H4K20me3 methyltransferase SUV420H2 shapes the chromatin landscape of pluripotent embryonic stem cells

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AUTHORS: Jiji T Kurup, Zhijun Han, Wenfei Jin, and Benjamin L Kidder

I have now received all the referees' reports on the above manuscript. 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.

In particular, Reviewer 1 raises concerns on the analysis of the chromatin conformation datasets and requests a more thorough description of the analyses performed, and in some instances, additional analysis to clarify and fully substantiate the conclusions drawn.If you are able to revise the manuscript along the lines suggested by the 2 Reviewers in full, I will be happy receive a revised version of the manuscript.

Your revised paper will be re-reviewed by 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.

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

H4K20me3 methyltransferase SUV420H2 shapes the chromatin landscape of pluripotent embryonic stem cells by Kurup et al.

This manuscript examines the role of H4K20me3 and the associated methyltransferase SUV420H2 in mediating changes in gene expression and chromatin organisation in embryonic stem cells (ESC). To do so, the authors perform comparisons of gene expression changes between wild-type (WT) mouse ESC, SUV420H2 knock-out (KO) and double SUV420H1/2 KO (DKO). These results suggest that the loss of H4K20me leads to a general disruption of the WT gene expression programme, delayed ESC differentiation to embryoid bodies and upregulation of specific families of repetitive elements, such as LTR/ERVs. The authors then perform Hi-C to examine changes in chromatin conformation in SUV420H2 KO cells, which leads to a potential alteration of the three-dimensional chromatin organisation in these cells, including the disruption of compartments and insulation and changes in local and distal chromatin interactions.

Overall the biological question examined in this manuscript is of interest. However, I have significant concerns regarding the analytical approach used to analyse and interpret the data. In particular the analysis of the chromatin conformation datasets is very unclear, and therefore, without a significant thorough re-analysis of the data it is difficult to determine whether these support the author's conclusions.

Comments for the author

In particular, I have the following major points:

1. I cannot find details in the methods regarding the calculation of the 'differentiation index' (Fig 1J). Without these details and with the data presented in Fig 1G, and the PCA plot in Fig 1I, I am unsure as to how the authors conclude that there is a developmental delay in the KO samples.

2. The manuscript does not contain information about the technical properties of the Hi-C data, such as the number of total reads per experiment (number of replicates), number of valid total pairs, etc. Without this information, it is very difficult to determine whether the samples are comparable. For example, the plots included in Fig 3A suggest that the Suv420h2 sample is technically significantly different from the WT ESC sample, and therefore any downstream comparisons might be artefactual.

3. Compartment analysis is significantly affected by the resolution of the Hi-C data (ie, sequencing depth and proportion of total reads that end up as valid pairs). Without information about the comparative resolution in both datasets, it is not possible to assess whether the changes in compartmentalisation presented in Fig 3J are meaningful. The authors should provide full details regarding the level of resolution of both datasets and resample these to the same resolution in case these wouldn't be comparable. The authors should also produce saddle plots, to examine global changes in compartmentalisation as in Flyamer et al. Nature 2017.

4. The analysis of changes in domain boundaries (Fig 4A) is also unclear, especially since the TAD calls presented in Fig 3A (lower panel) do not seem to be particularly robust (the yellow triangles depicting domains seem to not necessarily correlate with the visual perception of where those domains are present). A more robust determination of these domains is necessary for any interpretation of changes in domain boundaries. The manuscript will also benefit from aggregate TAD analysis to compare changes in TADs across the entire dataset.

5. A similar comment is relevant for loop calls. Loops were detected using the Juicer implementation of the HiCCUPS algorithm, but there is no evidence as to whether the loop calls are robust in these datasets. In addition, the authors could include aggregate loop analysis to compare overall changes in loop strength across samples.

6. I find it difficult to interpret the insulation score plotted in Fig 4F. The region depicted in the plot is 10MB in length, but the insulation score has a negative value throughout the entire region in SUV420H2 cells. How do the authors explain this negative value of the insulation score in such a long region? Negative insulation regions normally correspond to regions of high-level of insulation (e.g., TAD boundaries). Does the plot mean that there is a TAD boundary of 20MB? These results are difficult to understand given that the usual TAD size in mouse/human is ~0.5-1MB. These changes in insulation are also difficult to relate to those presented in Fig 4G, which includes regions of ~100kb.

7. Figure 4, 5 and 6 present analysis for data looking directly at paired-end tags (PETs) (please define the acronym in the text). However, the robustness of this analysis is unclear, especially in cases such as Fig 4G where a minimal number of PETs give support for the contacts compared between the samples. If the authors are interested in finding differentially contacting regions, they could perform this analysis using specialised tools such as diffHiC or HOMER, among others.

8. Direct comparisons of PETs are anyway likely to result in technical artefacts since contact data need to be normalised for biases including sequencing depth, chromatin accessibility, etc. This is of particular relevance in this work, since the authors show that removal of H4K20me3 is likely to result in significant changes in gene expression and therefore chromatin accessibility. Hence, I am unsure about the robustness of the reported results arising from comparisons based on the calculation of HCI (Fig 5), HCCI (Fig 5) or OCI (Fig 6) values and the validity of these results.

Reviewer 2

Advance summary and potential significance to field

In this body of work, Kurup et al describe the global loss of a critical methyltransferase in the precise maintenance of heterochromatin regions of pluripotent stem cells. Briefly, the authors demonstrate that upon loss of either SUV420H2 or both SUV420H1 and H2, ES cells exhibit an altered cellular morphology and delayed activation of differentiation transcriptional signatures. Loss of these enzymes resulted in a subsequent loss of its methylation mark, H4K20me3, and de-repression of retrotransposon elements that have previously been shown to be silenced by this epigenetic mark. Perhaps the most striking result of this work is the global reorganization of chromatin landscape and the dramatic switch in A/B compartments.
The authors note an increased number of loops and domains, albeit smaller in size, as well as an increase in local contacts and insulation patterns, perhaps explained by the general decondensation of these regions that allows for ectopic interactions. These results suggest that H4K20me3 normally acts to precisely regulate regions of heterochromatin that prevent any spurious interactions from forming.

