript.

Specific points:

1. Are the WT escape genes the authors define known escape genes? We assume so, but the authors should show it as a QC for their analysis.
2. Replicates would be great, then a t-test could be done comparing WT and MD/MD instead of the 10% cutoff. This is why doing a t-test or equivalent between WT and mutant would be better than using a cutoff: "It is worth mentioning, however, that genes depressed in Smchd1MD1/MD1 MEFs tended to have relatively high Xi-probability, even though they were classified as inactivated genes (% Xi < 10%) in Smchd1MD1/MD1 EpiSCs".
3. There is a general lack of quantifications. (A) The ChiP-seq data are displayed basically only displayed as enrichment tracks. It is nearly impossible to see what the authors are arguing. A quantitative approach needs to be implied to interpret the data. (B) The imaging data have no quantification (proportion of cells with Xi enrichment, intensity of Xi enrichment etc). There are some measurements of levels, but are those in the Xi or in the remainder of the nucleus? It seems it is not the Xi as the legend says "Signal intensity of the immunofluorescence produced by the antibodies against H3K27me3, H2AK119ub, and H3K9me3 were compared between wild-type (blue) and Smchd1MD1/MD1 (red) EpiSCs (top) and their MEF counterparts (bottom). p-value, Wilcoxon-Mann-Whitney test". We appreciate that the authors did not perform any manipulation of the image data, however, some processing and images of higher resolution with less pixelation would be helpful.
4. For the imaging data, we cannot find anywhere if all images are acquired under the same conditions particularly when intensities were compared. Similarly, it is not clear how many cells were analyzed.
5. It is not clear what the Lrif1 data add to the main idea of the manuscript. These data might be better to be included in a supplemental figure. As with H3K9me3, Lrif1 may have no enrichment on the Xi from immunostaining, but ChIP-seq would show us if it actually localized on the Xi?
6. In Figure 5D. it looks like H3K27me3 levels correlate with derepression in MEFs and EpiSCs. Genes derepressed in MEFs have lower H3K27me3 in MEFs, genes derepressed in EpiSCs have lower K27me3 in EpiSCs. But this suggests that Smchd1 affects H3K27me3 which correlates with silencing, not necessarily that it's mediated via H3K9me3.
7. In Figure 5B it looks like the regions that gain H3K27me3 in MD/MD still have H3K9me3 in MD/MD. Therefore, we don't agree with this statement: "The failure to form H3K9me3 blocks apparently allowed the expansion of H3K27me3 blocks into the adjacent regions normally occupied by H3K9me3." We do not see a clear link between H3K9me3 and maintenance/loss of H3K27me3. Moreover, the statement "In EpiSCs homozygous for Smchd1MD1, the distribution of H3K27me3 on the Xi was, at a glance, similar to that in wildtype, but, in fact, spread to some extent into the region normally occupied by H3K9me3 blocks" is not supported by the data as shown.
8. The discussion seems very speculative and should therefore be shortened. Most arguments made there are not supported by the data.
9. Please indicate significance on the boxplots (5D and S6).
10. In figure 1D, the authors argue that "all 5 genes examined were exclusively expressed from the B6 alleles in wild-type EpiSCs not only before but also after differentiation, suggesting stable silencing of genes on the Xi". We find this statement misleading as there is some activation on the Xi upon differentiation. This then raises the question of why the authors did not perform their

analysis in differentiated EpiSCs to directly assess how things change from the EpiSC to the differentiated state.
11. One question we have is whether the genes that are active on the Xi in MEFs and EpiSCs, are differentially expressed on the Xa in naïve pluripotent cells, EpiSCs and MEFs? This might explain why different genes appear are affected?
12. The authors suggest that accumulation of H3K9me3 in EpiSCs was distinct in shape and distribution in the nucleus from that in MEFs. How are we supposed to see that? As for most other points made in the paper, this is based on qualitative assessments that are difficult to even see.
13. It is somewhat unclear what the point of Figure 3 is supposed to be.
14. The western blot in Figure S2 should be quantified.
15. There are no quantifications for Figure 4 either - so unclear if the Xi accumulation is gone? Despite the lack of a critical assessment of the data, the authors make strong statements such as "Nonetheless, given the differences in the localization of Lrif1 on the Xi as well as the spatial distribution of H3K9me3 in the nucleus between MEFs and EpiSCs in wild type, it seems that neither constitutive heterochromatin nor the facultative heterochromatin, that is, the Xi, are yet fully established in EpiSCs".
16. How do the ChiP-seq data for H3K9me3 or H3K27me3 look on autosomes? Do the levels of the histone modifications change there, maybe in autosomal regions that are known to be regulated by SmcHD1? This statement is lacking support by quantifications - We are not necessarily seeing it "these H3K9me3 blocks were distributed roughly in the regions with relatively lower enrichment of H3K27me3, although they partially overlapped with each other, on the Xi".
17. Are the Chip-seq data controlled by spike in DNA so that the comparison can be done properly?
18. This is difficult to see - is coincided supposed to mean the exact same peak? "In addition, the regions that still retained some enrichment of H3K9me3 on the Xi in the mutant EpiSCs coincided with those showing enrichment of H3K9me3 on the Xa (Figure 5B)."
19. The authors state "the failure to form H3K9me3 blocks apparently allowed the expansion of H3K27me3 blocks into the adjacent regions normally occupied by H3K9me3." There is no quantification - what proportion of the X is regulated like that?
20. The authors argue that "These observations suggest that the X-linked genes that initially undergo XCI but become derepressed in the absence of SmcHD1 acquire H3K27me3 at early developmental stages but lose it during later development". How do we know that the gene acquired H3K27me3 during the initiation of X-inactivation, as the authors don't show us the Xa data in comparison or those for undifferentiated ESCs.
21. The authors suggest that "SmcHD1 plays a critical role in the propagation of H3K9me3 from the hubs on the Xi that has already been enriched with H2AK119ub and H3K27me3, and facilitates the establishment of the respective heterochromatin blocks of H3K27me3 and H3K9me3 on the Xi in the epiblast cells prior to differentiation" Time course data of H3K27me3 and H2Aub are available during the initiation of X-inactivation and could be used for comparison to actually support an argument like that.
22. The authors state: "Signal intensity of the Xi produced by immunofluorescence of antibodies against H3K27me3 and H2AK119ub was significantly enhanced in EpiSCs and MEFs homozygous for Smchd1MD1 compared to their wild-type counterparts. We are not sure where that is shown?

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary:

This manuscript by Sado and coworkers focusses on the role of Smchd1 on X chromosome inactivation by studying different Smchd1 knockout cell lines. They find that loss of Smchd1 leads to derepression of X linked genes which is more prominent in MEFs than in EpiSCs, and also describe loss of big epigenomic H3K9me3 blocks. Interestingly the loss of H3K9me3 is unrelated to derepression of silenced genes which in MEFs is related to loss of H3K27me3.

Major comments:

The major claim made by the authors e that the H3K9me3 blocks formed on the Xi facilitate robust heterochromatin formation in combination with H3K27me3, and the substantial loss of H3K9me3 caused by SmcHD1 deficiency leads to aberrant distribution of H3K27me3 on the Xi and derepression of X-inactivated genes is not supported by the data that is shown. Indeed there is a

relationship between loss of Smchd1 and loss of H3K9me3 but this is unrelated to the derepression phenotype. Derepression of silenced genes in MEFs appears related to loss of H3K27me3 but the mechanism remains unclear. I would recommend the authors to put more efforts in understanding the mechanism leading to derepression in more detail for instance by using the enormous sets of available data such as data interrogating the higher order structure or other epigenomic features (such as the subcompartments described by Lee and coworkers) related to the inactive X. Second Smchd1 fullfills many functions for instance related to DNA methylation this could be further interrogated as well. Finally, the authors describe ESC results in Figure 1, and could/should have used this model system to discriminate between loss of establishment of the inactive X or derepression of an inactive state.
Overall, the story needs a more detailed description and lacks statistics at many points/panels.

Minor comments:

Figure 1B, how are the ES cells differentiated, and to what? Please describe in methods section, I would have liked to see a gene that is derepressed.

Figure 1C, what is quantified here, list on the axis, plus that is is quantification of B?

Figure 1D, why not show Atrx here (to be consistent), and I would like to see an inverted picture.

Figure 2A, why was the arbitrary cut off set at 10%, and escaping genes should have number and percentage labels.

Figure 3C, what is shown here MEFs or EpiSCs?

Figure 4C, add Lrif on the side in the Westernblot shown.

Figure 5B, I would have liked to see more zoom in pictures of other representative areas (for instance as Sup Figure). In the single example shown in 5C there is no consistency between loss or gain of H3K9me3 or H3K27me3 with derepression of genes such as Rlim and Atrx...

Figure 5D and accompanying H3K9me3 panels in the sup figure miss statistics. Are these differences significant?

Figure 6, the model is totally unclear to me and doesn't provide a mechanism. The authors find a link between loss of H3K27me3 and derepression but what is Smchd1 doing here, this needs more explanation conceptually.

SIGNIFICANCE

This manuscript clearly needs some more experimental and conceptual work to be of interest for scientist working in the field of X inactivation or beyond. The finding that loss of Smchd1 appears to lead to a progressive loss of the silenced state is clearly interesting, but as mentioned above the mechanism behind this effect remains elusive and clearly needs more work to make this an appealing story for a more broader audience.

Reviewer #2 (Significance (Required)):

See above.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

In this study, Ichihara et al. describe the function of the chromosomal architectural protein SmcHD1 in formation of H3K9me3 heterochromatin blocks on the mouse inactive X chromosome. They use epiblast stem cells (EpiSCs) as a proxy for early embryonic cells at the beginning of X-

inactivation and mouse embryonic fibroblasts (MEFs) for cells of later stages, where X-inactivation has been completed and is maintained. The authors observe that in SmcHD1 mutant cells, H3K9me3 blocks are lost and H3K27me3 is redistributed, which results in a derepression of X-linked genes predominantly in MEFs. Thereby the authors provide further evidence for SmcHD1 as an important maintenance factor of X-inactivation and that this is achieved through its function as a modulator of heterochromatin.

Major comments:

1. page 5 introduction and Figure 1: "Many of the derepressed genes on the Xi in SmcHD1-deficient MEFs were repressed with enrichment of H3K27me3 in SmcHD1-deficient EpiSCs, suggesting that genes derepressed on the Xi at later developmental stages acquired H3K27me3 at an early phase of XCI but lost it later on in SmcHD1-mutant embryos."
This statement is based on cell lines, making it hard from it to infer what happened in embryos in vivo, from which they have been derived from. In their own paper (Sakakibara et al., Development 2018) for example, around 20% of epiblast cells in SmcHD1-mutant E7.5 embryos, which is a very close state to E6.5, from which the authors have derived EpiSCs, showed derepression of Atrx, while in figure 1, no derepression of Atrx is observed in EpiSCs. The epigenetic and gene silencing state could therefore have changed during establishment and prolonged culture of these cell lines, especially in the case of EpiSCs. The authors should therefore tone down this statement or provide evidence that what has been observed in MEFs and EpiSCs is reflecting accurately the epigenetic/gene silencing state in embryonic cells of the equivalent stages in vivo.

