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Histone variant macroH2A confers resistance to nuclear reprogramming

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 January 2011

Thank you very much for submitting your research paper for consideration to The EMBO Journal editorial office. Having received relatively consistent comments from two expert scientists, I am in the position to reach a final decision that should also prevent further unnecessary delays. Both referees appreciate in principle the novelty and general interest of your study on X-inactivation in epiblast stem cells. However, they also indicate that some additional support would be essential before they would finally recommend publication here. Importantly, ref#2 requests macroH2A knockdown in Mef's OR reprogramming of reported Xist-knockouts (that are shown to have reduced macroH2A at Xi). This would strengthen the major conclusion of macroH2A level determining X-inactivation. Conditioned on this crucial point and your attention to other relevant requests as outlined in the fairly detailed referee comments, we would be happy to re-assess a revised paper for potential publication here.

Please be also reminded that it is EMBO_J policy to allow a single round of revisions only and that the final decision entirely depends on the content within the last version of your manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this manuscript, the authors utilized a unique experimental system to investigate epigenetic state of various cell types, especially focusing on the maintenance of the inactive X chromosome (Xi). Interestingly, Xi of epiblast stem cells (EpiSCs) is reactivated upon injection into the germinal vesicles of Xenopus oocyte, suggesting that the establishment of Xi is incomplete in this particular cell type. The authors then examined molecular differences between EpiSCs and other cell types with irreversible Xi, and found that enrichment of macroH2A1 was closely correlated with the stability of the Xi. Together with other supporting evidence, the authors suggested that the combination of chromatin modification specify distinctive epigenetic state of the Xi. Considering growing interests on EpiSCs that show similar characteristics to human ES/iPS cells, this work is exceptionally interesting in that they have successfully revealed the uniqueness of the epigenetic state of this cell type with elegant experimental system developed in their laboratory. However, some of the evidence are fragmented and their conclusions are not fully supported because of a lack of several key experiments, which are detailed below.

Major points:
1. They have concluded that CG-methylation and accumulation of H3K27me3 cannot explain the difference in the stability of the Xi. However, they judge the inactive state of X chromosome solely by the expression of X-linked CMV-EGFP transgene. It is thus theoretically possible, although less likely, that expression of endogenous Xi-linked genes remain suppressed in the transferred EpiSCs even though the exogenous CMV promoter is re-activated. To exclude this possibility, it should be essential to examine the methylation state of the CMV promoter, or to examine bi-allelic expression of X-linked endogenous genes in the EpiSCs after nuclear transfer. The latter would be easily done by allele-specific RT-PCR, or in situ hybridization for the nascent transcripts using probes against intron sequences.
2. They examine the formation of macroH2A1 domain after nuclear transfer using C2C12 cells stably expressing macroH2A1-GFP. Since no data is available for the inactive state of the X chromosome in the transferred C2C12 nuclei, this experiment itself does not support correlation between the enrichment of macroH2A1 and the stability of Xi. To further support their conclusion, expression of X-linked genes should be examined in transferred C2C12 nuclei. Again, this could be done by FISH for nascent transcripts of the endogenous gene.
3. In figure 5, the expression of Xist is upregulated after nuclear transfer, whereas FISH signals for Xist are hardly detectable. It is true that this apparent discrepancy can be explained by the difficulty to detect diffused signals by FISH. However, another possibility is that truncated or aberrantly spliced form of Xist RNA is produced in the transferred nucleus, which cannot be detected by the FISH probes but can be detected by RT-PCR using primers designed against a short 5' region of the Xist RNA. Northern blot analysis would be an ideal method to confirm that full length Xist RNA is produced. At least, multiple primer sets should be used for the RT-PCR analysis. This issue is particularly important since mutant forms of Xist RNA lacking the localizing signals fail to coat Xi, raising an alternative possibility for the mis-locaization of Xist RNA in the transferred nucleus.
4. They have demonstrated that Xist-induced inactivation of PGK-puromycin transgene is not maintained in the transplanted nucleus even after the establishment of irreversible Xi in the differentiated ES cells. As the authors correctly pointed out, only small population of ES cells form macroH2A domain after induction of Xist transgene in differentiating ES cells (Rasmussen, 2001). Since this is such an important data, I would strongly recommend to show the expression of macroH2A1in the ES cells before nuclear transfer, which provides further evidence supporting the correlation between the macroH2A1 domain and stability of the transferred Xi.