Comments for the author

Overall, these results confirm previous findings and also add to our current understanding of the role of a heterochromatin associated mark in proactively regulating the chromatin landscape to maintain lineage identity. Despite the lack of functional evidence to validate these results, this work is acceptable for publication in Development, with some minor comments.

Some minor comments:
1. Given the global alteration in contact loops and domains shown in Fig 2A and B, a correlation with transcriptional changes of genes underlying the changed TADs would be interesting.
2. In Fig 4F, the authors show an example of decreased insulation scores at a genomic locus correlating with enriched CTCF occupancy. A more global analysis of this correlation between TAD boundaries demarcated by CTCF and regions of gained and lost insulation in the mutants may provide some insight about the regions that are susceptible to these alterations and the mechanism by which these domains are formed.
Is CTCF occupancy increased at the new TAD boundaries?

3. Minor spelling errors should be checked throughout the manuscript (eg: “connectivity” instead of “connectively”)

First revision

Author response to reviewers' comments

Response: DEVELOP/2020/188516 - Version 2 (Kurup et al. "H4K20me3 methyltransferase SUV420H2 shapes the chromatin landscape of pluripotent embryonic stem cells")

The authors appreciate the reviewer’s insightful comments that were used to improve the overall quality of the revised manuscript. All changes to our revised manuscript are labeled in RED. The reviewer’s comments are listed below in italics followed by the author’s response in bold.

Reviewer reports:

Reviewer 1

Reviewer 1 Advance Summary and Potential Significance to Field:

H4K20me3 methyltransferase SUV420H2 shapes the chromatin landscape of pluripotent embryonic stem cells by Kurup et al. This manuscript examines the role of H4K20me3 and the associated methyltransferase SUV420H2 in mediating changes in gene expression and chromatin organisation in embryonic stem cells (ESC). To do so, the authors perform comparisons of gene expression changes between wild-type (WT) mouse ESC, SUV420H2 knock- out (KO) and double SUV420H1/2 KO (DKO). These results suggest that the loss of H4K20me leads to a general disruption of the WT gene expression programme, delayed ESC differentiation to embryoid bodies and upregulation of specific families of repetitive elements, such as LTR/ERVs. The authors then perform Hi-C to examine changes in chromatin conformation in SUV420H2 KO cells, which leads to a potential alteration of the three-dimensional chromatin organisation in these cells, including the disruption of compartments and insulation and changes in local and distal chromatin interactions.

Overall the biological question examined in this manuscript is of interest. However, I have significant concerns regarding the analytical approach used to analyse and interpret the data. In particular the analysis of the chromatin conformation datasets is very unclear, and therefore, without a significant thorough re-analysis of the data it is difficult to determine whether these support the author’s conclusions.

In particular, I have the following major points:

1. I cannot find details in the methods regarding the calculation of the ‘differentiation index’ (Fig 1J). Without these details and with the data presented in Fig 1G, and the PCA plot in Fig 1I, I am unsure as to how the authors conclude that there is a developmental delay in the KO samples.

Response: We thank the reviewer for the comments. We have carefully addressed the reviewer’s concerns and comments below. We have included a description of the differentiation index (DI) calculation in the updated manuscript in the methods section as follows:

“Calculation of the differentiation index: We quantified the relative ratio of RNA-Seq expression (RPKM) of genes associated with the GO terms ‘development’ or ‘differentiation’, and whose expression was upregulated >1.5 fold in WT EBs (d8 or d12) relative to WT ES cells, in differentiated EBs relative to undifferentiated ES cells, which we have termed the

differentiation index (DI).”

Regarding Figure 1G, while WT EBs (d8 and d12) exhibit decreased expression of the pluripotency marker, Nanog, Suv420h2 KO and Suv420h1/h2 DKO EBs (day 8 and day 12) have elevated expression of Nanog. Moreover, expression of the differentiation marker, COL1A1, is elevated in WT EBs (d8 and d12) relative to Suv420h2 KO and Suv420h1/h2 DKO EBs (d8, d12). Combined, these findings suggest delayed differentiation of Suv420h2 KO and Suv420h1/h2 DKO EBs relative to WT EBs.

Regarding the PCA analyses, the PCA plot shows an altered trajectory of Suv420h2 KO and Suv420h1/h2 DKO EBs through the 3D space relative to WT EBs. Because Suv420h2 KO and Suv420h DKO EBs progress through the 3D space at an altered trajectory, we have removed the text related to delayed differentiation and focused on the altered trajectory of differentiation. The manuscript has been modified to reflect these changes.

“Principal component analysis (PCA) showed that SUV420H2 KO and SUV420H DKO ES cells and EBs exhibit an altered trajectory through the 3D space relative to WT ES cells and EBs (Figure 1I)”.

2. The manuscript does not contain information about the technical properties of the Hi-C data, such as the number of total reads per experiment (number of replicates), number of valid total pairs, etc. Without this information, it is very difficult to determine whether the samples are comparable. For example, the plots included in Fig 3A suggest that the Suv420h2 sample is technically significantly different from the WT ESC sample, and therefore any downstream comparisons might be artefactual.

Response: We have included a supplemental table (Table S1) which describes the technical properties and resolutions of the Hi-C datasets, including replicates and related information. Regarding Figure 3A, most plaid pattern plots for each chromosome exhibited similar trends between WT and SUV420H2 KO ES cells when viewed at the whole chromosome level (Figure 3A), but some visible differences were evident at higher resolution up to 5k for specific regions. In the updated manuscript we have provided coverage normalized plaid plots for all chromosomes between WT and SUV420H2 KO ES cells (Figure S5). Combined, these findings demonstrate that the technical properties of the WT and SUV420H2 KO ESC Hi-C datasets are similar.