2. As outlined in more detail in minor comments 4+5 below, conclusions on the differences between Xi heterochromatin between EpiSCs and MEFs are drawn from immunostainings, which are difficult to judge without proper quantifications. The authors should attempt to quantify and compare those differences in order to be able to make a more solid statement.
3. page 11-12 and Figure 5B: Similar to the immunostainings, also the ChIP-Seq experiments are mostly analyzed in a descriptive fashion based on the screen shot in figure 5B. It would aid the reader greatly and make the conclusions less subjective, if the effects of SmcHD1 deficiency on H3K27me3 and H3K9me3 blocks and their overlap were quantified. For example, what fraction of the X chromosome is covered by H3K27me3 and H3K9me3 in each lane (Xi vs Xa, EpiSCs vs MEF, wt vs SmcHD1 mutant), and to which degree are H3K27me3 and H3K9me3 mutually exclusive or overlapping? A quantitative comparison would make the conclusions drawn from the results more solid and easier to follow.
4. The authors should discuss their findings in light of papers, which describe the impact of SmcHD1 and Polycomb on X chromosome compartment structure (Wang et al. Cell 2018, Jansz et al. NSMB 2018, Wang et al. Nature Comm 2019 and Gdula et al. Nature Comm 2019, Bauer et al., Nature Comm 2021). As chromosome compartments and chromatin modification blocks for H3K27me3 and H3K9me3 are functionally interconnected, a discussion of compartment structure and how this might relate to the chromatin blocks observed in the current study could aid in the interpretation of the results and put it in context of the literature.

Minor comments:

1. The authors should describe in the methods section, how EpiSC differentiation for the results in Figure 1D was performed.
2. page 18: The cited paper on the threshold to define escapee genes (Peeters et al., 2014) is missing from the References list. Please add it.
3. Figure 3D: It is difficult to see the H3K9me3 signal only in a H3K9me3/Xist merge image. The individual channels for H3K9me3 and Xist should be shown.
4. Figure 3E/F: The statement from page 9 that the staining of H3K9me3 often appeared to spread in a larger area than DAPI-dense heterochromatin in EpiSCs (Figure 3E and 3F) and that this differs from the pattern in MEFs, is very subjective and hard to judge by purely looking at the images. It would strengthen the argument, if this could be somehow quantified, for example by measuring the overlap of H3K9me3 and DAPI dense regions and comparing the overlap between EpiSCs and MEFs.
5. Figure 4A/B: Similar to the statement above about H3K9me3 differences, also here it is difficult to see the differences by eye for Lrif1 on the Xi between EpiSCs and MEFs, especially as very few nuclei are shown. In order to make the statement on page 10 that Lirf1 localization is different on

the Xi between EpiSCs and MEFs a quantification of the Lirf1signal on the H3K27me3-marked Xi-territory should be made and compared between MEFs and EpiSCs.
6. Figure 5a: Statistical comparisons should be performed for the ChIP-QPCR experiments.
Reviewer #3 (Significance (Required)):

This paper builds upon previous studies by the group itself (Sakakibara, Development 2018) and several others, which elucidated the role of SmcHD1 as a factor important for X-inactivation maintenance. This study focuses predominantly on SmcHD1's function as a modulator of H3K9me3 and H3K27me3 chromatin blocks on the inactive mouse X. While these blocks have been mostly described on the human X, accumulating evidence now also has shown that they exist on the mouse X chromosome. This study provides evidence that SmcHD1 is important to maintain these chromatin blocks and that in its absence the chromatin separation changes, leading to reactivation of X-linked genes. This study therefore adds to our knowledge on different types of heterochromatin present on the inactive X and their potential function for maintenance of gene silencing.

The study appears technically sound, but would benefit from a more quantitative approach in data analysis, which would make the interpretation of the results more solid (major points 2+3). Also the discussion could place the study better in context with the existing literature on SmcHD1 and X-chromosome structure/epigenetics.

The study would be mostly of interest specifically to the X-inactivation field and overall to the epigenetics/chromatin community.

Keywords for my field of expertise: X-inactivation, stem cells, developmental biology.

Referee Cross-commenting

Looking at the comments of the other reviewers (in particular reviewer 1), it matches my own impression that the study is lacking quantitative and statistical data analysis in many parts (Immunofluorescence images, ChIP-Seq data), which weakens the confidence in the main claims of the study. I would therefore believe that it would be a minimum requirement to provide such improved analysis, in order for the study to be considered for publication. Furthermore an integration of published datasets or at a minimum a better discussion of their own data in light of the existing literature would enhance the study greatly to put it in context with what is already known about SmcHD1, X-inactivation and chromatin. This could be performed without additional major wet lab-experiment in a relatively short timeframe (3 months).

Original submission

First decision letter

MS ID#: DEVELOP/2021/200488

MS TITLE: SmcHD1 underlies the formation of H3K9me3 blocks on the inactive X chromosome in mice

AUTHORS: Saya Ichihara, Koji Nagao, Takehisa Sakaguchi, Chikashi Obuse, and Takashi Sado
ARTICLE TYPE: Research Article

Dear Dr. Sado,

I have now had the chance to read your paper and review the referee reports from Review Commons, and have reached a decision. The referees' comments are appended below, or

you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see from their reports, the referees recognise the potential of your work, but they also raise significant concerns about it. Given the nature of these concerns, I am afraid I have little choice other than to reject the paper at this stage.

However, having evaluated the paper, I do recognise the potential importance of this work. I would therefore be prepared to consider as a new submission an extension of this study that contains new experiments, data and discussions and that address fully the major concerns of the referees. The work required goes beyond a standard revision of the paper. Please bear in mind that the referees (who may be different from the present reviewers) will assess the novelty of your work in the context of all previous publications, including those published between now and the time of resubmission.

Yours sincerely,

Haruhiko Koseki
Handling Editor
Development

First revision

Author response to reviewers' comments

We appreciate constructive criticisms made by all the reviewers, especially suggestions to perform quantitative analyses of ChIP-seq and fluorescence images. We have attended all the comments and the response to them are shown in blue as you see below.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

There are major issues affecting the conclusions.

Reviewer #1 (Significance (Required)):

The manuscript by Ichihara and colleagues follows up on the role of SmcHD1 in the X chromosome inactivation process. Previous studies have shown that SmcHD1 is recruited to the inactive X chromosome via hnRNP-K and PRC recruitment by Xist and that this intriguing protein is needed for the silencing of some genes on the X chromosome. It was previously shown a relatively large fraction of genes on the inactive X chromosome is not silenced in SmcHD1 mutant embryos and in MEFs derived from these mutant embryos. There are also studies that describe X-inactivation defects in differentiating embryonic stem cells during the initiation of XCI. Yet, the question of whether most genes are first properly repressed early in development and later reactivate has not been directly addressed before it seems. To address this question, the authors derive EpiSCs, which capture a primed pluripotent state present in early post- implantation embryos, and address how gene silencing is altered by SmcHD1 mutations. They find that relatively few genes are not undergoing X-inactivation in EpiSCs, and that the number of affected genes is much larger in MEFs. These results suggest that most genes become reactivated later in development.

The authors then pursue the question of whether the histone marks H3K27me3 and H3K9me3 change in EpiSCs carrying the SmcHD1 mutation. They look at this by immunostaining and Chip-seq. The most obvious and interesting result is that H3K9me3 appears to be lost in mutant cells. A possible link between SmcHD1 was previously applied

by the association of SmcHD1 with HP1 and localization over H3K9me3 domains on the human inactive X chromosome, but whether ScmHD1 controls H3K9me3 levels on the X chromosome has not been shown before. Thus, this is potentially very interesting if it is properly done and if the causal relationship is revealed. Moreover, it remains unclear if this is a X chromosome specific result or affects the entire genome. It is implied that it only controls H3K9me3 on the Xi, raising the question of why. There are many other gene clusters that are known to be controlled by SmcHD1 - are those affected?

Overall, this manuscript is potentially interesting, but the ideas would need to be developed further and the analysis be altered. It is difficult to suggest more experiments and ask proper questions as most of the data are only qualitatively described. Therefore, it is unclear what is actually happening. Thus, most of the arguments are made based on a qualitative assessment of the data and it is very difficult to follow the conclusions or see the arguments. In addition, in many cases the authors could have explored questions much deeper.

Thus, at this point, I do not find the findings compelling. Even in Figures 1/2, the authors do not dig in a little more to better understand why some genes on the Xi apparently reactivate later in development in the absence of SmcHD1. For instance, are the active genes on the Xi that differ between EpiSCs and MEFs differentially expressed? There also have been prior papers that look at the effect of the SmcHD1 KO on the initiation of X-inactivation and the authors could have looked there as well for comparison. Lastly, there are no causal relationships between H3K27me3, H3K9me3, and silencing shown in this manuscript.
(Response)
Upon the criticisms mentioned above, we have carried out quantitative analyses of immunofluorescence and ChIP-seq as detailed below. As pointed out by reviewer 1, we have to admit what this study shows are correlations between the loss of SmcHD1 and diminished H3K9me3 levels on the Xi or aberrant distributions of H3K9me3 and H3K27me3 on the Xi, but do not show causal relationship between these observations. To our knowledge, however, potential links between them have not even been previously discussed before, and therefore, we believe that the findings in this study would provide further insights into our understanding about the mechanisms of how X-inactivated state is maintained.

As to the question if the active genes on the Xi in EpiSCs and MEFs are differentially expressed, please see the response to reviewer 1's comment 11. We have included some discussion based on the previous studies about the effect of SmcHD1 deficiency on the initiation of XCI on page 15 and 16.

Specific points:

1. Are the WT escape genes the authors define known escape genes? We assume so, but the authors should show it as a QC for their analysis.
(Response)
We have included Table S1 as a supplemental data to show genes classified as escapees in, at least, one of Smchd1^+/+ MEFs and two EpiSC lines for the QC of our allele-resolved analysis. Escapees common to all three cases, which are shown in red, are known constitutive escapees. Since many of them have been previously reported as escapees, some instances that escapees in one EpiSCs line is not necessarily escapees in the other may represent some variation.

2. Replicates would be great, then a t-test could be done comparing WT and MD/MD instead of the 10% cutoff. This is why doing a t-test or equivalent between WT and mutant would be better than using a cutoff: "It is worth mentioning, however, that genes depressed in Smchd1MD1/MD1 MEFs tended to have relatively high Xi-probability, even though they were classified as inactivated genes (% Xi < 10%) in Smchd1MD1/MD1 EpiSCs".
(Response)
10% cutoff has been used for defining escapees Peeters et al. (Bioessays 36, 746- 756) and others, and we thought this was reasonable. We intended to point out by original Figure 2C that although %Xi of many X-linked genes in mutant EpiSCs was below the threshold of the 10% cutoff, their expression status on the Xi in mutant EpiSCs correlates well with the expression status of those

genes in mutant MEFs. We have replaced the box plots of the former Figure 2C to new one as new Figure 2D, so that this message can be more readily transmitted.

3. There is a general lack of quantifications. (A) The ChiP-seq data are displayed basically only displayed as enrichment tracks. It is nearly impossible to see what the authors are arguing. A quantitative approach needs to be implied to interpret the data. B) The imaging data have no quantification (proportion of cells with Xi enrichment, intensity of Xi enrichment etc). There are some measurements of levels, but are those in the Xi or in the remainder of the nucleus? It seems it is not the Xi as the legend says "Signal intensity of the immunofluorescence produced by the antibodies against H3K27me3, H2AK119ub, and H3K9me3 were compared between wild-type (blue) and Smchd1MD1/MD1 (red) EpiSCs (top) and their MEF counterparts (bottom). p-value, Wilcoxon-Mann-Whitney test". We appreciate that the authors did not perform any manipulation of the image data, however, some processing and images of higher resolution with less pixelation would be helpful.
(Response)
We have carried out quantitative analyses of ChIP-seq (new Fig. 6, 7, Fig. S6, and S7) and imaging such as nuclear periphery-enrichment of H3K9me3 in EpiSCs as compared to MEFs (Figs. 3C, 3D) and Xi-enrichment of SmcHD1 and Lrif1 (Fig. 4). We apologize for the confusion of the Fig. 3B, which we assume the reviewer pointed out. Fig. 3B illustrates quantitative analysis for the comparison of the signal intensity of H3K27me3 and H2AK119ub on the inactive X between wild-type and SmcHD1-deficient EpiSCs and their counterparts of MEFs. We have amended the description and made it clearer in the legend for Fig. 3B. In newly added Fig. 3C and 3D, we have quantitatively evaluated enrichment of H3K9me3 at the nuclear periphery using an antibody against Lamin B1, which is a known nuclear lamina marker, in combination with an antibody against H3K9me3 (see response to comment 12). In new Fig. 4, we have included a quantitative analysis for comparison of the Xi-enrichment of SmcHD1 and Lrif1 between EpiSCs and MEFs (see response to comment 5).

