THE EMBO JOURNAL

Manuscript EMBO-2010-76003

Constitutive heterochromatin reorganization during somatic cell reprogramming

Eden Fussner, Ugljesa Djuric, Mike Strauss, Akitsu Hotta, Carolina Perez-Iratxeta, Fredrik Lanner, F. Jeffrey Dilworth, James Ellis and David P. Bazett-Jones

Corresponding author: David P. Bazett-Jones, The Hospital for Sick Children

Review timeline:

Submission date:	15 September 2010
Editorial Decision:	20 October 2010
Revision received:	24 December 2010
Editorial Decision:	25 January 2011
Revision received:	09 February 2011
Editorial Decision:	02 March 2011
Accepted:	03 March 2011

Transaction Report:

(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 20 October 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been evaluated by three referees and I enclose their reports below, as you will see they provide mixed recommendations regarding publication.

Overall the referees find the data regarding the organization of chromatin into 10nm fibres as determined by ESI to be one of the most interesting and important issues in the manuscript. However, the three referees all raise concerns with what is being detected using this approach and therefore, I would recommend that you focus on addressing these concerns. Referee #1 would like to see more evidence for the existence of the 10nm fibre structures including 3D information, while referee #2 and #3 would like to see further evidence that can be generalized and further data supporting the ESI analysis. A major concern is understanding if the the ESI technique is able to detect all the chromatin in a cell and if it is capable of detecting 30nm fibres in this after discussing this with referee #3 they suggested performing an in vitro control:

"Could they undertake an in vitro experiment mixing chromatin fibres in a proteinaceous matrix (to mimic the nucleus) and then look at them. This experiment could be done under different salt conditions altering chromatin structure (10-nm, low salt; 30-nm, 80 mM salt)."

As I mentioned above these are key concerns that need to be experimentally addressed in a revised version of a manuscript and for further consideration at The EMBO Journal. I hope that these are possible because the referees need to be convinced by the ESI data. Given the initial interest in the

study should you be able to address these issues, we would be wiling to consider a revised manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This ms by Fussner et al. contains a detailed examination of the chromatin structural changes that accompany the various stages in somatic cell reprogramming. Chromatin structure is analyzed by correlative light and electron microscopy, and ESI used to localize nitrogen versus phosphorus the TEM images.

The work builds on the well established technical expertise of the authors, and the conclusions regarding the relationships between levels of chromatin compaction in pericentromeric heterochromatin and their correlation with epigenetic programming stage are generally sound. There are, however, a number of points that need attention.

Much is made of the apparent absence of 30 nm chromatin fibers and presence of 10 nm fibers. In fact, clearly defined 30 nm fibers are seen only in specialized transcriptionally inactive nuclei such as avian erythrocytes, and, despite the widespread expectation that some 'higher order' chromatin organization is present in compact chromatin (based largely on the structure of isolated material), there is very little evidence for this. In the absence of any obvious higher order structure, the authors interpret and discuss their TEM images in terms of 10 nm chromatin fibers. In fact, only in Fig 4C are structures resembling fibers actually seen, and even here, the distinction between randomly folded arrays of nucleosomes and 10 nm fibers is not clear, especially since no stereo views or 3D information is presented. The micrographs clearly support the absence of well-defined chromatin higher order structures, but do not justify the repeated use of the term 10-nm fiber unless they define it as a randomly folded chain of nucleosomes. For most of the comparisons, it is more correct to phrase the differences simply in terms of compaction. For example, in the 2nd sentence of the Discussion, the word 'fibre' should simply be omitted.
In some of the box plots (e.g. 4B), it is not clear which comparisons show significant differences. Scale bars are missing or unexplained in many micrographs.

Much is made of the DAPI distribution, and it is often referred to as a 'counterstain'. It should be explained that DAPI preferentially binds A-T rich sequences, such as found in the repetitive pericentromeric DNA, and is the reason for its focal distribution in mouse cells. It's relative distribution is not a measure of compaction per se.