3. Compartment analysis is significantly affected by the resolution of the Hi-C data (ie, sequencing depth and proportion of total reads that end up as valid pairs). Without information about the comparative resolution in both datasets, it is not possible to assess whether the changes in compartmentalisation presented in Fig 3J are meaningful. The authors should provide full details regarding the level of resolution of both datasets and resample these to the same resolution in case these wouldn’t be comparable. The authors should also produce saddle plots, to examine global changes in compartmentalisation as in Flyamer et al. Nature 2017.

Response: We have provided information regarding the resolution of the Hi-C data as a response to the previous comment #2. We have provided a supplemental table (Table S1) which describes the technical properties of the Hi-C datasets including replicates and library resolutions.

Regarding compartment analysis, for A/B compartment analysis using Juicer, the KR normalized eigenvector was used to determine compartments (see methods). We have performed additional analyses to evaluate global changes in compartmentalization. To this end, we generated saddle plots which revealed variation in B-B compartment strength between WT and SUV420H2 KO ES cells, where B-B compartment strength was somewhat decreased in SUV420H2 KO ES cells. Results from these new analyses have been integrated into the modified manuscript as follows:

“To explore the relationship between decreased H4K20me3 and alterations in chromatin accessibility in SUV420H2 KO ES cells, we quantitated and visualized compartment strength by plotting interaction frequencies along the first eigenvector (PC1) to generate

compartmentalization saddle plots (Figure 5A), which have been described previously(Flyamer et al., 2017). The upper left quadrant represents B-B interactions while the lower right quadrant represents A-A interactions. These results reveal variation in strength of interactions as a function of the compartment vector between WT and SUV420H2 KO ES cells, where strength of B-B interactions was somewhat decreased in SUV420H2 KO ES cells. To further compare variation in compartment strength we generated a saddle plot by calculating the ratio of interaction frequencies as a function of the compartment vector between SUV420H2 KO to WT ES cells (Figure 5B). These results further highlight alterations in B-B interactions in SUV420H2 KO ES cells relative to WT ES cells."

Moreover, we have also utilized normalized and resampled datasets to the same resolution for diffHic analyses. Differential interactions between WT and SUV420H2 KO ES cells were evaluated using the Bioconductor diffHic R package (Lun and Smyth, 2015). Paired- end reads were mapped to the mm9 genome and resolution of WT and SUV420H2 KO ES cell datasets were matched by re-sampling to the same resolution. Please see response to comment #7 for further details regarding the diffHic analyses.

4. The analysis of changes in domain boundaries (Fig 4A) is also unclear, especially since the TAD calls presented in Fig 3A (lower panel) do not seem to be particularly robust (the yellow triangles depicting domains seem to not necessarily correlate with the visual perception of where those domains are present). A more robust determination of these domains is necessary for any interpretation of changes in domain boundaries. The manuscript will also benefit from aggregate TAD analysis to compare changes in TADs across the entire dataset.

Response: Regarding domain identification in our dataset, we used juicer to identify domains through Arrowhead transformation (see methods; Rao et al, 2014), and loops were called through the HICCUPS algorithm. Algorithms to detect domains or TADs exhibit variability between tools in number and size of domains (Dali et al, 2017), where detection of TAD boundaries is more reliable than assembly into composite TAD structures. We have updated Figure 3A with a representative view of robust differential domain boundaries between WT and SUV420H2 KO ES cells. In addition, we have performed aggregate domain analysis to compare changes in domains across the entire dataset. Results from aggregate domain analysis of all domains revealed similar patterning between WT and SUV420H2 KO ES cells. The domain patterns are consistent with robust domain calling using the juicer arrowhead transformation method. Moreover, overall similar patterning of domains between WT and SUV420H2 KO ES cells is consistent with results shown in Figure 3D, which demonstrate that most domains overlap between WT and SUV420H2 KO ES cells (Figure 3D). We also performed additional analyses to further evaluate differentiation domains between WT and SUV420H2 KO ES cells. We used TADCompare to identify domains with differential boundaries, and subsequently performed aggregate domain analysis using coolpuppy. Results from these analyses demonstrate that while domain strengths are overall similar between WT and SUV420H2 KO ES cells, a subset of domains are enriched in WT or SUV420H2 KO ES cells. Results from these new analyses can be found in the updated manuscript as follows:

"We also performed aggregate pile-up analysis of domains in WT and SUV420H2 KO ES cells using coolpuppy (Flyamer et al., 2020), which is based on a variation of this aggregate domain analysis approach (Flyamer et al., 2017) (Figure 3F, left). These results revealed overall consistent domain strengths between WT and SUV420H2 ES cell datasets. We used TADCompare to identify differential domain boundaries of Juicer Arrowhead called domains between WT and SUV420H2 KO ES cells, and subsequently performed pile-up domain analysis using coolpuppy. Using this approach, we observed increased domain strength at SUV420H2 KO ES cell-enriched domains (Figure 3F, middle) and minor decreases in domain strength at WT ES cell-enriched domains in SUV420H2 KO ES cells (Figure 3F, right). These findings are consistent with results described in Figure 3D, which demonstrate that while most contact domains overlap between WT and SUV420H2 KO ES cells, a subset of domains are enriched in WT or SUV420H2 KO ES cells."

5. A similar comment is relevant for loop calls. Loops were detected using the Juicer implementation of the HiCCUPS algorithm, but there is no evidence as to whether the loop calls are robust in these datasets. In addition, the authors could include aggregate loop analysis to

compare overall changes in loop strength across samples.