4. For the imaging data, we cannot find anywhere if all images are acquired under the same conditions particularly when intensities were compared. Similarly, it is not clear how many cells were analyzed.
(Response)
The statement about acquisition of the images has been included in Materials and methods. Information about the number of cells analyzed has been included in the main text (page 10) and the relevant Figure legends (Fig. 3 and 4).

5. It is not clear what the Lrif1 data add to the main idea of the manuscript. These data might be better to be included in a supplemental figure. As with H3K9me3, Lrif1 may have no enrichment on the Xi from immunostaining, but ChIP-seq would show us if it actually localized on the Xi?
(Response)
In the revised manuscript, we have altered the description of Lrif1 localization. Although we initially described that Lrif1 was largely off the Xi highlighted with H3K27me3, we have altered it trying to be more appropriate and now describe how much they overlap. We quantitated the extent of Lrif1 occupancy in the H3K27me3 domain representing the Xi between wild-type EpiSCs and MEFs and found significant differences between them (Fig. 4). We think this suggests the presence of some kind of differences in the Xi chromatin state between EpiSCs and MEFs and therefore should be shown in one of the main figures to support our idea that chromatin of EpiSCs is in a transitional state like that of the epiblast in the embryo.

We carried out ChIP-seq analysis using an antibody against Lrif1, which was used for western blotting shown in Fig. S5, and although the preliminary result demonstrated that slight enrichment of Lrif1 over the genome-wide average at some particular sites, which might correlate with enrichment of H3K9me3, on the Xi in EpiSCs, unfortunately it was not convincing enough due to low signal-to noise ratio. Since we could also see enrichment of Lrif1 on centromeric heterochromatin by ChIP-seq, however, we are skeptical of the idea that Lrif1 barely shows an enrichment on the Xi with immunostaining but does with ChIP-seq like H3K9me3 on the Xi.

6. In Figure 5D. it looks like H3K27me3 levels correlate with derepression in MEFs and EpiSCs. Genes derepressed in MEFs have lower H3K27me3 in MEFs, genes derepressed in

EpiSCs have lower K27me3 in EpiSCs. But this suggests that Smchd1 affects H3K27me3 which correlates with silencing, not necessarily that it's mediated via H3K9me3.
(Response)
Although we agree that data shown in the former Fig. 5D (current Fig. 6D) demonstrate correlation between H3K27me3 levels and derepression in a subset of Xi genes in SmcHD1-deficinet MEFs (groups of genes in green and orange in the left panel) and EpiSCs (groups of genes in red and orange in the right panel), those derepressed with lower enrichment of H3K27me3 in EpiSCs (groups of genes in red and orange on the right panel) consist of 72 (20%) in the 354 informative genes commonly silenced in wild-type EpiSCs and the remaining 282 (80%) are categorized in the repressed genes with enrichment of H3K27me3 in mutant EpiSCs. Of these 282 genes, 139 (49%) are shown to be derepressed in mutant MEFs with eventual loss of H3K27me3. We are focusing on these Xi genes consisting of a relatively large population, which we assume are kind of genes undergoing H3K27me3-mediated silencing at the early phase of XCI but becomes derepressed later on with a loss of previously deposited H3K27me3 in the SmcHD1-deficient embryo. We have modified description related to Fig 6D on page 13. Based on the finding that H3K9me3 blocks were not properly formed on the Xi in SmcHD1-deficient EpiSCs and MEFs, we raise the possibility that proper deposition of H3K9me3 on the Xi is required for sustaining H3K27me3 previously deposited at the bodies of these Xi genes. Although we have to admit that there is no evidence that the lack of H3K9me3 is causally related to derepression of X-inactivated genes and their loss of H3K27me3 as the reviewer pointed out, we think that the possibility we have raised would not be necessarily unacceptable and would rather provide intriguing insights into the future directions of the studies in the field of X inactivation and epigenetics.

7. In Figure 5B it looks like the regions that gain H3K27me3 in MD/MD still have H3K9me3 in MD/MD. Therefore, we don't agree with this statement: "The failure to form H3K9me3 blocks apparently allowed the expansion of H3K27me3 blocks into the adjacent regions normally occupied by H3K9me3." We do not see a clear link between H3K9me3 and maintenance/loss of H3K27me3. Moreover, the statement "In EpiSCs homozygous for Smchd1MD1, the distribution of H3K27me3 on the Xi was, at a glance, similar to that in wildtype, but, in fact, spread to some extent into the region normally occupied by H3K9me3 blocks" is not supported by the data as shown.
(Response)
We assume that the reviewer pointed out that he/she could not see clear link between H3K9me3 and maintenance/loss of H3K27me3 especially in MEFs. We have included a track in new Fig. 6A (the former Fig. 5B) showing an overlay of enrichment for respective histone modifications in wild-type (+/+) and Smchd1MD1/MD1 cells, so that one can readily recognize the expansion/increase of H3K27me3 and loss/decrease of H3K9me3 in the mutant cells. While loss of H3K9me3 is evident in mutant EpiSCs, the region showing enrichment of H3K9me3 over the average of the genome (blocks of H3K9me3) may not look very different between wild-type and mutant MEFs. As the overlay shows, however, that the extent of enrichment in the mutant MEFs (deep green) is lower than that in wild-type MEFs (pale green) in all H3K9me3 blocks. H3K27me3 in mutant MEFs invades into these H3K9me3 blocks characterized by diminished enrichment of H3K9me3 in mutant MEFs. One could say, therefore, that reduction of H3K9me3 enrichment is correlated with an expansion or invasion of H3K27me3 into these H3K9me3 blocks.

8. The discussion seems very speculative and should therefore be shortened. Most arguments made there are not supported by the data.
(Response)
We have removed some of the original discussion, which might have sounded too speculative, and have included discussion based on the time course and Hi-C analyses of differentiating ESCs in previous studies (Colognori et al., 2020; Gdula et al., 2019; Jansz et al., 2018a; Wang et al., 2018).

9. Please indicate significance on the boxplots (5D and S6).
(Response)
Statistical significance has been indicated in the new Fig. 6D (former Fig. 5.D) and new Fig S7E (former Fig. S6).

10. In figure 1D, the authors argue that "all 5 genes examined were exclusively expressed from the B6 alleles in wild-type EpiSCs not only before but also after differentiation, suggesting stable silencing of genes on the Xi". We find this statement misleading as there

is some activation on the Xi upon differentiation. This then raises the question of why the authors did not perform their analysis in differentiated EpiSCs to directly assess how things change from the EpiSC to the differentiated state.
(Response)
Our interpretation of the data shown in Fig. 1D is that there is no activation of genes on the inactive X^JF1 in wild-type EpiSCs during the course of differentiation for 3 weeks, and therefore, do not think the statement is misleading. The reason why we did not use differentiated EpiSCs for a series of experiments but took advantage of MEFs instead is that we assumed that the population of differentiated EpiSCs would contain cells at various stages of differentiation, which would cause variation in the state of derepression of genes on the inactive X and their chromatin state. In addition, we had already had some data obtained by detailed analyses using MEFs in our previous study (Sakakibara et al., 2018) to compare with.

11. One question we have is whether the genes that are active on the Xi in MEFs and EpiSCs, are differentially expressed on the Xa in naïve pluripotent cells, EpiSCs and MEFs? This might explain why different genes appear are affected?
(Response)
Thanks for pointing out this important issue. We have evaluated the potential bias of differential gene upregulation depending on developmental stages of cells for the differences in the list of drepressed genes, assuming differentiation proceeds from the state of ESCs to that of MEFs via the state of EpiSCs. As described on page 8 and shown in new Figure 2C, we compared in the wild-type context expression levels of these derepressed genes and those of genes stably repressed between EpiSCs and ESCs as well as between EpiSCs and MEFs. The results demonstrated that there was no significant difference in expression levels in EpiSCs relative to ESCs between the class of genes that became derepressed and the class of genes that remained repressed in the SmcHD1-deficient cells. Comparison of their expression levels between MEFs and EpiSCs showed, on the other hand, that there was a trend of higher expression in the former class of genes than the latter. Differential gene regulation between EpiSCs and MEFs may, therefore, partly contribute to an increase in the frequency of derepression in MEFs.

12. The authors suggest that accumulation of H3K9me3 in EpiSCs was distinct in shape and distribution in the nucleus from that in MEFs. How are we supposed to see that? As for most other points made in the paper, this is based on qualitative assessments that are difficult to even see.
(Response)
We have carried out quantitative analysis to show the differences in distribution of H3K9me3 in the nucleus between EpiSCs and MEFs. We took advantage of an antibody against Lamin B1 in combination with that against H3K9me3 for immunostaining and quantitated fluorescence of H3K9me3 overlapping with Lamin B1 in total fluorescence of H3K9me3 in the nucleus. Newly added Fig. 3C and 3D demonstrate that there are significant differences in the proportion of H3K9me3 at the nuclear periphery between EpiSCs and MEFs regardless SmcHD1 deficiency.

13. It is somewhat unclear what the point of Figure 3 is supposed to be.
(Response)
We carried out these immunostainings to examine if there were any differences in histone modifications between in the presence and absence of SmcHD1 in EpiSCs and MEFs and also between EpiSCs and MEFs in terms of localization on the Xi and distribution in the nucleus, etc. We found the intensity of H3K27me3 and H2AK119ub on the Xi was significantly higher in EpiSCs and MEFs in the absence of SmcHD1 than in the presence of SmcHD1. Although H3K9me3 localized on the Xi in neither EpiSCs nor MEFs, its distribution in the nucleus was distinct between them as mentioned in the response to comment 12 above. We think these are important findings for not only the effect of SmcHD1 loss in EpiSCs and MEFs but also the evaluation of EpiSCs as a model of the epiblast, and therefore, should be provided as one of main Figures.

14. The western blot in Figure S2 should be quantified.
(Response)
We have done it.

15. There are no quantifications for Figure 4 either - so unclear if the Xi accumulation is gone? Despite the lack of a critical assessment of the data, the authors make strong

statements such as "Nonetheless, given the differences in the localization of Lrif1 on the Xi as well as the spatial distribution of H3K9me3 in the nucleus between MEFs and EpiSCs in wild type, it seems that neither constitutive heterochromatin nor the facultative heterochromatin, that is, the Xi, are yet fully established in EpiSCs".