It also needs to be clarified that mouse cells are unusual in that the pericentromeric chromatin from different chromosomes tends to self-associate resulting in DAPI-bright. Human cells, for example, do not share this property.

The image processing steps used in Photoshop and ImageJ should be spelled out.
The ms needs to be carefully checked for typos - e.g. glutaraldehyde misspelled, 'plan' in Fig 8 legend should be 'plane'.

Referee #2 (Remarks to the Author):

Comments on Fussner et al., 'Heterochromatin reorganizes to dispersed 10 nm fibres at a late stage of somatic cell reprogramming'

Fusser et al study, using mostly fluorescence and electron spectroscopic imaging (ESI), changes in global chromatin configuration between MEFs, partial iPS cells and fully reprogrammed pluripotent iPS cells (and ES cells). Their main conclusions are that packed or condensed heterochromatin regions determined by bright DAPI or H3K9me3 signal (in relation to the nucleoplasmic background) are well delimited in MEFs and partial iPS cells and become less structured and less defined in ES cells and fully reprogrammed iPS cells. The authors correlate these heterochromatic regions seen in light microscopy with ESI and show that these chromocenter boundaries are poorly defined or even dispersed when chromatin fibres are observed in pluripotent cells (ES and full iPS cells). The authors also find that the only technically measurable chromatin fibres in MEF, partial iPS, full iPS and ES cells are the 10 nm fibers, challenging previous dogmas.

The most important discovery of the paper seems to be that the authors found prevalence of 10 nm fibres within the compact heterochromatin domains of MEFs, where, according to their own words "only 30 nm or higher-level fibre organization might be expected". If indeed true, this seems to be a significant and important observation but that has little to do with pluripotent cells or iPS cells.

Specific comments:

Title:
The title is problematic because it implies in a way that the chromatin fiber width changes during the somatic reprogramming and that is not what is shown in this paper. It also implies that 10 nm fibres can only be detected at a late stage during reprogramming. However, as noted above, the authors find that heterochromatin in MEF cells is also comprised of 10 nm fibres.

Abstract:
Their opening statement is portrayed as common knowledge, while there's in fact very little evidence at the moment for the global epigenetic changes in the field. I believe that it should be written with more caution, especially when referring to heterochromatin changes.

Results:
The CREST staining of the partial iPS cells (Fig S1) should be referred to in the results section.

Figure 1: The results for each panel are on a different set of cells. Perhaps not all 5 cell types are crucial for every experiment, but at least add J1 ES cells to panel C: pMX-LTR (bottom right).

Page 2 (of the Results section - please add page numbers!): the authors compare the numbers of differentially expressed genes between the different cell types. According to the microarray analyses, it seems that the partial iPS cells are more similar to MEFs than the ES cells. If this is the case, then why do the authors conclude that reorganization of heterochromatin is a late event during reprogramming?

Page 3: it is very important to show the correlation between DAPI and H3K9me3 enrichment that is stated in page 3 of the Results section (data not shown), especially since in Fig.3A the DAPI-H3K9me3 correlation is not very clear for pluripotent cells (ES and full iPS cells).

Page 3 (of the Results section): Please add additional references regarding chromocenters in pluripotent and differentiated cells in addition to Ahmed et al., 2009.

In order to demark the constitutive heterochromatin domains and to correlate them with ESI, H3K9me3 immunostaining is used (Fig. 4). In this case the analysis of the chromocenters by scan plot and analysis of the bright regions in relationship to the nuclear background should be made on the H3K9me3 stained nuclei and not on the DAPI signal (Fig. 3). Alternatively, one can correlate the ESI images to the DAPI signal. This is important to get coherent and strong evidences for these correlations.

Figure 3B: The figure is labeled incorrectly. I believe 'MEF' should be at the right?

In the Materials and Methods part, please provide a description of the scan plot analysis and the calculation of the background to chromocenter ratio shown in Fig.3B for example.

Page 4 of Results: the authors discuss their findings of 10 nm fibres in constitutive heterochromatin of MEFs. Since this is a crucial part of the paper it is important to strengthen this point. Do other somatic cells show the same 10 nm fibres? Is the 30 nm fibre never detectable? Do all iPS cells show prevalence of 10 nm fibres? Some additional cell measurements and statistics would be appropriate.