Response: Loop domains were identified using the Juicer (Durand et al., 2016b) implementation of the HiCCUPS algorithm (Rao et al., 2014). We performed aggregate loop analyses to compare overall changes in loops between WT and SUV420H2 KO ES cells. Aggregate loop analysis for all loops showed similar loop strengths between WT and SUV420H2 KO ES cells. To evaluate loop strength at differential loops we first identified differential loops using juicer HiCCUPSDiff. We then used coolpuppy software to evaluate loop strengths at WT-enriched loops and SUV420H2 KO ES cell-enriched loops. These results demonstrate robust and distinct loop enrichment at differential loop regions.
Results from these new analyses have been included in the updated manuscript as follows:

"To evaluate loop strengths between WT and SUV420H2 KO ES cells, we performed aggregate pile-up analysis of loops using coolpuppy (Flyamer et al., 2017; Flyamer et al., 2020), which revealed consistent loop strengths for all loops in WT and SUV420H2 KO ES cells (Figure 3I, left). We also performed pile-up analysis of differential loop domains identified between WT and SUV420H2 KO ES cells using juicer HiCCUPSDiff. These results highlight increased loop strength of WT-enriched loops in WT ES cells (Figure 3I, middle) and SUV420H2 KO ES cell-enriched loops in SUV420H2 KO ES cells (Figure 3I, right). These results demonstrate that while overall loop strengths are consistent between WT and SUV420H2 KO ES cells, a subset of loops are enriched in WT or SUV420H2 KO ES cells."

6. I find it difficult to interpret the insulation score plotted in Fig 4F. The region depicted in the plot is 10MB in length, but the insulation score has a negative value throughout the entire region in SUV420H2 cells. How do the authors explain this negative value of the insulation score in such a long region? Negative insulation regions normally correspond to regions of high-level of insulation (e.g., TAD boundaries). Does the plot mean that there is a TAD boundary of 20MB? These results are difficult to understand given that the usual TAD size in mouse/human is ~0.5- 1MB. These changes in insulation are also difficult to relate to those presented in Fig 4G, which includes regions of ~100kb.

Response: We appreciate this reviewer for inquiring about the interpretation of lengthy regions of negative insulation scores. While Figure 4F showed a ~10 MB region with a flipped insulation score in SUV420H2 KO ES cells relative to control cells, the length of most regions of positive or negative insulation scores is shorter than 10 MB. The average length of regions with positive or negative insulation scores in WT ES cells is 389 kb and 359 kb, respectively, while the average length of regions with positive or negative insulation scores in SUV420H2 KO ES cells is 342 kb and 315 kb, respectively. Moreover, the length of the longest regions with positive insulation scores in WT and SUV420H2 KO ES cells is 11.5 MB and 5.7 MB, respectively, while the length of the longest regions with negative insulation scores in WT and SUV420H2 KO ES cells is 7.4 MB and 9.4 MB, respectively. It is possible that extended regions with negative insulation scores could be heterochromatin regions, which have relatively homogenous features. To avoid confusion related to interpretation of lengthy regions with positive or negative insulation scores, the example browser view of the region with flipped insulation scores in SUV420H2 KO ES cells in Figure 5 has been replaced with a representative view of a region of typical length. Because lengthy regions with negative or positive insulation scores may represent outliers, we have focused on changes in insulation scores at regions of typical length.

We have included a summary of these findings in the updated manuscript as follows:

"We also evaluated the length of regions with positive or negative insulation scores in WT and SUV420H2 KO ES cells. Results from these analyses show that the average length of regions with positive insultation scores in WT and SUV420H2 KO ES cells is 389 kb and 342 kb, respectively, while the average length of regions with negative insultation scores in WT and SUV420H2 KO ES cells is 359 kb and 315 kb, respectively (p=6.3e-6) (Figure S7A- B)."

7. Figure 4, 5 and 6 present analysis for data looking directly at paired-end tags (PETs) (please define the acronym in the text). However, the robustness of this analysis is unclear, especially in cases such as Fig 4G where a minimal number of PETs give support for the contacts compared

between the samples. If the authors are interested in finding differentially contacting regions, they could perform this analysis using specialised tools such as diffHiC or HOMER, among others.

Response: We have defined the PET acronym in the updated manuscript. Moreover, we agree with the importance of utilizing a robust method to evaluate differential contact regions. Therefore, we have removed aspects of Figure 4 related to direct evaluation of PETs, and we have replaced Figure 5 with results from diffHic analyses. By performing additional analyses using diffHic, which detects differential chromatin interactions between genomic anchor points, on re-sampled Hi-C datasets to the same resolution, we identified 6564 differential anchor points consisting of 3235 regions with decreased chromatin interactions, and 3322 regions with increased chromatin interactions. Findings from these new analyses have been included in the modified manuscript as follows:

"To study the changes in chromatin interactions between WT and SUV420H2 ES cells we used the Bioconductor diffHic R package (See methods) (Lun and Smyth, 2015), which uses the generalized linear model (GLM) functionality of edgeR(Robinson et al., 2009), and performs normalization for library-specific biases. Paired-end reads aligned to the mm9 reference genome were processed into interaction counts in bin pairs, which were filtered for low abundance and normalized for library-specific biases. Counts for bin pairs were subsequently modeled using edgeR (Lun and Smyth, 2015; Robinson et al., 2009). Using this approach, we identified 6,564 differential anchor points, where anchor points are defined as genomic regions where chromatin interactions are present(Lun and Smyth, 2015), including 3,235 differential anchor points that exhibited decreased interactions, and 3,323 differential anchor points which exhibited increased interactions in SUV420H2 KO ES cells relative to WT ES cells (Figure 4A-B). We also found that 100% of differential anchor points with increased interactions overlap H4K20me3 ChIP-enriched peaks, while 80% of differential anchor points with decreased interactions overlap H4K20me3 peaks (Figure 4C), further suggesting that depletion of SUV420H2 and loss of H4K20me3 is associated with dysregulated chromatin interactions. We next sought to investigate whether loss of H4K20me3 in SUV420H2 KO ES cells is associated with differential anchor points. A comparison of H4K20me3 levels at differential anchor points in WT and SUV420H2 KO ES cells demonstrated that H4K20me3 levels decrease at differentiation anchor points with increased or decreased chromatin interactions (Figure 4D). We also found that several GO terms of differentially expressed genes between WT and SUV420H2 KO ES cells were strongly enriched around differential anchor points (within 10 kb). DAVID GO annotation of upregulated or downregulated genes located around differential anchor points in SUV420H2 KO ES cells were found to be associated with GO terms such as development, differentiation, and morphogenesis (Figure 4E). A representative region of differential chromatin interactions and decreased H4K20me3 in SUV420H2 KO ES cells is shown in Figure 4F."