(Response)

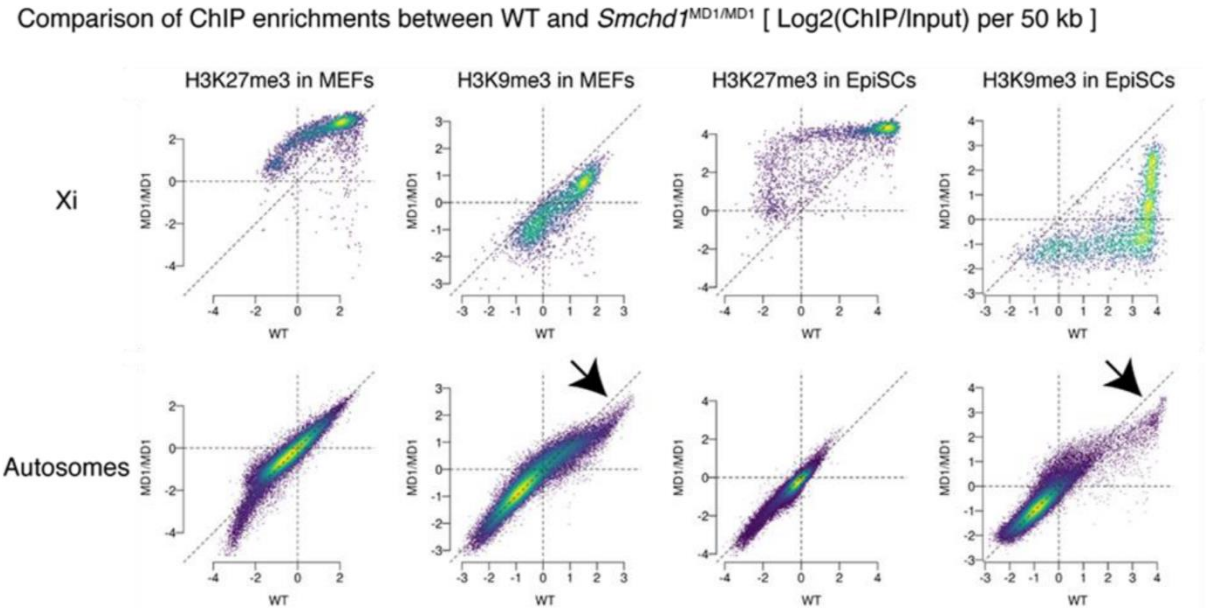
We have carried out quantitative analysis to show differences in the occupancy of Lrif1 in the H3K27me3 domain representing the Xi between EpiSCs and MEFs. The occupancy of SmcHD1 was also analyzed in the same way. This analysis demonstrated that Lrif1 occupied rather limited region of the Xi in EpiSCs as compared to MEFs regardless the presence or absence of SmcHD1. There was also a slight difference in the occupancy of SmcHD1 between EpiSCs and MEFs as well.

These results, which have been included as new Fig. 4B and 4D, suggest some differences in the chromatin state of the Xi between EpiSCs and MEFs.

16. How do the ChiP-seq data for H3K9me3 or H3K27me3 look on autosomes? Do the levels of the histone modifications change there, maybe in autosomal regions that are known to be regulated by SmcHD1? This statement is lacking support by quantifications
- We are not necessarily seeing it "these H3K9me3 blocks were distributed roughly in the regions with relatively lower enrichment of H3K27me3, although they partially overlapped with each other, on the Xi".

(Response)

Consistent with the observation for the Xi, the levels of H3K9me3 also decreased at autosomal regions enriched with H3K9me3 in both MD1/MD1 EpiSCs and MEFs as shown in Fig. S7B and the following Rebuttal Figure (see arrows). The changes of H3K27me3 on autosomes seem to be subtle. It is worth noting that SmcHD1 on autosomes is mainly localized to H3K9me3 domains, although SMCHD1 on the Xi is localized to both H3K9me3 and H3K27me3 domains in human (Nozawa et al., 2013). We think SmcHD1 acts on histone modifications on both the Xi and autosomes. We would, however, like to focus on the Xi in this study and detailed analyses of the autosomes should be reported as separate work in the future. To avoid ambiguous description, the sentence pointed out has been removed.



Rebuttal Figure

17. Are the Chip-seq data controlled by spike in DNA so that the comparison can be done properly?

(Response)

Although we did not include spike-in DNA for ChIP-seq, we have carried out reasonable evaluation to conclude that our method for normalization is comparably reliable to the spike-in method for comparison of the enrichment of H3K9me3 and H3K27me3. We assumed that enrichment of the respective modifications in most of autosomal regions are comparable between EpiSCs and MEFs as

well as wild-type and MD1/MD1 cells. As described in Material and methods, we performed normalization so that the large fraction of autosomal region indicated in yellow in the scatter plots shown in Rebuttal Figure above can be aligned along the diagonal when the enrichment in wildtype is plotted against MD1/MD1. In general, normalization using spike-in is performed to make a certain region with comparable enrichment between the samples be the same assuming most of autosomal regions were comparable. These two methods would, therefore, be considered to be essentially the same.

18. This is difficult to see - is coincided supposed to mean the exact same peak? "In addition, the regions that still retained some enrichment of H3K9me3 on the Xi in the mutant EpiSCs coincided with those showing enrichment of H3K9me3 on the Xa (Figure 5B)."
(Response)
We agree that the word “coincided” is not appropriate. We have not only altered the sentence pointed out to “the regions that still retained some enrichment of H3K9me3 on the Xi in the mutant EpiSCs harbored those showing slight enrichment of H3K9me3 on the Xa” but also included a new figure showing an overlay of H3K9me3 enrichment tracks of Xi and Xa as Fig. S6B.

19. The authors state "the failure to form H3K9me3 blocks apparently allowed the expansion of H3K27me3 blocks into the adjacent regions normally occupied by H3K9me3." There is no quantification - what proportion of the X is regulated like that? **(Response)**
We have carried out quantitative analysis of ChIP-seq data and have provided
additional figures showing the extent of changes in the occupancy of H3K9me3 and H3K27me3 on the Xi in the wild-type and mutant EpiSCs and MEFs (Fig. 6B, C, Fig. S7A). We think that these new data support our description pointed out by the reviewer.

20. The authors argue that "These observations suggest that the X-linked genes that initially undergo XCI but become derepressed in the absence of SmcHD1 acquire H3K27me3 at early developmental stages but lose it during later development". How do we know that the gene acquired H3K27me3 during the initiation of X-inactivation, as the authors don't show us the Xa data in comparison or those for undifferentiated ESCs. **(Response)**
Although we did not examine H3K27me3 state on Xa in undifferentiated cells ourselves, it has been shown by Colognori et al. (2020) that H3K27me3 is essentially absent on the Xa in undifferentiated ESCs and is acquired on the X undergoing inactivation during differentiation. The statement in Introduction pointed out here has been deleted and the similar statement in Discussion has been modified with citing the study by Colognori et al. and toned down (page 15).

21. The authors suggest that "SmcHD1 plays a critical role in the propagation of H3K9me3 from the hubs on the Xi that has already been enriched with H2AK119ub and H3K27me3, and facilitates the establishment of the respective heterochromatin blocks of H3K27me3 and H3K9me3 on the Xi in the epiblast cells prior to differentiation" Time course data of H3K27me3 and H2Aub are available during the initiation of X- inactivation and could be used for comparison to actually support an argument like that. **(Response)**
We have reanalyzed time course data of ChIP-seq using differentiating female ESCs reported by Colognori et al (2020) in our hands, so that we can not only align these data with our ChIP-seq data in parallel for direct comparison (new Fig. 7A) but also evaluate correlation of histone modifications between their differentiating ESCs and either of our EpiSCs and MEFs (Fig. 7B). We took these data thus generated into account for further discussion of how our new findings in this study can be put in the current view of the SmcHD1-meidated epigenetic regulation of the Xi (page 15 and 16).

22. The authors state: "Signal intensity of the Xi produced by immunofluorescence of antibodies against H3K27me3 and H2AK119ub was significantly enhanced in EpiSCs and MEFs homozygous for Smchd1MD1 compared to their wild-type counterparts. We are not sure where that is shown?
(Response)
This statement is based on the results shown in Fig, 3A and B. We apologize that the legend for Fig. 3B in the original submission contained some irrelevant words about the analysis we had excluded, which apparently caused this unnecessary confusion. The legend has been appropriately amended.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary:

This manuscript by Sado and coworkers focusses on the role of Smchd1 on X chromosome inactivation by studying different Smchd1 knockout cell lines. They find that loss of Smchd1 leads to derepression of X linked genes which is more prominent in MEFs than in EpiSCs, and also describe loss of big epigenomic H3K9me3 blocks. Interestingly the loss of H3K9me3 is unrelated to derepression of silenced genes which in MEFs is related to loss of H3K27me3.
Major comments:

The major claim made by the authors e that the H3K9me3 blocks formed on the Xi facilitate robust heterochromatin formation in combination with H3K27me3, and the substantial loss of H3K9me3 caused by SmcHD1 deficiency leads to aberrant distribution of H3K27me3 on the Xi and derepression of X-inactivated genes is not supported by the data that is shown. Indeed there is a relationship between loss of Smchd1 and loss of H3K9me3 but this is unrelated to the derepression phenotype. Derepression of silenced genes in MEFs appears related to loss of H3K27me3 but the mechanism remains unclear. I would recommend the authors to put more efforts in understanding the mechanism leading to derepression in more detail for instance by using the enormous sets of available data such as data interrogating the higher order structure or other epigenomic features (such as the subcompartments described by Lee and coworkers) related to the inactive X. Second Smchd1 fullfills many functions for instance related to DNA methylation this could be further interrogated as well. Finally, the authors describe ESC results in Figure 1, and could/should have used this model system to discriminate between loss of establishment of the inactive X or derepression of an inactive state.
Overall, the story needs a more detailed description and lacks statistics at many points/panels.
(Response)
As pointed out here, we have shown causal relationship neither between the lack of SmcHD1 and the substantial loss of H3K9me3 on the Xi nor between the substantial loss of H3K9me3 on the Xi and derepression of hitherto inactivated genes on the Xi with loss of H3K27me3 nearby. Loss of H3K9me3 on the Xi was, however, one of the most prominent phenotypes observed in EpiSCs lacking SmcHD1. Although it is not clear if this is direct or indirect consequence of loss of SmcHD1, this finding is unexpected and has never been described before, and therefore, should provide valuable insights into our understanding of how SmaHD1 contributes to the stable maintenance of heterochromatin and higher order chromatin structure. We agree that enormous sets of data are available to discuss the possible mechanism leading to derepression of X-inactivated genes, but we did not discuss enough from such point of view. In the revised manuscript, we have included some discussion of how our new findings can be incorporated into the scenario that has been described for the role of SmcHD1 in the regulations of histone modifications and higher order chromatin structure of the Xi. As reviewer 2 pointed out the importance to consider the functional relevance of SmcHD1 in DNA methylation, we have also included an additional analysis of our RNA-seq data set in view of CGI methylation kinetics reported by Gendrel et al. (2012) (Fig. S1E). This revealed that genes derepressed in both EpiSCs and MEFs in SmcHD1-deficient background were highly associated with slow methylating CGIs, which Gendrel et al. suggest to be regulated by SmcHD1-dependent pathway.

What we described in Figure 1 was results of EpiSCs not ESCs. Nonetheless, we agree that ESC differentiation system is an alternative to address the issue we have addressed using EpiSCs and MEFs. One potential problem with using differentiating ESCs is the heterogeneity of the population containing differentiated and undifferentiated cells. Since cells to be analyzed have to have undergone XCI once as prerequisite to address the maintenance mechanism of the Xi, such heterogenous population would not be suitable. In contrast, EpiSCs, which has undergone XCI, can be maintained in a relatively unique cell population under the condition described by Sugimoto et al. (2015) and therefore, we thought they would be a better model for our purpose than differentiating ESCs.

Minor comments:

Figure 1B, how are the ES cells differentiated, and to what? Please describe in methods section, I would have liked to see a gene that is derepressed.
(Response)
Figure 1B shows a result of RNA-FISH in EpiSCs, which had been maintained in an undifferentiated state. If the reviewer was referring to the experiment shown in Figure 1D, we differentiated EpiSCs by forming embryoid bodies and subsequently growing them on a coverslip as outgrowths according to Sugimoto et al. (2015), which we expected gave rise to tissues of all three germ layers. Since the method how we differentiate them was missing in Materials and methods, we have included it in the revised manuscript.