In addition, as mentioned above for the title of the paper, the last sentence of this same section (page 5 of Results section): 'acquisition of the dispersed 10 nm fibre meshwork correlates with the fully reprogrammed state' does not reflect the data and should be rephrased.

Figure 7A: the DAPI panel is too small to discern. Please show the DAPI panel at the same size as the Nanog panel. Figure 7B: how many nuclei were analyzed? Please indicate in figure legend. Figure 7C: the figure is not very convincing. Please provide the merged images and quantify Nanog positive / GFP-positive cells. (if the merged images do not provide more clarity, please remove it and the associated conclusions).

Figure 8B, the six low and high Nanog panels are bleary (out of focus??) in comparison with the Cdx2 and the GATA6 images. This is important as it may affect the measurements shown. Please provide other representative pictures (and corresponding measurements), which will be clearer.

Discussion:
Third sentence "... we show here that heterochromatin specifically enriched in H3K9me3 is composed of 10 nm fibers in ES and full iPS cells." Once again, more cautious is warranted, since 10 nm fibers are also reported in the partial iPS and MEF cells, the statement may cause confusion.

Overall, an interesting, albeit descriptive study which strengthens previous works showing the differences in chromatin architecture between pluripotent and differentiated cells (Meshorer et al., 2006; Aoto et al., 2006; Efroni et al., 2008; Bartova et al., 2008), while adding an important finding that heterochromatin, even in MEF cells, is comprised of 10 nm fibres.

Referee #3 (Remarks to the Author):

Heterochromatin reorganizes to dispersed 10 nm fibres at a late stage of somatic cell reprogramming

The chromatin structure of stem cells and differentiated cells are still poorly characterised and in the literature there is much conflicting data. Also, the role chromatin structure plays in stem cell differentiation is poorly understood. It is also unknown how chromatin structure changes when differentiated cells are converted back to stem cells (iPS cells).

In this study Bazett-Jones and colleagues have investigated the chromatin structure of stem cells, iPS cells and differentiated cells by light microscopy (DAPI staining) and LM/ESI. I think this is a very interesting topic and most of the data presented is good and shows a clear change in chromatin architecture of the cells. However, I have major concerns about the interpretation of the ESI data and the fact that the two main techniques used (DAPI staining and LM/ESI) are related to each other in the manuscript but are looking at very different levels of nuclear organisation.

For me the authors need to further address what the ESI approach is showing and to further develop the DAPI line scan assay so that it is more quantifiable.

The authors use of the term heterochromatin is quite loose. In the abstract they both refer to the silencing of genes as heterochromatinisation (line 3) and then talk about studying heterochromatin domains (line 4) although these are both quite different. At the end of the abstract they imply retroviral silencing is heterochromatinisation and then talk about constitutive heterochromatin structures. In the first line of the introduction they talk about heterochromatinisation of tissue specific genes. Later in the introduction (start of paragraph 3) the authors talk about changes in heterochromatin marks (presumably at genes) but then talk about heterochromatin domains (presumably constitutive heterochromatin) but these structures are entirely different in cells.

As this study focuses on constitutive heterochromatin domains I think the authors need to be more clear about this and make the distinction that constitutive heterochromatin is very different from the "heterochromatinisation" that occurs during stable gene silencing. It could also be misleading to relate what is found at constitutive heterochromatin to the silencing of genes - as the processes and chromatin structures involved will be entirely different.

In the introduction the authors say evidence for 30-nm fibres in vivo is weak. This is true but there are two key pieces of data (i) low angle x-ray scattering clearly indicates that chromatin in cells is packaged into 30-nm fibres and (ii) chromatin isolated from cells adopts a 30-nm fibre structure under physiological conditions.