Minor points:
1. They examined the formation of macroH2A1 domain using antibody in Figure 7A, whereas they used EGFP-macroH2A1 to detect the same domain in transferred C2C12 nucleus. Is there any particular reason for not using antibody staining? If not, it sounds more natural to check the expression of macroH2A1 in the transferred nuclei prepared from MEF or EpiSCs by immunostaining. This issue should be mentioned at least in the materials and methods section.
2. They used a number of antibodies to detect Xi-related signals, but only two antibodies were specified in the manuscript. Since information of primary and secondary antibodies are extremely important, they should be clearly provided.
3. In figure 7A, they present two columns of macroH2A1 expression in differentiated EpiSCs. The

left column seems to show differentiated EpiSCs without macroH2A1 domain, which is not mentioned in the figure legends nor in the main text. Is there any particular reason for providing these additional images?
4. Page 12, line 7: H2K27me3 marks Xi territory, but not the X-chromosome territory. X-chromosome territories can only be visualized by chromosome painting probes.
5. Page 15, line 15: "100% of MEF nuclei" should be "100% of C2C12 nuclei".
6. Page 18, line 18: SATB1 is not required for the chromosomal localization of Xist RNA. Rather, SATB1 expression induces dispersed Xist RNA signals in lymphocyte (Figure 3G, H in Agrelo et al., 2009). Discussion should be modified.

Referee #2:

Gurdon and colleagues test the stability of the somatically silent X chromosome in female differentiated cells of the mouse upon transfer into xenopus oocytes. Intriguingly, an X-linked GFP transgene reactivated when epiblast stem cells were transferred, but not when embryonic fibroblasts or trophoblast stem cells were transplanted. In an attempt to understand the cause of the difference in reactivation capacity, the authors define the chromatin state of the inactive X in MEFs and EpiSCs and find that H3K27 methylation and DNA methylation do not differ, but that macroH2A is enriched on the inactive x in MEFs but not EpiSCs, suggesting that the lack of macro H2A corresponds to the improved reprogramming capabilities of EpiSCs. While these data are interesting, it is not clear whether macoH2A indeed affects reprogramming of the XI. Many other marks of the inactive X were not tested (or are still unknown), thus, it is not clear whether macroH2A enrichment is the key event in X-inactivation that explains the differences in X reactivation. To test this idea, a knockdown of macroH2A should be performed in MEFs, or Xist knockout MEFs (Xist 2lox/Ko mefs published by the Jaenisch lab) be reprogrammed that lack macroH2A enrichment on the Xi. Before seeing some of these functional experiments I would be hesitant to recommend publication in the EMBO journal.

In addition, the points listed below should be addressed:

1. It remains unclear whether the reactivation of the X in xenopus oocytes relates to the reactivation of the X when the ICM is established. Could the authors please speculate on this?

2. Figures 1 and 2 nicely demonstrate X reactivation in EpiSCs but not MEFs. However, why is GFP from the Xa detected on day 0 where it should be washed out due to permeabilisation as described for Sox2 in the text (for EpiSCs). In any case, do endogenous X-linked genes reactivate, i.e. could Fish or RT-PCR (taking advantage of polymorphisms) be performed to detect expression of genes from the Xi?

3. Xi reactivation appears very efficient, almost to levels of the Xa - which is rather surprising. Do all transplanted cells reactivate or just a few?

4. In Figure 4 it is demonstrated that H3K27methylation is maintained upon SCNT. However, much fewer cells have an Xi enrichment in the case of transplanted MEFs (comparing to MEFS in culture). For transplanted EpiSCs the number is missing (4B). Could the authors please explain this observation - is it just technical in nature that the Xi is difficult to detect upon transplantation? Nevertheless, it is surprising that H3K27 methylation on the X is maintained in the absence of Xist coating (Figures 4 and 5), given that H3K27methylation is Xist dependent on the X in MEFs. The same is true for macroH2A. Therefore I wonder whether cell division is required for loss of K27 methylation/macroH2A but not Xist. Do these cells divide upon transplantation?

5. The tet-inducible Xist experiment in Figure 6 is not well explained and does not help to explain the difference between MEFs and EpiSCs as it is not clear how this tet inducible system relates to these cell states. Thus I did not find the figure very helpful, nor explained sufficiently so that lanes 1-6 can be understood easily.

6. It is not clear why the authors switch to the C2C12 system, for which we don't know whether the Xi reactivates upon SCNT, to image macroH2A. As for H3K27methylation in Figure 4, macroH2A

could have been stained at different time points upon transplantation of MEFs and EpiSCs. Therefore Figure 7B is not very helpful.

1st Revision - Authors' Response 08 April 2011

Referee #1:

In this manuscript, the authors utilized a unique experimental system to investigate epigenetic state of various cell types, especially focusing on the maintenance of the inactive X chromosome (Xi). Interestingly, Xi of epiblast stem cells (EpiSCs) is reactivated upon injection into the germinal vesicles of Xenopus oocyte, suggesting that the establishment of Xi is incomplete in this particular cell type. The authors then examined molecular differences between EpiSCs and other cell types with irreversible Xi, and found that enrichment of macroH2A1 was closely correlated with the stability of the Xi. Together with other supporting evidence, the authors suggested that the combination of chromatin modification specify distinctive epigenetic state of the Xi. Considering growing interests on EpiSCs that show similar characteristics to human ES/iPS cells, this work is exceptionally interesting in that they have successfully revealed the uniqueness of the epigenetic state of this cell type with elegant experimental system developed in their laboratory. However, some of the evidence are fragmented and their conclusions are not fully supported because of a lack of several key experiments, which are detailed below.