Figure 1C, what is quantified here, list on the axis, plus that is is quantification of B?
(Response)
As described in Figure legend, Fig. 1C shows proportions of the nuclei with a single pinpoint signal for Atrx, which did not overlap with the Xist cloud, in wild-type and Smchd1MD1/MD1 EpiSCs.

Figure 1D, why not show Atrx here (to be consistent), and I would like to see an inverted picture.
(Response)
We did not include Atrx here because SNP overlapping a restriction site was not available at the Atrx locus. We actually prepared an inverted image of PCR shown in Figure 1D before initial submission, however, it did not seem to improve the visibility of the bands and we ended up thinking of the images originally submitted being better.

Figure 2A, why was the arbitrary cut off set at 10%, and escaping genes should have number and percentage labels.
(Response)
10% cutoff has been used to define escapees by Peeters et al. (Bioessays 36, 746- 756) and others, and we thought this was reasonable. The number of escapees and the percentage of them among the informative genes have been added in Figure 2A. We have also included a new Table S1 in the revised manuscript, which lists up genes referred to as escapees in wild-type MEFs and two EpiSCs lines.

Figure 3C, what is shown here MEFs or EpiSCs?
(Response)
As described in the figure legend, the original Figure 3C had shown Immunofluorescence of wild-type and *Smchd1*$^{MD1/MD1}$ EpiSCs for H3K9me3 and H3K27me3 in comparison with that of wild-type and *Smchd1*$^{MD1/MD1}$ MEFs. After performing an additional experiment for quantitative analysis using an antibody against Lamin B1 in combination with the antibody against H3K9me3, the original Figure 3C (and3D), have been moved to Supplemental data as Fig. S3.

Figure 4C, add Lrif on the side in the Westernblot shown.
(Response)
Labeling was added on the side of western blot on original Figure 4C, which has been moved to Supplemental data as Fig. S5.

Figure 5B, I would have liked to see more zoom in pictures of other representative areas (for instance as Sup Figure). In the single example shown in 5C there is no consistency between loss or gain of H3K9me3 or H3K27me3 with derepression of genes such as Rlim and Atrx...
(Response)
We have modified the way of displaying the correlation between derepression of genes on the Xi and enrichment of H3K27me3 and moved the figure to supplemental data as Fig. S7D. Although we showed a simple blow-up of the enrichment tracks that spanned a 4-Mb region containing different categories of genes in previous Figure 5C, the correlation between them might not be very clear because the resolution of the enrichment tracks was not high enough and many irrelevant genes were also aligned. Accordingly, in new Fig. S7D, we have picked up representatives in each category of genes, that are, those repressed in EpiSCs but derepressed in MEFs (Rlim and Atrx),

derepressed in both EpiSCs and MEFs (Ogt), stably repressed in both EpiSCs and MEFs (Fndc3c1), and expressed/escaped in both EpiSCs and MEFs (Xist and Jpx). While expression of each category of genes on the Xi in the mutant EpiSCs and MEFs shows clear correlation with the extent of H3K27me3 enrichment, such correlation was not evident between their expression and H3K9me3 enrichment.

Figure 5D and accompanying H3K9me3 panels in the sup figure miss statistics. Are these differences significant?
(Response)
p-value has been included in the figures pointed out, which have been shown as new Fig. 6D and Fig. S7E

Figure 6, the model is totally unclear to me and doesn't provide a mechanism. The authors find a link between loss of H3K27me3 and derepression but what is Smchd1 doing here, this needs more explanation conceptually.
(Response)
We have withdrawn the model.

SIGNIFICANCE

This manuscript clearly needs some more experimental and conceptual work to be of interest for scientist working in the field of X inactivation or beyond. The finding that loss of Smchd1 appears to lead to a progressive loss of the silenced state is clearly interesting, but as mentioned above the mechanism behind this effect remains elusive and clearly needs more work to make this an appealing story for a more broader audience. (Response)
We have carried out some additional quantitative analyses for ChIP-seq and imaging data as suggested by reviewers. We have shown causal relationship neither between the lack of SmcHD1 and the substantial loss of H3K9me3 on the Xi nor between the substantial loss of H3K9me3 on the Xi and derepression of hitherto inactivated genes on the Xi with loss of H3K27me3 nearby. Loss of H3K9me3 on the Xi was, however, one of the most prominent phenotypes observed in EpiSCs and MEFs lacking SmcHD1. Although it is not clear if this is direct or indirect consequence of loss of SmcHD1, this finding is unexpected and has never been described before, and therefore, should provide valuable insights into our understanding of how SmaHD1 contributes to the stable maintenance of heterochromatin and higher order chromatin structure.

Reviewer #2 (Significance (Required)):

See above.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

In this study, Ichihara et al. describe the function of the chromosomal architectural protein SmcHD1 in formation of H3K9me3 heterochromatin blocks on the mouse inactive X chromosome. They use epiblast stem cells (EpiSCs) as a proxy for early embryonic cells at the beginning of X-inactivation and mouse embryonic fibroblasts (MEFs) for cells of later stages, where X-inactivation has been completed and is maintained. The authors observe that in SmcHD1 mutant cells, H3K9me3 blocks are lost and H3K27me3 is redistributed, which results in a derepression of X-linked genes predominantly in MEFs. Thereby the authors provide further evidence for SmcHD1 as an important maintenance factor of X-inactivation and that this is achieved through its function as a modulator of heterochromatin.

Major comments:

1. page 5 introduction and Figure 1: "Many of the derepressed genes on the Xi in SmcHD1-deficient MEFs were repressed with enrichment of H3K27me3 in SmcHD1- deficient EpiSCs, suggesting that genes derepressed on the Xi at later developmental stages acquired H3K27me3 at an early phase of XCI but lost it later on in SmcHD1- mutant embryos."
This statement is based on cell lines, making it hard from it to infer what happened in embryos in vivo, from which they have been derived from. In their own paper (Sakakibara et al., Development 2018) for example, around 20% of epiblast cells in SmcHD1-mutant E7.5 embryos, which is a very close state to E6.5, from which the authors have derived EpiSCs, showed derepression of Atrx, while in figure 1, no derepression of Atrx is observed in EpiSCs. The epigenetic and gene silencing state could therefore have changed during establishment and prolonged culture of these cell lines, especially in the case of EpiSCs. The authors should therefore tone down this statement or provide evidence that what has been observed in MEFs and EpiSCs is reflecting accurately the epigenetic/gene silencing state in embryonic cells of the equivalent stages in vivo.
(Response)
We agree that the statement pointed out was too strong given the fact that our study was based on cell lines. We have deleted the statement in Introduction pointed out (page5) and have modified the similar statement in Discussion and toned down (page 15).

2. As outlined in more detail in minor comments 4+5 below, conclusions on the differences between Xi heterochromatin between EpiSCs and MEFs are drawn from immunostainings, which are difficult to judge without proper quantifications. The authors should attempt to quantify and compare those differences in order to be able to make a more solid statement.
(Response)
As described in response to comments 3, 5, 12 and 15 by reviewer 1, we have performed quantitative analyses of immunofluorescence images and now think that the results provided in new figures (Fig. 3C, 3D, 4B, and 4D) support our conclusions.

3. page 11-12 and Figure 5B: Similar to the immunostainings, also the ChIP-Seq experiments are mostly analyzed in a descriptive fashion based on the screen shot in figure 5B. It would aid the reader greatly and make the conclusions less subjective, if the effects of SmcHD1 deficiency on H3K27me3 and H3K9me3 blocks and their overlap were quantified. For example, what fraction of the X chromosome is covered by H3K27me3 and H3K9me3 in each lane (Xi vs Xa, EpiSCs vs MEF, wt vs SmcHD1 mutant), and to which degree are H3K27me3 and H3K9me3 mutually exclusive or overlapping? A quantitative comparison would make the conclusions drawn from the results more solid and easier to follow.
(Response)
We have carried out some quantitative analyses for ChIP-seq data. The occupancies of the respective histone modifications were quantified according to the extent to which the enrichment of H3K9me3 and H3K27me3 over the genome-wide average of wild-type MEFs was consecutively spread along the chromosome. Fig. 6B illustrates the differences in the occupancies of either modification alone or both on the Xi between wild-type and SmcHD1 mutant cells of EpiSCs and MEFs. The coverages of H3K9me3 and H3K27me3 on the Xi was shown in comparison with those on the Xa and autosomes in EpiSCs and MEFs in Fig S7A and S7B. Fig. S6B displays an overlay of H3K9me3 enrichment on the Xa and Xi in the respective cell-type. We believe that all these quantitative measurements of the enrichment of H3K9me3 and H3K27me3 have indeed made the results more solid and the arguments much clearer.

4. The authors should discuss their findings in light of papers, which describe the impact of SmcHD1 and Polycomb on X chromosome compartment structure (Wang et al. Cell 2018, Jansz et al. NSMB 2018, Wang et al. Nature Comm 2019 and Gdula et al. Nature Comm 2019, Bauer et al., Nature Comm 2021). As chromosome compartments and chromatin modification blocks for H3K27me3 and H3K9me3 are functionally interconnected, a discussion of compartment structure and how this might relate to the chromatin blocks observed in the current study could aid in the interpretation of the results and put it in context of the literature.

(Response)
We have made some discussion of how the new findings in this study can be put in the current view of the SmcHD1-mediated regulation of histone modifications and higher order chromatin structure based on the previous studies (Wang et al. Cell 2018, Jansz et al. NSMB 2018, Wang et al. Nature Comm 2019 and Gdula et al. Nature Comm 2019) especially focusing on the findings that that H3K27me3 and H3K9me3 enrichment on the Xi in EpiSCs and MEFs show striking correlation with S1/S2 compartments reported by Wang et al. (2018). Although the arguments based on Hi-C data and subsequent PCA during reprogramming process of iPSC could have been included for discussing the potential impact of H3K9me3 on the regulation and kinetics of compartmentalization of the Xi, we decided not to extend the discussion to that far without time course data of H3K9me3 enrichment and Hi-C in differentiating ESCs. Accordingly, we incorporated the data reported by four of the five previous studies the reviewer suggested into our discussion on page 16 and 17.

Minor comments:

1. The authors should describe in the methods section, how EpiSC differentiation for the results in Figure 1D was performed.
(Response)
We thank the reviewer for pointing out that we did not describe the method for EpiSC differentiation. We have included it in Materials and methods.

2. page 18: The cited paper on the threshold to define escapee genes (Peeters et al., 2014) is missing from the References list. Please add it.
(Response)
Thanks again for pointing out this missing reference. We have added it in Reference.

3. Figure 3D: It is difficult to see the H3K9me3 signal only in a H3K9me3/Xist merge image. The individual channels for H3K9me3 and Xist should be shown.
(Response)
We have replaced the images as suggested and have moved the original Figure 3C and 3D to Supplemental data as Fig. S3.

4. Figure 3E/F: The statement from page 9 that the staining of H3K9me3 often appeared to spread in a larger area than DAPI-dense heterochromatin in EpiSCs (Figure 3E and 3F) and that this differs from the pattern in MEFs, is very subjective and hard to judge by purely looking at the images. It would strengthen the argument, if this could be somehow quantified, for example by measuring the overlap of H3K9me3 and DAPI dense regions and comparing the overlap between EpiSCs and MEFs.
(Response)
As mentioned in response to comment 12 by Reviewer 1, we have carried out quantitative analysis to show the difference in distribution of H3K9me3 in the nucleus between EpiSCs and MEFs. We took advantage of an antibody against Lamin B1 in combination with that against H3K9me3 for immunostaining and quantitated fluorescence of H3K9me3 overlapping with Lamin B1 in total fluorescence of H3K9me3 in the nucleus. Newly added Figure 3D demonstrates that there are significant differences in the proportion of H3K9me3 at the nuclear periphery between EpiSCs and MEFs regardless SmcHD1 deficiency.