8. Direct comparisons of PETs are anyway likely to result in technical artefacts since contact data need to be normalised for biases including sequencing depth, chromatin accessibility, etc. This is of particular relevance in this work, since the authors show that removal of H4K20me3 is likely to result in significant changes in gene expression and therefore chromatin accessibility. Hence, I am unsure about the robustness of the reported results arising from comparisons based on the calculation of HCI (Fig 5), HCCI (Fig 5) or OCI (Fig 6) values and the validity of these results.

Response: Regarding comparison of PETs between samples, we have removed analyses utilizing a direct comparison of PET counts between samples in Figures 4 and 5. While the HCI and HCCI calculation is conceptually similar to the OCI, we have removed analyses related to the HCI and HCCI and focused on OCI-related analyses. The open chromatin index (OCI) calculation has been used to calculate the frequency of distal and intrachromosomal interactions (external) to local (internal) contacts (Chandra et al., 2015). Seqmonk software was used to calculate the OCI (see methods), which normalizes for bias across the chromosome.

Reviewer 2

Reviewer 2 Advance Summary and Potential Significance to Field:

In this body of work, Kurup et al describe the global loss of a critical methyltransferase in the precise maintenance of heterochromatin regions of pluripotent stem cells. Briefly, the authors demonstrate that upon loss of either SUV420H2 or both SUV420H1 and H2, ES cells exhibit an altered cellular morphology and delayed activation of differentiation transcriptional signatures. Loss of these enzymes resulted in a subsequent loss of its methylation mark, H4K20me3, and de-repression of retrotransposon elements that have previously been shown to be silenced by this epigenetic mark. Perhaps the most striking result of this work is the global reorganization of chromatin landscape and the dramatic switch in A/B compartments. The authors note an increased number of loops and domains, albeit smaller in size, as well as an increase in local contacts and insulation patterns, perhaps explained by the general decondensation of these regions that allows for ectopic interactions. These results suggest that H4K20me3 normally acts to precisely regulate regions of heterochromatin that prevent any spurious interactions from forming.

Reviewer 2 Comments for the Author:

Overall, these results confirm previous findings and also add to our current understanding of the role of a heterochromatin associated mark in proactively regulating the chromatin landscape to maintain lineage identity. Despite the lack of functional evidence to validate these results, this work is acceptable for publication in Development, with some minor comments.

Some minor comments:

1. Given the global alteration in contact loops and domains shown in Fig 2A and B, a correlation with transcriptional changes of genes underlying the changed TADs would be interesting.

Response: We thank this reviewer for the constructive comments. We agree that evaluating transcriptional changes that occur nearby domains would be interesting. To this end, we evaluated differential domain analysis of Juicer Arrowhead domains between WT and SUV420H2 KO ES cells using TADCompare. First, we identified differential domain boundaries using TADCompare, and then we identified differential boundaries enriched in WT or SUV420H2 KO ES cells. Second, we evaluated the expression of genes nearby differential domain boundaries (within 10kb). For genes associated with WT ES cell enriched domains, SUV420H2 KO ES cell enriched domains, all WT domains, or all KO domains, boxplots revealed that the expression level of genes that were upregulated in SUV420H2 KO ES cells was lower in WT ES cells relative to genes that were downregulated in SUV420H2 KO ES cells. These findings suggest that alterations in domain boundaries is associated with dysregulated gene expression in SUV420H2 KO ES cells. GO annotation of genes associated with altered domain boundaries using DAVID and GoSemSim revealed enrichment of multiple GO terms including development. Results from these analyses can be found in the updated manuscript:

"Next, we performed differential analysis of Juicer Arrowhead domain boundaries using TADCompare(Cresswell and Dozmorov, 2020) to identify differential domain boundaries between WT and SUV420H2 KO ES cells. We then explored the expression profile of genes nearby differential domain boundaries (within 10 kb) by integrating RNA-Seq data. For genes associated with WT ES cell enriched domains, SUV420H2 KO ES cell enriched domains, or all WT or KO domains, boxplots revealed that the expression level of genes that were upregulated in SUV420H2 KO ES cells was lower in WT ES cells relative to genes that were downregulated in SUV420H2 KO ES cells (Figure S6A). Moreover, to evaluate the biological significance of boundary changes, we annotated GO terms of genes nearby differential boundaries (within 10 kb) using DAVID(Dennis et al., 2003). GO annotation of genes associated with altered domain boundaries revealed enrichment of multiple GO terms including development (Figure S6B). These findings suggest that alterations of domain boundaries are associated with dysregulated gene expression in SUV420H2 KO ES cells."

2. In Fig 4F, the authors show an example of decreased insulation scores at a genomic locus correlating with enriched CTCF occupancy. A more global analysis of this correlation between TAD boundaries demarcated by CTCF and regions of gained and lost insulation in the mutants may

provide some insight about the regions that are susceptible to these alterations and the mechanism by which these domains are formed.
Is CTCF occupancy increased at the new TAD boundaries?

Response: We have performed a global analysis to investigate a correlation between CTCF binding, altered domain boundaries, and gained or lost insulation (flipped insulation scores). Results from these analyses demonstrate that CTCF occupancy is elevated at regions of decreased insulation in SUV420H2 KO ES cells. Moreover, CTCF binding is also elevated at regions of gained domain boundaries with flipped insulation scores relative to lost domain boundaries in SUV420H2 KO ES cells. Findings from these new analyses have been integrated into the manuscript as follows:

"To investigate a correlation between domain boundaries demarcated by CTCF and gained or lost insulation, we evaluated CTCF binding (Chen et al., 2008) at regions with flipped insulation between WT and SUV420H2 KO ES cells. These findings demonstrate that CTCF binding is higher at regions with decreased insulation relative to regions of increased insulation in SUV420H2 KO ES cells (Figure 5K). We also evaluated CTCF occupancy at differential domain boundaries, identified using TADCompare, with gained or lost insulation (flipped insulation scores) between WT and SUV420H2 KO ES cells.
These findings demonstrate that CTCF binding is higher at regions with increased enrichment of domain boundaries relative to regions with decreased enrichment of domain boundaries in SUV420H2 KO ES cells (Figure 5L)."