We would like to thank the Referee for very well informed and constructive comments that have greatly helped us to improve our manuscript.

Major points:

1. They have concluded that CG-methylation and accumulation of H3K27me3 cannot explain the difference in the stability of the Xi. However, they judge the inactive state of X chromosome solely by the expression of X-linked CMV-EGFP transgene. It is thus theoretically possible, although less likely, that expression of endogenous Xi-linked genes remain suppressed in the transferred EpiSCs even though the exogenous CMV promoter is re-activated. To exclude this possibility, it should be essential to examine the methylation state of the CMV promoter, or to examine bi-allelic expression of X-linked endogenous genes in the EpiSCs after nuclear transfer. The latter would be easily done by allele-specific RT-PCR, or in situ hybridization for the nascent transcripts using probes against intron sequences.

As suggested by the reviewer, we have undertaken allele-specific expression analysis of endogenous X-linked genes in EpiSCs after nuclear transfer. This is based on known single nucleotide polymorphisms between *Mus musculus musculus* and *Mus musculus castaneus* alleles (Huynh & Lee, 2003). We were able to obtain *X-GFP Mus/Cast* embryos from which we derived MEFs. After genotyping individual embryos for sex and transgene transmission, we identified female *X-GFP Mus/Cast* MEFs preparations and used flow cytometry to separate *Xi-GFP Mus/Cast* MEFs from *Xa-GFP Mus/Cast* MEFs. In this way we obtained pure populations of female MEFs with the X chromosome of one species exclusively inactive (*Xi-GFP Mus/Cast*) or active (*Xa-GFP Mus/Cast*). The *Xi-GFP* EpiSCs used previously had already been derived from *X-GFP Mus Musculus* / *Mus castaneus* embryos. As shown in Figure 2C, allele-specific expression of *Rlim* can be carried out due to the presence of a single nucleotide polymorphism generating a HaeIII restriction site in the *Mus musculus*-derived *Rlim* transcript, but not in the ones derived from *Mus castaneus (Huynh & Lee, 2003)*. Hence, allelic origin of RT-PCR products can be distinguished based on restriction enzyme patterns. We carried out this analysis on nuclei transplanted into *Xenopus* oocytes, which did not interfere with the assay (Figure 2C). As shown in Figure 2D, we found that *Rlim* expression is reactivated from the *musculus* (Xi) allele in transplanted EpiSC nuclei but not in transplanted MEF nuclei. Therefore, there is indeed nuclear transfer-mediated activation of an endogenous Xi gene in transplanted EpiSCs.

Regarding DNA methylation, we have now analyzed the DNA methylation state of the *X-GFP* transgene regulatory and coding regions in female *Xi-GFP* EpiSCs, MEFs and TS cells. We found these regions consistently highly methylated in all cell types analysed. In agreement with our previous conclusion, DNA methylation at these regions does not correlate with the irreversible or reversible state of the Xi after nuclear transfer to oocytes, and therefore does not help explain the differences in *X-GFP* reactivation seen between EpiSCs, MEFs and TS cells. We have now included these results in Figure 3. DNA methylation changes after nuclear transfer are too complex to be examined in depth in this manuscript. We will study them in detail as our work progresses.

2. They examine the formation of macroH2A1 domain after nuclear transfer using C2C12 cells stably expressing macroH2A1-GFP. Since no data is available for the inactive state of the X chromosome in the transferred C2C12 nuclei, this experiment itself does not support correlation between the enrichment of macroH2A1 and the stability of Xi. To further support their conclusion, expression of X-linked genes should be examined in transferred C2C12 nuclei. Again, this could be done by FISH for nascent transcripts of the endogenous gene.

We agree with the reviewer, and now show that macroH2A-GFP also remains associated with chromatin of the Xi of transplanted female MEF nuclei (Figure 7B). The reason for our choice to work with C2C12 was several fold. First, macroH2A immunostaining on transplanted nuclei cannot be performed with available macroHA1 antibodies due to the presence of an abundant and unidentified epitope present in the *Xenopus* oocyte GV. We have tried very hard to detect macroH2A localization in transplanted nuclei using various protocols but without success. With the aim of testing macroH2A localization in transplanted nuclei, we have created a reporter macroH2A-GFP cell line. We used the C2C12 system because we failed to generate a stable macroH2A-GFP expressing female MEF cell line. C2C12 seemed a good cell type as this mouse myogenic precursor is likely to have undergone XCI and to possess a fully inactive X chromosome. In addition, a recent high profile paper reported the use of C2C12 cells to study Xi replication timing control (Casas-Delucchi et al, 2011). In accord with the view that XCI is complete in C2C12 cells, macroH2A-GFP indeed localises to the Xi in C2C12 cells (Figure 7C; Supplementary Figure S7A,B,C; Supplementary Movie S1; Supplementary Movie S2). Since we obtained identical results with MEFs and C2C12, we have maintained both sets of results in the manuscript and therefore support our conclusion that mH2A1-GFP remains associated with the chromatin of the Xi in transplanted nuclei (New Figure 7B,C).