5. Figure 4A/B: Similar to the statement above about H3K9me3 differences, also here it is difficult to see the differences by eye for Lrif1 on the Xi between EpiSCs and MEFs, especially as very few nuclei are shown. In order to make the statement on page 10 that Lirf1 localization is different on the Xi between EpiSCs and MEFs a quantification of the Lirf1signal on the H3K27me3-marked Xi-territory should be made and compared between MEFs and EpiSCs.
(Response)
As mentioned in response to comment 15 by Reviewer 1, we have carried out quantitative analysis to show differences in the occupancy of Lrif1 in the H3K27me3 domain representing the Xi between EpiSCs and MEFs. The occupancy of SmcHD1 was also analyzed in the same way. This analysis demonstrated that Lrif1 occupied rather limited region on the Xi in EpiSCs as compared to MEFs regardless the presence or absence of SmcHD1. There was also slight but significant differences in the occupancy of SmcHD1 between EpiSCs and MEFs as well. These results, which have been

presented as new Figure 4B and 4D, suggest some differences in the chromatin state of the Xi between EpiSCs and MEFs.

6. Figure 5a: Statistical comparisons should be performed for the ChIP-QPCR experiments.
(Response)
Statistical significance between wild-type and mutant has been included in new Fig. 5.

Reviewer #3 (Significance (Required)):

This paper builds upon previous studies by the group itself (Sakakibara, Development 2018) and several others, which elucidated the role of SmcHD1 as a factor important for X-inactivation maintenance. This study focuses predominantly on SmcHD1's function as a modulator of H3K9me3 and H3K27me3 chromatin blocks on the inactive mouse X. While these blocks have been mostly described on the human X, accumulating evidence now also has shown that they exist on the mouse X chromosome. This study provides evidence that SmcHD1 is important to maintain these chromatin blocks and that in its absence the chromatin separation changes, leading to reactivation of X-linked genes. This study therefore adds to our knowledge on different types of heterochromatin present on the inactive X and their potential function for maintenance of gene silencing.

The study appears technically sound, but would benefit from a more quantitative approach in data analysis, which would make the interpretation of the results more solid (major points 2+3). Also the discussion could place the study better in context with the existing literature on SmcHD1 and X-chromosome structure/epigenetics.
The study would be mostly of interest specifically to the X-inactivation field and overall to the epigenetics/chromatin community.

Keywords for my field of expertise: X-inactivation, stem cells, developmental biology.

Referee Cross-commenting

Looking at the comments of the other reviewers (in particular reviewer 1), it matches my own impression that the study is lacking quantitative and statistical data analysis in many parts (Immunofluorescence images, ChIP-Seq data), which weakens the confidence in the main claims of the study. I would therefore believe that it would be a minimum requirement to provide such improved analysis, in order for the study to be considered for publication. Furthermore an integration of published datasets or at a minimum a better discussion of their own data in light of the existing literature would enhance the study greatly to put it in context with what is already known about SmcHD1, X-inactivation and chromatin. This could be performed without additional major wet lab- experiment in a relatively short timeframe (3 months).
(Response)
We thank these constructive comments and suggestions. We have carried out quantitative analyses for images of immunostaining and ChIP-seq as detailed above, which we think fulfill the minimum requirements suggested to be provided and have strengthened our interpretation of the data. We also reanalyzed and utilized published dataset of Colognori et al. (2020) and Wang et al. (2018), which are incorporated as new Figure 7, for evaluating the importance of our findings in the context of what is known about SmcHD1, XCI, and chromatin.

Resubmission

First decision letter

MS ID#: DEVELOP/2022/200864

MS TITLE: SmcHD1 underlies the formation of H3K9me3 blocks on the inactive X chromosome in mice

AUTHORS: Saya Ichihara, Koji Nagao, Takehisa Sakaguchi, Chikashi Obuse, and Takashi Sado

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. In addition, I must say that quality of some immuno-fluorescence images is not good enough. They should be replaced by more appropriate ones. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript entitled "SmcHD1 underlies the formation of H3K9me3 blocks on the inactive X chromosome in mice" by Ichihara et al. investigates the function of SmcHD1 for regulating chromatin modification of the inactive X in epiblast stem cells and embryonic fibroblasts from female mice.
The authors observe several X-linked genes that are derepressed in SmcHD1 mutant MEFs remain repressed and H3K27me3 modified on Xi in EpiSCs. This is shown by allele specific RNAseq and by RNA FISH of Atrx in SmcHD1 mutant EpiSCs.
RT-PCR analysis of Pdha1, G6pd, and Hprt additionally demonstrates that some X-linked genes that are repressed become reactivated upon entry of differentiation of SmcHD1 mutant EpiSCs. This is a very nice result which is illustrating the prevailing idea that initiation of XCI is largely unaffected by SmcHD1 mutation.
The mai observation is of a loss of H3K9me3 from the Xi in SmcHD1 mutant EpiSCs. This is a novel finding that is of general interest for Xi heterochromatin and maintenance. However, the amount of H3K9me3 on Xi and the mechanistic relevance remain unclear. The discussion suggests a correlation between S1/S2 compartments on Xi and H3K9/K27me3 which is also very interesting but is not further explored.
Mechanistically the authors propose that a loss of H3K9me3 in SmcHD1 mutant cells maybe affected the distribution and amount of H3K27me3 (and H2AK119ub) on Xi. This would make a nice explanation but without removal of H3K9me3 specifically remains speculative.

In conclusion, the study makes several interesting observations that further clarify the role of SmcHD1 in XCI.

Comments for the author

A number of points below limit the mechanistic advance and might be further explored by the authors.

Specific points:
1. The relevance of H3K9me3 in XCI in mice remains undefined - is an active mechanism engaged to establish or is it a consequence of transcriptional silencing and lack of acetylation on Xi? The authors note that there is no correlation with H3K9me3 at gene promoters on Xi and derepression upon loss of SmcHD1 (page 13 last sentence). See also point 4 below. This would make the report of H3K9me3 changes in SmcHD1 mutant cells an interesting observation, which nonetheless could be of a minor importance for the mechanism.
2. Page 17 last paragraph: Substantial loss of H4K9me3 in SmcHD1 mutant EpiSCs on the Xi could become enriched as a consequence of DNA methylation. Is there regional overlap between DNA methylation and H3K9me3 enrichment?
3. Page13, first paragraph: H3K9me3 and H3K27me3 overlap on Xi - this would suggest that these are not automatically mutually exclusive. Hence, the loss of H3K9me3 would not necessarily explain why H3K27me3 expands in SmcHD1 mutant EpiSCs. H3K9me3 does apparently not block H3K27me3 in the double-modified regions.
4. It is not clear why immunofluorescence identified H3K27me3 on the Xi in EpiSCs clearly but H3K9me3 appears barely enriched (page 9 last paragraph). The antibody detects H3K9me3 on the pericentric heterochromatin. This would suggest that there is actually very little H3K9me3 on the Xi even if the ChIP would suggest extensive blocks - maybe only in a low percentage of cells where the ChIP enrichment comes from.

Minor points:
a) The authors perform allele resolved analysis using the Mus musculus molossinus polymorphisms. It is unclear why mm9 has been chosen as the reference genome assembly. GRCm39 is the current, and GRCm28 would correspond to mm10. mm9 appears
outdated and might be a questionable choice as it might contain a higher number of assembly errors on top of a less complete annotation.
b) The in text citations are strangely formatted in some places within the text. This should be corrected, eg page 6 start of first paragraph.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed some of the prior comments. I still have several concerns outlined below and believe that addressing them would be useful. The most interesting finding in the manuscript is that much of H3K9me3 enrichment on the inactive X chromosome is dependent on SMCHD1, but how this regulation occurs is less clear.

Comments for the author

Specific points:

In Figure 2, it is important to display the expression level of genes on the Xa in MEFs and EpiSCs for the genes commonly derepressed (62), derepressed in EpiSCs only or in MEFs only (2B). This will show if the 139 genes specifically derepressed in MEFs are only highly expressed at this state of development or in this cell type. Moreover, the Xi ratio should be shown for the 139 genes derepressed specifically in MEFs, for both ESCs and MEFs Without this information it is not clear if these genes were initially repressed on the Xi. In my opinion displaying the data as in 2C does not make it straight forward to assess this problem, and it does not differentiate the Xi and Xa. Importantly, Figure 2C appears to imply already that those genes that appear repressed specifically

repressed in MEFs and not EpiSCs are more highly expressed in MEFs than EpiSCs (but it would be nice to see these data in absolute and not relative terms for the Xa and Xi).

The data in Figure 2D imply that these genes are not completely repressed in EpiSCs – thus it is not clear how solid the argument of initial inactivation in the absence of SMCHD1 really is. Therefore, the concept of derepression later in development is a unclear.

Figure 3B should show the levels in the nucleus outside of the Xi. It is unclear if the levels are generally higher in the absence of SMCHD1 or specifically on the Xi. Moreover, the global levels of H3K27me3 in Figure S2 seem to change, albeit in different directions in MEFs and EpiSCs, raising the question if the data of this assay are reproducible. Thus, in my opinion, it is difficult to conclude there are no global changes based on these data.

The H3K9me3 images in Figure S3 are of low quality and should be replaced. It also remains unclear if H3K9me3 is weaker in the wt Xi than in the chromatin around it.

It is difficult to see this in Figure 3C: “While immunofluorescence signals produced by the antibody in MEFs were discrete and located more interiorly in the nucleus, forming several distinct chromocenters, they were distributed at the periphery of the nucleus in EpiSCs (Fig. 3C, D).” Are these 3D images and the measurements based on projections? Overall, it is unclear how this finding fits the story line of studies of the Xi.

In Figure 4C it is difficult to appreciate the LRIF enrichment on the Xi. It would be important to add zoom-in pictures of several Xi’s for LRIF for better display. The differential staining pattern between MEFs and EpiSCs

FOR LRIF AND SMCHD1 is also not very clear based on the images provided.

What is the role of LRIF in mediating H3K9me3 on the mouse Xi?

Figure 6A legend – typo “Enrichments or depletions of the respective histone modifications over or blow”

Fig 7. Since the main point of this figure is to argue that H3K9me3 correlates with S2, the PC1 tracks should be directly below the ChIP tracks from this study. Additionally, a pairwise scatterplot would be nice to see as well as the pearson correlation for at least some of the key comparisons. Can you really argue that H3K9me3 correlates with S2, or is it that H3K9me3 is anticorrelated with H3K27me3, and H3K27me3 is correlated with the S1 compartment, since this is the stronger correlation. Would this be a meaningful distinction?

Fig S2 replicates and statistical analysis of this data would be preferred

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First revision

Author response to reviewers' comments

We would like to thank the reviewers for their critical comments. We have attended all of them and the response to them are shown in blue as you see below. Since some figures are included in this rebuttal letter, we would like the reviewers to see the PDF version of Rebuttal letter.