3. Minor spelling errors should be checked throughout the manuscript (eg: "connectivity" instead of "connectively")

Response: We have corrected the spelling of "connectivity" throughout the manuscript.

Second decision letter

MS ID#: DEVELOP/2020/188516

MS TITLE: H4K20me3 methyltransferase SUV420H2 shapes the chromatin landscape of pluripotent embryonic stem cells

AUTHORS: Jiji T Kurup, Zhijun Han, Wenfei Jin, and Benjamin L Kidder

I have now received all the referees reports on your revised manuscript. The manuscript was re-reviewed by the same reviewers who reviewed the work initially. 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.

You will see that Reviewer 2 is fully satisfied with your responses. While Reviewer 1 remains positive about your work, the reviewer has some remaining concerns and discusses points that he/she feels have left unanswered.

I have gone through all the points myself, as well as through the manuscript and your cover and rebuttal letter. Since I am not in favour of multiple rounds of revisions, and because I think you can address the points by clarifying and discussing additional points, I would like to provisionally accept the manuscript, but at the same time would ask you to please incorporate the remaining Reviewer's 1 main comments in a form of a revised manuscript.

In order to facilitate this process, I have highlighted below, the points that I believe need attention (5 points only, in total). It would be great if you could address those and resubmit a main manuscript document with those few changes incorporated highlighted, so that we can expedite

the final decision. If there is any of the points that you would like to discuss, or you think is inaccurate, please do so in a cover letter.

Points to revise (based on the comments provided by Reviewer 1, which you will find after the email from the Editorial Office, below):

1. Please improve the labelling of the figure 3A or the figure legend and provide clearer explanation of what the domains are and how they were calculated in the figure legend.

2. Please revise conclusion related to Figure 6A, with regards to gene expression changes in WT and KO enriched domains

3. Please provide clarification, related to Fig. 5G-h as to whether the domains analysed are enriched in K20me3 in wt cells, and therefore the changes in insulation do reflect a loss of K20me3 in KO cells.

4. Please insert a paragraph/few lines in the discussion to include the alternative interpretations on point 8 (unless you disagree, in which case I will ask you to write in a couple of sentences in your letter to the editor, the argumentation)

5. Please revise and/or discuss in the text of the paper the point 13 of the reviewer.

I believe that the points above can all be incorporated and addressed by minimal text changes in the manuscript, and therefore it would be great, if I could receive your revised manuscript in the next 2 weeks.

Reviewer 1

Advance summary and potential significance to field

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Comments for the author

The authors have addressed some, but not all the questions that I raised in my first review. I appreciate the inclusion of the necessary details regarding the technical properties of the Hi-C datasets and the additional analyses. Unfortunately, I still have significant concerns regarding the analysis of the Hi-C datasets and the conclusions that the authors draw from these, that I will review in turn. I think these aspects need to be properly evaluated to be able to determine whether SUV420H2 shapes the chromatin landscape of embryonic stem cells.

Major points:

1. I still have doubt regarding the calculation of chromatin domains (e.g., those depicted in Figure 3A bottom panels). Are these all the domains that the authors determine in these regions? Or is this a representation of the “altered” domains?
This is not clear from the labelling of the figure or the figure legend. As I mentioned in my previous review, the calculation of these domains is crucial for the rest of the analysis. However, looking at the panel, I am still unsure that the domains called in these samples are comparable. Overall the two datasets look qualitatively different (e.g., the width of the signal at the diagonal), which would be simple to evaluate looking at a contact decay plot. Therefore, using the same parameters to determine domains might not result in comparable sets. To me this is obvious from the zoom in plots in the bottom panel of Figure 3A since domains not identified by the authors are clearly distinguishable (assuming that the yellow rectangles depict all domains in that region are shown). This suggests that a number of domains are missed, presumably because of the choice of parameters, which then cast doubts on the comparative analysis between domains in both samples (e.g., the comparison of size of domains in Figure 3B). Because a significant amount of the follow up analysis is based on the computation of these domains, and since the robustness of these domains is not examined, I am concerned that some of the subsequent conclusions examining the biological significance of changes in domain structure are affected by technical biases arising from

the parameter choice in determining these. This needs to be properly evaluated in a revised version of this analysis.

2. In Figure 3D the authors show a depletion of H4K20me3 at domain boundaries. Since the authors report a global loss of H4K20me3 (e.g., Figure 5D), is this loss specifically associated to domain boundaries? The authors can test this by a random permutation analysis shifting the position of domain boundaries but maintaining the original separation between domain boundaries.

3. I am afraid that I don't understand the relevance of the analysis presented in Figure S6A and how the authors conclude that "alterations of domains boundaries are associated with dysregulation gene expression". The patterns of gene expression changes are the same in WT enriched domains than in KO enriched domains. Therefore it is not clear to me why the authors conclude that there is a link between the two.
For example, do the majority of changes in gene expression occur at regions that contain KO-specific or WT-specific boundaries? This could be tested through Chi-squared or Fisher's exact tests. In addition, if the authors want to associate changes in domain boundaries with changes in gene expression, to show this conclusively, they should include a zoomed in example of such a change.

4. I find surprising that 100% and 80% of differential anchor points with increased or decreased interactions, respectively, overlap with H4K20me3 (Figure 4C). To me this would be a very significant finding. However, I cannot really see such associations when I look at the data shown in Figure 4F. Some interactions overlap but some don't. In addition, there are a number of H4K20me3 peaks that do not seem to be associated with any changed interactions. It would be useful if the authors would compare the statistical significance of the overlaps by random permutation analysis of the HhK20me3 peaks, for example. This is important because since the authors observe associations with both increased and decreased interactions, this might suggest that the changes in interactions and H4K20me3 are not directly associated with each other.