3. In figure 5, the expression of Xist is upregulated after nuclear transfer, whereas FISH signals for Xist are hardly detectable. It is true that this apparent discrepancy can be explained by the difficulty to detect diffused signals by FISH. However, another possibility is that truncated or aberrantly spliced form of Xist RNA is produced in the transferred nucleus, which cannot be detected by the FISH probes but can be detected by RT-PCR using primers designed against a short 5' region of the Xist RNA. Northern blot analysis would be an ideal method to confirm that full length Xist RNA is produced. At least, multiple primer sets should be used for the RT-PCR analysis. This issue is particularly important since mutant forms of Xist RNA lacking the localizing signals fail to coat Xi, raising an alternative possibility for the mis-locaization of Xist RNA in the transferred nucleus.

While Northern blot analysis is not feasible in our system due to the low amount of material available, we were able to address *Xist* splicing in nuclei transplanted into *Xenopus* oocytes (Supplementary Figure S4C). Defects in splicing would indeed not be surprising following interspecies nuclear transfer. We assayed splicing of *Xist* between exon 3 and 6, as our RNA FISH probes are located in exon 6. Surprisingly, we found that *Xist* was efficiently spliced from EpiSC and MEF nuclei transplanted into *Xenopus* oocytes, even up to 3 days after nuclear transfer (Supplementary Figure S4C). Moreover, expression of *Xist* cDNA (spliced) from the clone 36 *Xist* inducible cell line was not able to maintain or induce silencing in oocytes. Therefore, defects in *Xist* splicing are not likely to explain *Xist* mislocalization to the Xi in transplanted nuclei. More likely, *Xist* RNA dispersion into the oocyte GV prevents efficient Xi coating. Truncation of *Xist* transcripts is also unlikely given that perfectly intact transcripts are detected between exon 3 and 6 spanning

almost 2 kb of immature *Xist* transcript.

4. They have demonstrated that Xist-induced inactivation of PGK-puromycin transgene is not maintained in the transplanted nucleus even after the establishment of irreversible Xi in the differentiated ES cells. As the authors correctly pointed out, only small population of ES cells form macroH2A domain after induction of Xist transgene in differentiating ES cells (Rasmussen, 2001). Since this is such an important data, I would strongly recommend to show the expression of macroH2A1in the ES cells before nuclear transfer, which provides further evidence supporting the correlation between the macroH2A1 domain and stability of the transferred Xi.

We have tested the incorporation of macroH2A into chromatin of the Xi in differentiated ES cells after induction of *Xist* for 4 days. We have scored the proportion of nuclei possessing a macroH2A labelled territory in ES cells exposed to *Xist* induction for 4 days (Supplementary Figure S6D). As reported, (Rasmussen, 2001; Pullirsch 2010), only a small proportion (9%) of nuclei contained macroH2A, thereby providing additional correlative evidence between the ability to reactivate the Xi after nuclear transfer and the absence of macroH2A on the Xi. We have now included these additional results in Supplementary Figure S6D.

Minor points:

1. They examined the formation of macroH2A1 domain using antibody in Figure 7A, whereas they used EGFP-macroH2A1 to detect the same domain in transferred C2C12 nucleus. Is there any particular reason for not using antibody staining? If not, it sounds more natural to check the expression of macroH2A1 in the transferred nuclei prepared from MEF or EpiSCs by immunostaining. This issue should be mentioned at least in the materials and methods section.

As mentioned above, macroH2A immunofluorescence on transplanted nuclei is not possible with the available antibodies due to the presence of unidentified epitope in the *Xenopus* oocyte GV. We added a note on this issue in the Supplementary Material and Method (Immunoflurescence section).

2. They used a number of antibodies to detect Xi-related signals, but only two antibodies were specified in the manuscript. Since information of primary and secondary antibodies are extremely important, they should be clearly provided.

We have now included additional antibody information in the Supplementary Material and Method section.

3. In figure 7A, they present two columns of macroH2A1 expression in differentiated EpiSCs. The left column seems to show differentiated EpiSCs without macroH2A1 domain, which is not mentioned in the figure legends nor in the main text. Is there any particular reason for providing these additional images?

We wanted to report our observations that macroH2A becomes expressed in EpiSCs upon their differentiation and subsequently enriched on the Xi. The left column of the right panel of Figure 7A shows induction of macroH2A in differentiating EpiSCs, identified by the loss of pluripotency marker SSEA1 (lower image). The images in this column therefore demonstrate an increase in macroH2A protein in differentiating EpiSCs. The images in the right column show that macroH2A becomes enriched on the Xi in differentiating EpiSCs. This macroH2A domain corresponds to the Xi as identified by overlap with ubH2A immunostaining (lower image), a known marker of the Xi. Moreover, some ubH2A positive stained cells do not possess enrichment in macroH2A, corresponding to the undifferentiating EpiSCs in the culture. We have now included more explanatory details in the figure legend of Figure 7A.