Reviewer 1 Advance Summary and Potential Significance to Field:
The manuscript entitled "SmcHD1 underlies the formation of H3K9me3 blocks on the inactive X chromosome in mice" by Ichihara et al. investigates the function of SmcHD1 for regulating chromatin modification of the inactive X in epiblast stem cells and embryonic fibroblasts from female mice. The authors observe several X-linked genes that are derepressed in SmcHD1 mutant MEFs remain repressed and H3K27me3 modified on Xi in

EpiSCs. This is shown by allele specific RNAseq and by RNA FISH of Atrx in SmcHD1 mutant EpiSCs. RT-PCR analysis of Pdha1, G6pd, and Hprt additionally demonstrates that some X-linked genes that are repressed become reactivated upon entry of differentiation of SmcHD1 mutant EpiSCs. This is a very nice result which is illustrating the prevailing idea that initiation of XCI is largely unaffected by SmcHD1 mutation. The main observation is of a loss of H3K9me3 from the Xi in SmcHD1 mutant EpiSCs. This is a novel finding that is of general interest for Xi heterochromatin and maintenance. However, the amount of H3K9me3 on Xi and the mechanistic relevance remain unclear. The discussion suggests a correlation between S1/S2 compartments on Xi and H3K9/K27me3 which is also very interesting but is not further explored. Mechanistically the authors propose that a loss of H3K9me3 in SmcHD1 mutant cells maybe affected the distribution and amount of H3K27me3 (and H2AK119ub) on Xi. This would make a nice explanation but without removal of H3K9me3 specifically remains speculative. In conclusion, the study makes several interesting observations that further clarify the role of SmcHD1 in XCI.

Reviewer 1 Comments for the Author:
A number of points below limit the mechanistic advance and might be further explored by the authors.

Specific points:
1. The relevance of H3K9me3 in XCI in mice remains undefined - is an active mechanism engaged to establish or is it a consequence of transcriptional silencing and lack of acetylation on Xi? The authors note that there is no correlation with H3K9me3 at gene promoters on Xi and derepression upon loss of SmcHD1 (page 13 last sentence). See also point 4 below. This would make the report of H3K9me3 changes in SmcHD1 mutant cells an interesting observation, which nonetheless could be of a minor importance for the mechanism.
RESPONSE
We have to admit that the relevance of H3K9me3 in XCI in mice still remains undefined. However, given that H3K9me3 has been extensively lost in EpiSCs, in which the majority of genes on the Xi are silenced, we speculate that the loss of H3K9me3 is not a result of derepression. We will try to address functional relevance of H3K9me3 in the maintenance of XCI in the future study anyway. Nonetheless, we believe that this study should make the community recognize the functional importance of H3K9me3 for the maintenance of XCI in mice and facilitate further investigation.

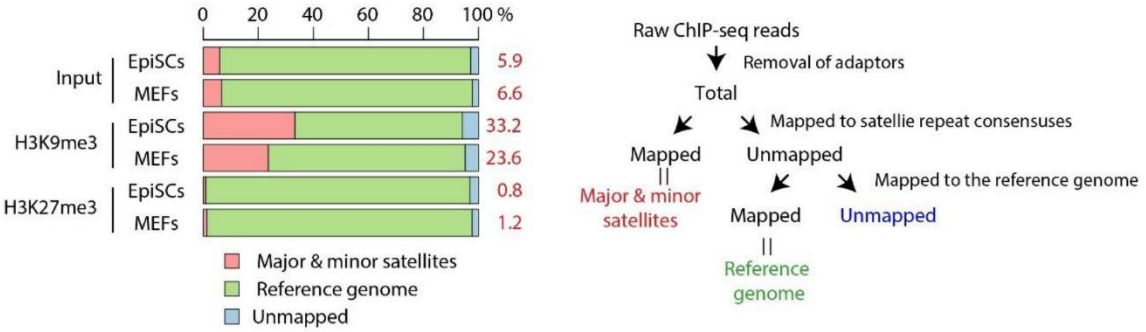
2. Page 17 last paragraph: Substantial loss of H4K9me3 in SmcHD1 mutant EpiSCs on the Xi could become enriched as a consequence of DNA methylation. Is there regional overlap between DNA methylation and H3K9me3 enrichment?
RESPONSE
Regional overlap between DNA methylation and H3K9me3 enrichment in EpiSCs cannot be addressed at the moment due to the lack of information about distribution of DNA methylation on the Xi in EpiSCs. We agree, however, that it would be of interest to explore the possible relationship between H3K9me3 and DNA methylation in the establishment of the X- inactivated state that can be stably maintained in the future experiments.

3. Page13, first paragraph: H3K9me3 and H3K27me3 overlap on Xi - this would suggest that these are not automatically mutually exclusive. Hence, the loss of H3K9me3 would not necessarily explain why H3K27me3 expands in SmcHD1 mutant EpiSCs. H3K9me3 does apparently not block H3K27me3 in the double-modified regions.
RESPONSE
We agree that the loss of H3K9me3 would not necessarily explain why H3K27me3 expands in SmcHD1 mutant EpiSCs and we might have made rather strong expression about this one possibility for description in Results. We have deleted not only the relevant sentence in the paragraph pointed out but also similar description in the last part of Results on page 13.

4. It is not clear why immunofluorescence identified H3K27me3 on the Xi in EpiSCs clearly but H3K9me3 appears barely enriched (page 9 last paragraph). The antibody detects H3K9me3 on the pericentric heterochromatin. This would suggest that there is actually very little H3K9me3 on the Xi even if the ChIP would suggest extensive blocks
- maybe only in a low percentage of cells where the ChIP enrichment comes from.
RESPONSE

To evaluate the fraction of the centromeric heterochromatin in ChIP-seq, we mapped the ChIP-seq reads of wild-type EpiSCs and MEFs to the major and minor satellite consensus sequences (GSAT_MM and SATMIN in Repbase, respectively) as shown in Rebuttal Figure 1. The satellite repeats were found in about 5.9 % and 6.6% of the Input reads in EpiSCs and MEFs, respectively; this is consistent with the previous estimates that the major and minor satellites comprise about 5.5% and 0.2~0.4% of the mouse genome, respectively (Hastie 1989).As shown in here, major and minor satellites occupies as much as 33.2% and 23.6% of the reads recovered by ChIP using an anti-H3K9me3 antibody in EpiSCs and MEFs, respectively. We speculate that the presence of this huge amount of H3K9me3 blocks at the centromeric and pericentromeric heterochromatin in mice might be one reason why we fail to detect H3K9me3 enrichment on the Xi but detect it at pericentromeric heterochromatin. Overwhelming fluorescence detected at centromeric and pericentromeric heterochromatin could compromise the resolution in differentiating enrichment of H3K9me3 between two regions in the nucleus exhibiting much lower fluorescence than centromeric and pericentromeric heterochromatin due to a reduction in the signal-noise ratio.

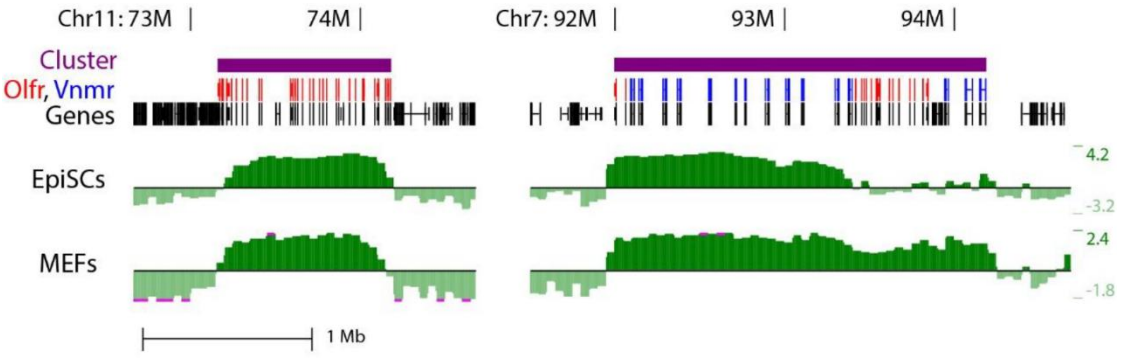
Hastie, N. D. Highly repeated DNA families in the genome of Mus Musculus. Genetic Variants and Strains of the Laboratory Mouse (eds Lyon, M. F. & Searle, A. G.) 559-573 (Oxford Univ. Press, Oxford, 1989).



Rebuttal Figure 1

In addition, we think the possibility that ChIP enrichment comes from only in a low percentage of cells is less likely. Rebuttal Figure 2 below shows gene cluster of vomeronasal (Vnmr) and olfactory (Olfr) receptors, which are known as facultative H3K9me3 domain on autosomes (Magklara et al., 2011). We compared enrichment levels of H3K9me3 in our wild- type EpiSCs and MEFs at the Olfr/Vnmr clusters with those on the Xi using the same y-scale as Fig. 6A. Enrichment levels of H3K9me3 at Olfr/Vnmr clusters were comparable on the Xi. Given that these regions are likely to be enriched with H3K9me3 in the majority of the cell population, it is unlikely that the enrichment of H3K9me3 seen on the Xi represents only a small percentage of the population.

Magklara, A., Yen, A., Colquitt, B. M., Clowney, E. J., Allen, W., Markenscoff-Papadimitriou, E., et al. (2011). An epigenetic signature for monoallelic olfactory receptor expression. Cell, 145(4), 555-570. http://doi.org/10.1016/j.cell.2011.03.040



Rebuttal Figure 2

Minor points:

a) The authors perform allele resolved analysis using the Mus musculus molossinus polymorphisms. It is unclear why mm9 has been chosen as the reference genome assembly. GRCm39 is the current, and GRCm28 would correspond to mm10. mm9 appears outdated and might be a questionable choice as it might contain a higher number of assembly errors on top of a less complete annotation.
RESPONSE
Since much of the NGS data so far reported in previous studies of XCI are processed using mm9 as a reference genome, we have also been making use of mm9, so that we can directly compare the data obtained in our hands with those reported previously. In fact, the use of mm9 allowed us to show that H3K27me3 and H3K9me3 blocks correlates well with S1 and S2 compartments, respectively, by direct comparison between our ChIP-seq data and Hi-C data reported by the Lee lab. In addition, a series of SNPs and indels set we identified in the JF1 (Mus m. molossinus) genome in our hands is also based on mm9 and has been extensively used for the analyses of B6/JF hybrid in the either case of the JF1-derived X being unanimously active (Sakata et al., 2017) or unanimously inactive (Sakakibara et al., 2018). Evaluation of the data in these studies is valuable and used in this study as well.

b) The in text citations are strangely formatted in some places within the text. This should be corrected, eg page 6 start of first paragraph.
RESPONSE
We apologize this mess, which has been amended.

Reviewer 2 Advance Summary and Potential Significance to Field:
The authors have addressed some of the prior comments. I still have several concerns outlined below and believe that addressing them would be useful. The most interesting finding in the manuscript is that much of H3K9me3 enrichment on the inactive X chromosome is dependent on SMCHD1, but how this regulation occurs is less clear.

Reviewer 2 Comments for the Author:
Specific points:
In Figure 2, it is important to display the expression level of genes on the Xa in MEFs and EpiSCs for the genes commonly derepressed (62), derepressed in EpiSCs only or in MEFs only (2B). This will show if the 139 genes specifically derepressed in MEFs are only highly expressed at this state of development or in this cell type. Moreover, the Xi ratio should be shown for the 139 genes derepressed specifically in MEFs, for both ESCs and MEFs. Without this information it is not clear if these genes were initially repressed on the Xi. In my opinion displaying the data as in 2C does not make it straight forward to assess this problem, and it does not differentiate the Xi and Xa. Importantly, Figure 2C appears to imply already that those genes that appear repressed specifically repressed in MEFs and not EpiSCs are more highly expressed in MEFs than EpiSCs (but it would be nice to see these data in absolute and not relative terms for the Xa and Xi).
RESPONSE
In response to reviewer's suggestion, we have replaced a box plot in Fig 2C to new ones, which show expression levels of derepressed genes in MEFs, EpiSCs, and both on the active X in ESCs, EpiSCs, and MEFs in the wild-type background. All 354 genes except for two in those derepressed in neither EpiSCs nor MEFs were expressed in any of ESCs, EpiSCs, and MEFs (FPKM > 0). This indicates that most of the genes derepressed in MEFs are not a kind of those that become upregulated as cells differentiate for the first time, but those that had been expressed on the Xa but repressed on the Xi in EpiSCs. We hope new Fig. 2C eliminates the concern raised by the reviewer and satisfies him/her with the idea that genes that have been inactivated on the Xi in EpiSCs were derepressed in MEFs in the absence of SmcHD1. As reviewer says, there is a trend that genes derepressed only in MEF are more highly expressed in MEFs than EpiSCs, and we have mentioned this possibility in the text (page 8). As to the Xi ratio for 139 derepressed genes specifically in MEFs had been shown in Fig. 2D in the previous round of review.