5. I do not understand why the authors look at changes in the insulation score in Figure 5F rather than evaluating these when comparing changes in domain structure.
Changes in the insulation score would reflect changes in domain structure (eg, the split of a domain into two or more, or the fusion of two domains). From the data shown in Figure 5F, I cannot appreciate regions with significantly altered insulation. Indeed, most of the regions showed in the plot seem to have negative values of the insulation score (representing high insulation or a domain boundary) in both samples. There is a level of scatter in the plot, but I suspect that most of the outliers would be due to regions close to unmappable regions that would bias the calculation of this index. The strongly correlated values of insulation suggest that the domain boundaries are very similar between the samples, which directly contradict the authors' conclusions from Figure 3 of an altered domain structure.

6. Related to point §5, the authors refer to regions of flipped insulation in Figure 5G-H, but it is not clear which regions are included here. Is the effect of loss of H4K20me3 a specific feature of these regions, or is this a genome-wide effect? It would be important to determine this to assess the relevance of this association specifically in these regions.

7. It is also unclear to me why the authors would look at changes in insulation at regions that display loops (Figure 5I). Presumably most of these loops would occur within loop domains, and therefore, I would expect a strong overlap between the data presented in Figure 5F and 5I. Similarly as per my point above, in my opinion, the data presented suggest that insulation remains fairly constant at these regions between the two samples, rather than highlighting the alterations in genome-wide chromatin insulation interpreted by the authors.

8. I do not understand the rationale behind comparing the length of regions with positive or negative insulation scores presented in Figure S7A-B. The insulation score is usually employed to detect local minima of the value, around which domain boundaries are called. The overall sign of the score depends of the parameters used to calculate this and I do not understand the biological meaning of this comparison.
In fact, looking at Figure 5J, it looks like overall the entire insulation score is shifted between WT and H2 KO samples (even areas with positive insulation score).

This strongly argues against a direct effect of the loss of H4K20me3 in changes of insulation at domain boundaries. On the contrary, in my opinion, this either reflects an indirect effect, or technical differences between the datasets (as mentioned in point §1 above).

9. The authors state that “Combined, these results suggest that cis interactions between proximal H4K20me3 interacting regions may serve as a chromatin insulator,
[…]”. I am not sure that I understand the sentence properly, but it is unclear to me which part of the analysis in this manuscript looks at cis interactions between H4K20me3 regions, and how would these then serve as chromatin insulators. The next part of this sentence continues “[…], where loss of SUV420H2 leads to reduced H4K20me3 and altered insulation”. I do agree with the authors on this conclusion, but based on the data presented, I do not see evidence for a direct link with loss of H4K20me3 based on the current analysis.

10. I am still unsure about the conclusions drawn from the OCI analysis in Figure 6.
First, SeqMonk does not normalise the data to take into consideration that the raw Hi-C reads might have different biases. In my opinion, a proper evaluation of these biases is important since a significant difference between the two datasets seems to come from regions with repetitive elements. Second, the authors state that (page 18; first paragraph) “These results demonstrate that regions with increased OCI values in SUV420H2 KO ES cells have lower GC content, further suggesting localized decondensation of chromatin in SUV420H2-depleted ES cells.” I am unsure as to how the lower GC content suggests localised decondensation of chromatin in SUV420H2 ES cells. To me the link with the GC content is indicative of the decondensation of the repetitive elements shown in Figure 2. Finally, it would be important to show in the analysis presented in Figure 6D that the change in H4K20me3 in regions with “flipped” OCI values is specific to these regions for example by permutation analysis. The authors show a comparison against all regions as a control, but I find these results (no change in H4K20me3) difficult to reconcile given the H4K20me3 patterns in Figure 5D.

Minor points:

11. The authors should double-check that the Log2 (DNase I) scale in Figure 6B is correct.

12. I cannot find the details of how “flipped” regions (for the different measures) are calculated.

13. The authors suggest changes nearby pericentromeric regions (Figure 6C). However for some chromosomes, the changes seem to expand up to 40Mb, almost a third of a chromosome. I wonder whether the conclusion of a pericentromeric associated change is then valid.

Reviewer 2

Advance summary and potential significance to field

The authors have satisfactorily addressed my comments.

Comments for the author

The authors have satisfactorily addressed my comments.

Second revision

Author response to reviewers' comments

Response: DEVELOP/2020/188516 - Version 3 (Kurup et al. "H4K20me3 methyltransferase SUV420H2 shapes the chromatin landscape of pluripotent embryonic stem cells")

The authors appreciate the reviewer's comments that were used to improve the quality of the revised manuscript. All changes to our revised manuscript are labeled in BLUE. The reviewer / editorial comments are listed below in italics followed by the author's response in bold.

Editorial Summary: *Points to revise (based on the comments provided by Reviewer 1, which you will find after the email from the Editorial Office, below):*

9. Please improve the labelling of the figure 3A or the figure legend and provide clearer explanation of what the domains are and how they were calculated in the figure legend.

Response: We have updated the labeling of Figure 3A legend and main text to describe the contact domains. Juicer Arrowhead (Durand et al., 2016b) was used to identify chromatin domains. Figure 3A (bottom) shows all contact domains identified in the region shown. This representative region shows a decreased size of a domain in SUV420H2 KO ES cells.

"Juicer Arrowhead (Durand et al., 2016b) was used to identify chromatin domains…. Visualization of Hi-C data using Juicebox (Durand et al., 2016a) showed altered boundaries of a contact domain between WT and SUV420H2 KO ES cells (Figure 3A)."

Figure 3 legend:

(A) Contact matrices showing observed normalized Hi-C signal from WT and SUV420H2 KO ES cells from the entire chromosome 6 at 250 kb resolution (top) and 5kb resolution (bottom) as evaluated using Juicer and Juicebox(Durand et al., 2016a; Durand et al., 2016b). Bottom panels depict contact matrices at 5 kb resolution and all contact domains identified in this region are shown in yellow. Juicer Arrowhead (Durand et al., 2016b) was used to identify chromatin domains. Pixel intensity represents the normalized number of contacts between two loci. The max intensity is shown in each panel.