4. Page 12, line 7: H2K27me3 marks Xi territory, but not the X-chromosome territory. X-chromosome territories can only be visualized by chromosome painting probes.

Thank you very much. We have now modified the text accordingly (page 12, line 16).

5. Page 15, line 15: "100% of MEF nuclei" should be "100% of C2C12 nuclei".

Thank you for spotting this error, we have now corrected the text (page 16, line 13.) and changed it to "100% of MEF and C2C12" according to our new data.

6. Page 18, line 18: SATB1 is not required for the chromosomal localization of Xist RNA. Rather, SATB1 expression induces dispersed Xist RNA signals in lymphocyte (Figure 3G, H in Agrelo et al., 2009). Discussion should be modified.

We have modified the discussion (page 20, line 25).

Referee #2:

Gurdon and colleagues test the stability of the somatically silent X chromosome in female differentiated cells of the mouse upon transfer into xenopus oocytes. Intriguingly, an X-linked GFP transgene reactivated when epiblast stem cells were transferred, but not when embryonic fibroblasts or trophoblast stem cells were transplanted. In an attempt to understand the cause of the difference in reactivation capacity, the authors define the chromatin state of the inactive X in MEFs and EpiSCs and find that H3K27 methylation and DNA methylation do not differ, but that macroH2A is enriched on the inactive x in MEFs but not EpiSCs, suggesting that the lack of macro H2A corresponds to the improved reprogramming capabilities of EpiSCs. While these data are interesting, it is not clear whether macoH2A indeed affects reprogramming of the XI. Many other marks of the inactive X were not tested (or are still unknown), thus, it is not clear whether macroH2A enrichment is the key event in X-inactivation that explains the differences in X reactivation. To test this idea, a knockdown of macroH2A should be performed in MEFs, or Xist knockout MEFs (Xist 2lox/Ko mefs published by the Jaenisch lab) be reprogrammed that lack macroH2A enrichment on the Xi. Before seeing some of these functional experiments I would be hesitant to recommend publication in the EMBO journal.

We appreciate the Reviewer's careful evaluation of our manuscript and his/her suggestions to improve it.

We have now knocked-down macroH2A1, or macroH2A2, or both macroH2A1 and macroH2A2 together (Figure 8A, B) by generating stable *Xi-GFP* female MEFs stably expressing shRNAs. We show that these shRNAs efficiently, and specifically, knock-down mH2A both at the mRNA (mH2A1 and 2) and protein level (mH2A1) (Figure 8A and B). Consistent with previous reports (Barzily-Rokni et al, 2011; Hernandez-Munoz et al, 2005), macroH2A knock-down alone was not sufficient to induce *Xi-GFP*, *Sox2* or *Oct4* reactivation (Supplementary Figure S8A,B), except for an interesting 2.5-fold increase over background in *Oct4* transcripts upon co-depletion of macroH2A1 and macroH2A2 (Supplementary Figure S8B). However, macroH2A depletion in donor nuclei did lead to a little, but statistically significant increase in transcriptional reactivation of *Xi-GFP* from transplanted MEFs (Figure 8C). This increase was comparable to that seen upon inhibition of histone deacetylases by using the well-known inhibitor Trichostatin A (TSA), (Figure 8C).

Interestingly, macroH2A depletion together with TSA treatment had the combined effect of both treatments alone, namely a 3-fold increase in detected *GFP* transcripts (Figure 8C). Moreover, the effect of macroH2A depletion and TSA treatment was even more pronounced on the reactivation of pluripotency genes *Sox2* and *Oct4* (Figure 8D,7- and 16-fold increase, respectively). Our results therefore demonstrate that macroH2A restricts transcriptional reprogramming of repressed genes. macroH2A alone is, however, not sufficient to explain all the restriction, implicating the role of other mechanisms. Our conclusion agrees with several other studies. During XCI, genes become silent before macroH2A is incorporated onto the Xi. Therefore, macroH2A is not required for silencing but acts more as a mechanism to stably keep genes in a repressed state.

We read with much interest that this is precisely the role macroH2A is thought to play in restricting cancer progression (Kapoor et al, 2010; Sporn et al, 2009). macroH2A was recently shown to become aberrantly silenced in melanoma, leading to the derepression of CDK8 resulting in increased cell proliferation and progression to metastasis (Kapoor et al, 2010). macroH2A had already been linked to cancer following the observation that low macroH2A in lung (and possibly breast) tumours is a good indicator or cancer recurrence (Sporn et al, 2009). These observations fit very well with our results. macroH2A incorporation into chromatin serves as a way to lock genes in a repressed state, restricting their reactivation. In this way, macroH2A both restricts reprogramming and inhibits the reactivation of tumour suppressor genes (our study and (Barzily-Rokni et al, 2011)). The mechanisms by which macroH2A restricts reprogramming and transcriptional activation are likely to be caused by one or a combination of the following. Biochemical studies have shown that macroH2A can inhibit the binding of certain transcription factors to macroH2A containing nucleosomes (Angelov et al, 2003). Luger and colleagues have also shown that the H2A-like portion of macroH2A contains a slightly different L1 interaction loop leading to an increase in macroH2A-containing nucleosome stability (Chakravarthy et al, 2005). In addition to interacting with HDAC1 and HDAC2 (Chakravarthy et al, 2005), macroH2A has also been shown to restrict p300-mediated histone acetylation (Doyen et al, 2006). macroH2A has also been shown to prevent efficient recruitment of the SWI/SNF chromatin remodelling complex (Angelov et al, 2003; Chang et al, 2008), and to promote DNA compaction upon DNA damage (Timinszky et al, 2009). Altogether, these studies strongly suggest that macroH2A uses multiple mechanisms to impede spurious transcriptional activity when incorporated into chromatin of the promoters of repressed genes, and thereby reinforces the repressed state, and therefore contributes to the extraordinary stability of the differentiated state.