The data in Figure 2D imply that these genes are not completely repressed in EpiSCs – thus it is not clear how solid the argument of initial inactivation in the absence of SMCHD1 really is. Therefore, the concept of derepression later in development is a unclear.

RESPONSE
RNA-seq analysis is highly sensitive to reveal that genes on the inactive X are not necessarily completely silenced and their transcripts, although at much lower levels than those produced by their counterparts on the active X, are sometimes detected. Accordingly, to evaluate whether such minor expression should be referred to as being barely repressed or escaping inactivation, a threshold has been set at 10% in previous studies. Although Xi probability (%Xi) of 139 genes was significantly higher in mutant EpiSCs than wild-type EpiSCs and therefore, silencing seemed to be affected to some extent in the former, the fact that their %Xi was lower than the threshold still indicates that genes on the Xi was suppressed, at least, as low as 1/9 of the expression of the active copy on the active X. Their %Xi in MEFs, on the other hand, was far beyond the threshold in mutant MEFs, suggesting that Xi silencing in mutant MEFs was less effective than that in EpiSCs. Although there may be a slight trend that differential expression between EpiSCs and MEFs could contribute to derepression in MEF as we mentioned in the text (page 7), it would be hard to assume that many of the 139 genes on the Xi in MEFs were not derepressed following inactivation early on but simply upregulated on the Xi as cells differentiate.

Figure 3B should show the levels in the nucleus outside of the Xi. It is unclear if the levels are generally higher in the absence of SMCHD1 or specifically on the Xi. Moreover, the global levels of H3K27me3 in Figure S2 seem to change, albeit in different directions in MEFs and EpiSCs, raising the question if the data of this assay are reproducible. Thus, in my opinion, it is difficult to conclude there are no global changes based on these data. RESPONSE
As described in Materials and methods, the intensity of immunofluorescence produced by respective antibodies on the Xi was normalized as follows: (area of a selected domain produced by either antibody x mean fluorescence intensity of selected domain) - (area of the selected domain x mean fluorescence intensity of the nucleoplasm surrounding the selected domain). This should eliminate the concern raised here. We agree the images provided in Fig. S2 was not convincing enough, especially, due to a lack uniform signal in a band of pan H3 and have been replaced with now ones in new Fig. S2.

The H3K9me3 images in Figure S3 are of low quality and should be replaced. It also remains unclear if H3K9me3 is weaker in the wt Xi than in the chromatin around it.
RESPONSE
We have replaced Fig. S3.

It is difficult to see this in Figure 3C: “While immunofluorescence signals produced by the antibody in MEFs were discrete and located more interiorly in the nucleus, forming several distinct chromocenters, they were distributed at the periphery of the nucleus in EpiSCs (Fig. 3C, D).” Are these 3D images and the measurements based on projections? Overall, it is unclear how this finding fits the story line of studies of the Xi.
RESPONSE
In response to the similar criticism in the previous round of review, we have quantitatively analyzed the overlap of immunofluorescence signals produced by antibodies against H3K9me3 and LaminB1, which is a marker for the nuclear lamina. The resultant data, which is less subjective than the description based on the figure images, support our conclusion. As mentioned in the Materials and methods, the intensity of immunofluorescence on single plane images obtained by confocal microscopy was measured. We think that this finding reflects the difference in the chromatin states between EpiSCs and MEFs and supports the idea that chromatin state of EpiSCs is in a transitional state between epiblast and differentiated cells.

In Figure 4C it is difficult to appreciate the LRIF enrichment on the Xi. It would be important to add zoom-in pictures of several Xi’s for LRIF for better display. The differential staining pattern between MEFs and EpiSCs FOR LRIF AND SMCHD1 is also not very clear based on the images provided.
RESPONSE
The merged image shown in Fig. 4C demonstrates that the immunofluorescence signal for Lrif1 (green) occupies only a part of the H3K27me3 domain (magenta) in wild-type EpiSCs but essentially entire region of the H3K27me3 domain in wild-type MEFs, which we think is evident as an appearance of a white signal produced by an overlay of green for Lrif1 and magenta for H3K27me3. It was, however, pointed out in the previous round of review that such description could not be properly evaluated without a quantitative analysis. Since our quantitative analysis substantiates the

significance of differential staining pattern of Lrif1 and SmcHD1 between EpiSCs and MEFs, which would be less subjective than showing the relevant images, we think that our conclusion should be accepted. In response to reviewer's suggestion, however, we have also provided some blowups of the Xi images in Fig. S4B.

What is the role of LRIF in mediating H3K9me3 on the mouse Xi?
RESPONSE
This is currently unknown and we would like to address this issue in the future study.

Figure 6A legend – typo "Enrichments or depletions of the respective histone modifications over or blow"
RESPONSE
We thank the reviewer for pointing out this typo, which has been amended.

Fig 7. Since the main point of this figure is to argue that H3K9me3 correlates with S2, the PC1 tracks should be directly below the ChIP tracks from this study. Additionally, a pairwise scatterplot would be nice to see as well as the pearson correlation for at least some of the key comparisons. Can you really argue that H3K9me3 correlates with S2, or is it that H3K9me3 is anticorrelated with H3K27me3, and H3K27me3 is correlated with the S1 compartment, since this is the stronger correlation. Would this be a meaningful distinction?
RESPONSE
We agree that the PC1 track should be directly below the ChIP-seq track and have amended accordingly. We also included a pairwise scatterplot as additional Supplemental Fig. S8. The correlation or anticorrelation issue pointed out here is hard to differentiate and we cannot say if this is meaningful or not. Nonetheless, the data should provide valuable information.

Fig S2 replicates and statistical analysis of this data would be preferred
RESPONSE
We have provided new Fig. S2.

Second decision letter

MS ID#: DEVELOP/2022/200864

MS TITLE: SmcHD1 underlies the formation of H3K9me3 blocks on the inactive X chromosome in mice

AUTHORS: Saya Ichihara, Koji Nagao, Takehisa Sakaguchi, Chikashi Obuse, and Takashi Sado

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the Reviewer 2 is not necessarily positive to accept the manuscript as it is. I went through the manuscript with my colleague and came across a few concerns.In this manuscript, Although we found this study presents several novel findings such as the control of H3K9me3 accumulation and the following H3K27me3 accumulation at several X-linked genes on the Xi by Smchd1, there are a few points that are not consistent with previous studies, which need to be explained in the manuscript.

1. The authors argue that the loss of H3K9me3 from the Xi does not seem to be directly involved in derepression of Smchd1-dependent X-linked genes on the Xi. Instead, the loss of H3K27me3 from these genes is likely to be involved in the silencing of these genes. However, the loss of H3K27me3 could be a consequence of the expression of these genes by other causes. This relates to the point 2 raised below.
2. Terry Magnuson's group previously demonstrated that a X-linked GFP transgene is maintained as silent state in the epiblast of ΔEed (ΔPRC2) post implantation embryos (Wang et al, 2001,

Kalantry and Magnuson, 2006). How the findings in this study reconcile with these previous papers? This point should be more discussed in the manuscript.
3. It is not clear that the Smchd1-dependent genes found in this study lack the silencing of initiation of random XCI and are being kept as expressed from the beginning or that they are once silenced in the absence of Smchd1 then reactivated at later stages of development.

We thus recommend further revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The revised version of the manuscript entitled "SmcHD1 underlies the formation of H3K9me3 bliocks on the inactive X chromosome in mice" by Ichihara et al. contains additional data and clarifications of the text that have further improved the study. In particular, the authors have provided a reasonable explanation as to why it might be more difficult to observe H3K9me3 on the Xi in mouse cells by immunofluorescence experiments. Indeed, the expanded pericentromeric hetercochromatin might be blinding to a less concentrated area of H3K9me3 and the authors approach by ChIP is certainly consistent with this notion. Therefore the study confirms a role of H3K9me3 in XCI and implicates SmcHD1 in this process. Although the relevance remains to be fully explored the data will be of high interest to the community working in X chromosome inactivation and chromatin biology.

Comments for the author

The revision has addressed my earlier concerns in a satisfactory manner and can now be considered for publication.

Reviewer 2

Advance summary and potential significance to field

The author study the role of SMCHD1 in gene expression regulation and histone mark levels on the Xi

Comments for the author

it seems the authors addressed some of the concerns - I wish they had done more to look into how"derepressed" the genes on the Xi in MEFs really are.

Second revision

Author response to reviewers' comments

We would like to thank you and reviewers for some additional input for improving the manuscript. We have responded to all the concerns raised in the previous round of review as you see blow. Please find PDF version of the rebuttal letter uploaded as supplemental data.

As you will see, the Reviewer 2 is not necessarily positive to accept the manuscript as it is. I went through the manuscript with my colleague and came across a few concerns in this manuscript. Although we found this study presents several novel findings such as the control of H3K9me3 accumulation and the following H3K27me3 accumulation at several X-linked genes on the Xi by Smchd1, there are a few points that are not consistent with previous studies, which need to be explained in the manuscript.

1. The authors argue that the loss of H3K9me3 from the Xi does not seem to be directly involved in derepression of Smchd1-dependent X-linked genes on the Xi. Instead, the loss of H3K27me3 from these genes is likely to be involved in the silencing of these genes. However, the loss of H3K27me3 could be a consequence of the expression of these genes by other causes. This relates to the point 2 raised below.
2. Terry Magnuson's group previously demonstrated that a X-linked GFP transgene is maintained as silent state in the epiblast of ΔEed (ΔPRC2) post implantation embryos (Wang et al, 2001, Kalantry and Magnuson, 2006). How the findings in this study reconcile with these previous papers? This point should be more discussed in the manuscript.

RESPONSE

We understand these concerns raised in comment 1 and 2. We agree that loss of H3K27me3 would not be an only reason for depression of X-inactivated genes. Since our arguments were essentially confined to the role of H3K27me3 for stable silencing of X-inactivated genes as you and your colleague pointed out, we have mentioned other modifications such as DNA methylation and H2AK119ub about their possibly partially redundant function in stable maintenance of X-inactivated state in Discussion.

3. It is not clear that the Smchd1-dependent genes found in this study lack the silencing of initiation of random XCI and are being kept as expressed from the beginning or that they are once silenced in the absence of Smchd1 then reactivated at later stages of development.

RESPONSE

Although we could not differentiate whether genes expressed on the Xi in SmcHD1-deficient EpiSCs were derepressed following inactivation early on or failed to be inactivated from the beginning as we described in the text, this does not affect the discussion and conclusion of the study and therefore, we believe that this can be left behind.

Reviewer 2 Comments for the Author:
it seems the authors addressed some of the concerns - I wish they had done more to look into how"derepressed" the genes on the Xi in MEFs really are.

RESPONSE

Although reviewer 2 might have not be satisfied enough our revision as we could not address the reason why SmcHD1-deficiency causes extensive loss of H3K9me3, we believe that it is still important to show the involvement of SmcHD1 in establishment of H3K9me3 blocks on the Xi and the lack of such H3K9me3 blocks likely compromises stable silencing of the X-inactivated genes on the Xi. We agree that it would be important to elucidate how SmcHD1 mediates the proper formation of H3K9me3 blocks on the Xi and stable silencing of the Xi, but these would be something we should address in the future study.

Third decision letter

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ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Development through Review Commons.

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.