10. Please revise conclusion related to Figure S6A, with regards to gene expression changes in WT and KO enriched domains.

Response: We have revised the conclusion related to Figure S6A. For genes associated with WT ES cell enriched domains, SUV420H2 KO ES cell enriched domains, or all WT or KO domains, the expression level of genes that were upregulated in SUV420H2 KO ES cells was lower in WT ES cells relative to genes that were downregulated in SUV420H2 KO ES cells. While the overall patterns of gene expression changes are similar between WT and KO enriched domains, we observed differences in GO term enrichment for differentially expressed genes associated with WT or KO domains. We have updated the manuscript to highlight differential enrichment of GO terms associated with altered domain boundaries.

"GO annotation of genes associated with altered domain boundaries revealed enrichment of multiple GO terms including development, gene expression, transport and morphogenesis (Figure S6B). Genes upregulated in SUV420H2 KO ES cells and associated with decreased domain boundary strength were enriched with the GO term 'gene expression' while downregulated genes were enriched with the GO term 'transport'. In addition, genes downregulated in SUV420H2 KO ES cells and associated with increased domain boundary strength were enriched with the GO terms 'development' and 'morphogenesis' (Figure S6B). These findings suggest that alterations of domain boundaries are associated with dysregulated expression of genes enriched with multiple GO terms in SUV420H2 KO ES cells."

11. Please provide clarification, related to Fig. 5G-h as to whether the domains analysed are enriched in K20me3 in wt cells, and therefore the changes in insulation do reflect a loss of K20me3 in KO cells.

Response: The regions analyzed in Figure 5G-H include regions of flipped insulation nearby WT ES cell domain boundaries. Results described in Figure 5G-H demonstrate that H4K20me3 levels are similarly depleted at regions of altered insulation in SUV420H2 KO ES cells relative

to genome-wide levels of H4K20me3 in SUV420H2 KO ES cells. These results also demonstrate that H4K20me3 levels are comparably high in WT ES cells at regions of flipped insulation in SUV420H2 KO ES cells (Figure 5G) relative to genome- wide levels of H4K20me3 in WT ES cells (Figure 2C). Our results also demonstrate that 72% of WT domains were enriched with H4K20me3 at boundary regions in WT ES cells.
Localized changes in insulation nearby regions of depleted H4K20me3 in SUV420H2 KO ES cells may result in a disruption of long-range interactions. We have updated the manuscript with this information as follows:

"H4K20me3 levels were similarly depleted at regions of altered insulation in SUV420H2 KO ES cells relative to genome-wide levels of H4K20me3 in SUV420H2 KO ES cells (Figure 2C). Moreover, 72% of WT domains were enriched with H4K20me3 at boundary regions in WT ES cells. These results demonstrate that depletion of SUV420H2 is associated with dysregulated insulation at a subset of regions with decreased H4K20me3."

12. Please insert a paragraph/few lines in the discussion to include the alternative interpretations on point 8 (unless you disagree, in which case I will ask you to write in a couple of sentences in your letter to the editor, the argumentation)

Response: The rationale for comparing the length of regions with positive or negative insulation scores was in response to a comment regarding a long region with a negative insulation score. To avoid confusion related to interpretation of lengthy regions with positive or negative insulation scores, the example browser view of the region with flipped insulation scores in SUV420H2 KO ES cells in Figure 5 was replaced in our previous revision with a representative view of a region of typical length. We also included Figure S7 to demonstrate the average length of regions with positive or negative insulation scores.
Results from these findings demonstrate that the length of regions with negative and positive insulation scores decrease in SUV420H2 KO ES cells. These findings are consistent with results shown in Figure 3C, which demonstrate an increase in the number of contact domains in SUV420H2 KO ES cells relative to WT ES cells. As the insulation score is calculated from the average of interaction frequencies crossing over it (within a genomic range), where the local minima is used to determine insulation regions and domain boundaries, a decrease in the length of regions with positive/negative insulation scores is consistent with an increased number of domains and insulators. The increased number of insulators and domains may interfere or reduce long range interactions and lead to dysregulated insulation at domain boundaries. We have updated the manuscript with a discussion on these results:

"Results from our analyses demonstrate that the length of regions with negative and positive insulation scores decreases in SUV420H2 KO ES cells (Figure S7). These findings are consistent with results shown in Figure 3C, which demonstrate an increase in the number of contact domains in SUV420H2 KO ES cells relative to WT ES cells. As the insulation score is calculated from the average of interaction frequencies crossing over it (within a genomic range), where the local minima is used to determine insulation regions and domain boundaries(Lajoie et al., 2015), a decrease in the length of regions with positive/negative insulation scores is consistent with an increased number of domains and insulators. The increased number of insulators and domains may interfere or reduce long range interactions and lead to dysregulated insulation at domain boundaries."

13. Please revise and/or discuss in the text of the paper the point 13 of the reviewer.

Response: We have modified the text to reflect that OCI changes occur at both pericentromeric and surrounding regions. The manuscript has been revised as follows:

"Visualization of OCI calculations revealed increased OCI values nearby pericentric regions and surrounding regions for multiple chromosomes in SUV420H2 KO ES cells (Figure 6C), suggesting decreased local interactions resulting from chromatin decondensation and increased long-range contacts."

Third decision letter

MS ID#: DEVELOP/2020/188516

MS TITLE: H4K20me3 methyltransferase SUV420H2 shapes the chromatin landscape of pluripotent embryonic stem cells

AUTHORS: Jiji T Kurup, Zhijun Han, Wenfei Jin, and Benjamin L Kidder
ARTICLE TYPE: Research Article

Thank you for revising your manuscript along the points I highlighted in my last letter. I have gone now through the changes and the manuscript, and I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.