To conclude, we have carried out the knock-down of macroH2A requested by the reviewer. Our results therefore support our conclusion that macroH2A contributes to resistance towards reprogramming. Together, our new results identify macroH2A as a component of heterochromatin that helps to keep genes in a repressed state, and by doing so to restrict transcriptional reprogramming.

In addition, the points listed below should be addressed:

1. It remains unclear whether the reactivation of the X in xenopus oocytes relates to the reactivation of the X when the ICM is established. Could the authors please speculate on this?

Based on existing literature and experiments recently obtained in our laboratory, we believe that some, but not all, of the molecular mechanisms leading to X reactivation in the ICM may be operative in *Xenopus* oocytes. Firstly, we know that homologous pluripotency factors such as the *Oct4* homolog Oct-60 are highly abundant in *Xenopus* oocytes (Hinkley et al, 1992). We have also observed increased pluripotency gene reactivation from transplanted nuclei after overexpression of other pluripotency factors of the *Klf* family, some of which are also present in *Xenopus* oocytes. However, the whole mammalian pluripotency network is clearly not fully conserved between mouse and *Xenopus*. We comment on this point in the discussion (page 18, line 25).

2. Figures 1 and 2 nicely demonstrate X reactivation in EpiSCs but not MEFs. However, why is GFP from the Xa detected on day 0 where it should be washed out due to permeabilisation as described for Sox2 in the text (for EpiSCs). In any case, do endogenous X-linked genes reactivate, i.e. could Fish or RT-PCR (taking advantage of polymorphisms) be performed to detect expression of genes from the Xi?

During the preparation of donor nuclei (Streptolysin-O mediated cytoplasmic membrane permeabilisation), mRNAs are mostly retained in nuclei. However, certain transcripts, such as *Sox2* transcripts, quickly disappear because they have a very short half-life. Hence time point 0 samples invariably have a low level of *Sox2* transcripts, especially when *Sox2* is highly transcribed in the donor cell type used, as for example with TS cells (Figure 1C, day 0). Most genes make transcripts of much longer half-life, such as *GFP* and others; hence, their transcripts are retained during the permeabilisation procedure and can be readily detected on day 0 (Figure 1B,C; lower graphs, day 0). We realize that this point was not made clear in our initial manuscript and we have now removed the statement about *Sox2* transcript loss upon donor nuclei preparation from our manuscript in order to avoid any ambiguity for the reader (page 7).

To address the reviewer's comment regarding transcriptional reactivation of endogenous X-linked genes, we have carried out allele-specific RT-PCR against X-linked gene *Rlim* using previously validated protocols (Huynh & Lee, 2003). We derived *X-GFP* MEFs from individual embryos obtained from a cross between *X-GFP Mus musculus* and *Mus castaneus* mice. After genotyping individual embryos for sex and transgene transmission, we identified female *X-GFP Mus/Cast* MEFs preparations and separated by flow cytometry *Xi-GFP Mus/Cast* MEFs from *Xa-GFP Mus/Cast* MEFs. In this way we obtained pure populations of female MEFs with the X chromosome of one species exclusively inactive (*Xi-GFP Mus/Cast*) or active (*Xa-GFP Mus/Cast*). The *Xi-GFP* EpiSCs used previously had already been derived from *X-GFP Mus Musculus* / *Mus castaneus* embryos. As shown in Figure 2C, allele-specific expression of *Rlim* can be carried out due to the presence of a single nucleotide polymorphism generating a HaeIII restriction site in the *Mus musculus*-derived *Rlim* transcript, but not in the *Mus castaneus* derived one (Huynh & Lee, 2003). Hence, the allelic origin of RT-PCR products can be distinguished based on restriction enzyme patterns. We carried out this analysis on nuclei transplanted into *Xenopus* oocytes, which did not interfere with the assay. As shown in Figure 2D, we found that *Rlim* expression is reactivated from the *musculus* (Xi) allele in transplanted EpiSC nuclei but not in transplanted MEF nuclei. Therefore, there is indeed nuclear transfer-mediated activation of an endogenous Xi gene in transplanted EpiSCs.

3. Xi reactivation appears very efficient, almost to levels of the Xa - which is rather surprising. Do all transplanted cells reactivate or just a few?

The Reviewer brings up an interesting question. Based on several experiments, we believe that most transplanted nuclei undergo reactivation when transplanted into *Xenopus* oocyte GVs. The first observation comes from nuclear transfer of retinoic-acid differentiated ES cells (ESRA). Transcription of pluripotency genes *Oct4* and *Sox2* is rapidly downregulated in these cells upon RA treatment. However, *Oct4* and *Sox2* are efficiently reactivated upon nuclear transfer of ESRA cells (Halley-Stott et al, 2010). The transcript level of these genes in transplanted ESRA nuclei is as high as for transplanted ES cells, indicating full gene reactivation (Halley-Stott et al, 2010; Jullien et al, 2010). This suggests that most if not all nuclei are induced to reprogram in our system. The second observation comes from microscopic analysis of real time exchange of oocyte-derived reprogramming factors, such as histone B4. Histone B4, required for pluripotency gene reactivation after *Xenopus* oocyte nuclear transfer, is seen to be incorporated in over 90% of transplanted nuclei (Jullien et al, 2010). We have also recently observed induction of active transcription as judged by immunofluorescence of the elongating form of RNA Polymerase II (Serine 2 phosphorylated RNA Polymerase II) in nearly 100% of transplanted nuclei (unpublished results). We conclude that the *Xi-GFP* of EpiSC reactivates to levels comparable to *Xa-GFP* of MEFs just after nuclear transfer, and indicates a very efficient transcriptional reactivation (Figure 2B). We note however that this level is still half the level of transplanted *Xa-GFP* MEFs 3 days after nuclear transfer.

4. In Figure 4 it is demonstrated that H3K27methylation is maintained upon SCNT. However, much fewer cells have an Xi enrichment in the case of transplanted MEFs (comparing to MEFS in culture). For transplanted EpiSCs the number is missing (4B). Could the authors please explain this observation - is it just technical in nature that the Xi is difficult to detect upon transplantation? Nevertheless, it is surprising that H3K27 methylation on the X is maintained in the absence of Xist coating (Figures 4 and 5), given that H3K27methylation is Xist dependent on the X in MEFs. The same is true for macroH2A. Therefore I wonder whether cell division is required for loss of K27 methylation/macroH2A but not Xist. Do these cells divide upon transplantation?

The lower proportion of nuclei seen to also possess a H3K27me3 domain in transplanted female MEF nuclei is readily explained by the fact that this dataset represent single plane Z-section confocal images (Figure 4B), as opposed to projections of multiple Z-sections across each sample (Figure 4A). In the absence of projection of several Z-sections, covering entire nuclei, a reduced proportion of H3K27me3 labelled Xi domains are seen (Figure 4B, time 0, 51% compared to Figure 4A, 98%). We have now imaged transplanted female MEFs using Z-sectioning followed by projection of all planes onto a single plane and reassessed the proportion of nuclei with a H3K27me3 labelled Xi. We find that >90% of transplanted nuclei have a H3K27me3 labelled Xi, and this lasts for 3 days after nuclear transfer (Supplementary Figure S1 for Reviewers). In addition, we note that the proportion of transplanted nuclei with an H3K27me Xi domain obtained in our original dataset presented in Figure 4B does not decrease significantly over time after nuclear transfer (Figure 4B, 51% at time 0 versus 48% 72 hours after nuclear transfer). We think that our results convincingly show that H3K27me3 is not lost from the Xi after female MEFs nuclear transfer to *Xenopus* oocytes. We have now included a statement in each figure legend of figures containing miscroscopic images, indicating whether they represent projected Z-sections or single Z-section (Legends of Figure 4A,B; Figure 5A,B,C; Figure 7A,B,C; Supplementary Figure S1D, Supplementary Figure S3, Supplementary Figure S4A,B; Supplementary Figure S6D and Supplementary Figure S7A,B,C). We have included a number for EpiSCs (Figure 4B).

We fully agree with the reviewer that the maintenance of H3K27me3 and macroH2A in the absence of *Xist* RNA is surprising. Transplanted nuclei do not divide upon transplantation in our system. All changes seen after nuclear transfer are therefore independent of cell division as well as DNA synthesis. It was shown that conditional deletion of *Xist* leads to the loss of macroH2A from the Xi (Csankovszki et al, 1999), although how quickly this occurs after *Xist* deletion was not addressed in their study. As the reviewer points out, conditional deletion of *Xist* also results in loss of H3K27me3 on the Xi (Plath et al, 2004). Therefore, the maintenance of H3K27me3 as well as macroH2A on the Xi in our experiments highly suggests that cell division is required for their loss upon conditional deletion of *Xist*. It remains an interesting question how *Xist* coating of the Xi is linked with epigenetic inheritance of macroH2A and H3K27me3 upon cell division. We have added a comment on this point in the discussion (page 20, line 9 and page 21, line19).

5. The tet-inducible Xist experiment in Figure 6 is not well explained and does not help to explain the difference between MEFs and EpiSCs as it is not clear how this tet inducible system relates to these cell states. Thus I did not find the figure very helpful, nor explained sufficiently so that lanes 1-6 can be understood easily.

We fully agree with the reviewer that this figure had not been sufficiently explained in the original version of the manuscript. This is because the *Xist*-inducible system has been extensively used and characterised elsewhere (Kohlmaier et al, 2004; Leeb & Wutz, 2007; Wutz & Jaenisch, 2000; Wutz et al, 2002). We have now re-written the paragraph in relation to Figure 6 and with have referred to *Xist*-dependent and *Xist*-independent, stable states to avoid confusion with the reversible and irreversible states referred to in other parts of our manuscript. We have also added an explanation of the importance of these experiments in leading us to investigate macroH2A. We hope that these changes will ease the understanding of this figure.

6. It is not clear why the authors switch to the C2C12 system, for which we don't know whether the Xi reactivates upon SCNT, to image macroH2A. As for H3K27methylation in Figure 4, macroH2A could have been stained at different time points upon transplantation of MEFs and EpiSCs. Therefore Figure 7B is not very helpful.

The reason for switching to the C2C12 system is because we found that the mH2A1 antibody binds to an unknown epitope in the *Xenopus* GV. Immunofluorescence against mH2A1 could not be used in transplanted nuclei, even when transplanted nuclei were fixed immediately after nuclear transfer using several different fixing conditions. We used the C2C12 system because we failed to generate a stable *macroH2A1-GFP* female MEFs cell line. C2C12 seemed a good cell type as this mouse myogenic precursor is likely to have undergone complete XCI and to possess a fully inactive X chromosome. Accordingly, *macroH2A-GFP* indeed localises to the Xi in C2C12 cells (Figure 7C; Supplementary Figure S7A,B,C; Supplementary Movie S1; Supplementary Movie S2). In addition, a recent high profile paper reported the use of C2C12 cells to study Xi replication timing control (Casas-Delucchi et al, 2011). We have now added detailed explanations about this issue in the manuscript (page 15, lane 15) and Supplementary information (page 4, lane 23)**.** In addition, we have followed mH2A1-GFP on the Xi of female MEFs transiently expressing *mH2A1-GFP* and obtained the same conclusion as with C2C12: mH2A1-GFP remains associated with chromatin of the Xi in transplanted nuclei (New Figure 7B). We have moved the old Figure 7B to the Supplementary Information (Supplementary Figure S7C).

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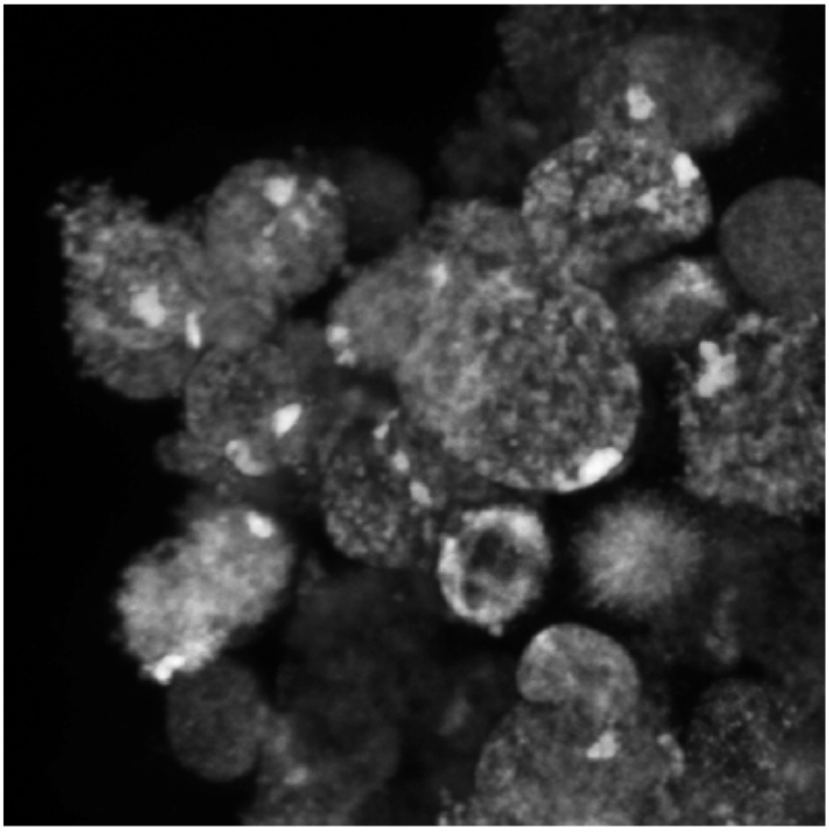
Supplementary Figure S1 For Reviewers. H3K27me3 is maintained on the Xi after nuclear transfer of female MEF nuclei. Immunofluorescence against H3K27me3 of MEF nuclei 3 days after nuclear transfer to *Xenopus* oocyte GVs. The image is a projected series of Z-sections.



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