In the introduction (paragraph 4) the authors refer to one of their previous studies (Efroni et al., 2008) showing that "ES cells display a meshwork of 10 nm chromatin fibres through out the nucleus, and a paucity of the blocks of condensed chromatin observed in somatic cells". This was originally published as supplementary data and although there is a clear difference between stem cells and differentiated cells by ESI I feel describing it as a 10-nm chromatin fibre mesh is too prescriptive. At the end of paragraph 4 the authors also suggest that it is accepted that ES cell chromatin is organised as a 10-nm fibre structure, which I feel might be overstated.

Figure 2
C. The authors show considerable differences in expression by qRT-PCR (4 orders of magnitude). Can I just confirm the scale is correct. The authors also do not mention what the error bars are.

D. Would a Western blot show the differences in expression more quantitatively than IF?

Figure 3.
The authors use DAPI and antibody staining to show that chromocentres are different between stem cells and differentiated cells. This analysis is very dependent on where the line is drawn through the nucleus. Rather than quantifying this as background:chromocentre DAPI ratio can the authors measure signal variance across the entire nucleus? From the data you would expect increased variance in differentiated cells.

I feel the authors need to emphasise that DAPI staining is both affected by chromatin packaging (e.g. can see a Barr body using DAPI in female cells) and by DNA sequence (preferentially binds AT DNA). This therefore makes it difficult to interpret what DAPI staining is actually showing us about chromatin compaction.

If the DAPI linescan assay is reflective of a change in chromocentre structure then as the chromocentre structure changes with differentiation so will the area the chromocentres occupy in the nucleus. As DAPI staining is both reflective of chromatin compaction and AT-binding maybe another technique could be used. It would be possible to stain chromocentres using FISH for major satellite and quantifying the chromocentre area in the nucleus. Alternatively the cells could be transfected with GFP-H3 (or equivalent) to mark the "compact" heterochromatin and then quantify the area they occupy in the nucleus.

The authors need to label the y axis of the graph in B and the size of the scale bar in A is not mentioned in the figure.

Figure 4
For me this is really the crux of the paper. The authors use LM/ESI to visualise the chromocentres by H3K9me3 staining and analyse the phosphorus/nitrogen content of the chromocentres as a measure of chromatin compaction. The results clearly show there is a difference in chromocentre architecture by ESI and the authors interpret this to mean chromatin structure has changed and that stem cells have a dispersed 10-nm fibre structure.

In fig 4A the authors show considerable phosphate/nitrogen enrichment (yellow) in the chromocentre for MEFs which they interpret to indicate they have a high density of chromatin (i.e. compact chromatin). In contrast they show that stem cell like chromocentres (e.g. EOS3F-28) have the same chromatin compaction as euchromatin.

Previously GFP-H3 (or equivalent) has been visualised in a number of stem cells and differentiated cells. By fluorescence microscopy the chromocentres in ES cells clearly have more GFP-H3 associated with them. This would argue that chromocentres do have a more compact structure than euchromatin. If this is not seen by ESI could this mean that ESI is visualising something different to chromatin structure.

These figures need scale bars

Fig 4B. I am not sure this analysis is very informative as it depends on where the analysis line is drawn through the nucleus.

Fig 4C
This panel is highly significant. The authors say they are visualising individual chromatin fibres, including 10-nm fibres, within the chromocentres. If these are individual chromatin fibres it should be possible to calculate how much chromatin is in the chromocentres.

As a quick calculation 8% of the mouse genome is constitutive heterochromatin giving 430 Mb. There are approximately 10 chromocentres per nucleus giving 43 Mb of DNA per chromocentre. For this the DNA length is approximately 2.3 x10(7) nm.

From the images in panel C the MEFs chromocentre is approximately 320 nm in diameter and from Bazett-Jones et al., (1999) sections are approximately 30 nm thick. From figure 3A (assuming scale bar is 5 microns) chromocentres are normally 800 nm in diameter. Assuming the chromocentre is a sphere and the slice shown in Fig3C is from this sphere then this slice corresponds to 1% volume of the chromocentre sphere. Therefore this slice should contain 430 kb DNA. One quarter of this slice (as shown in the zoomed part of fig 4C) would then contain 100 kb DNA.

100 kb packaged into a 10 nm fibre (assuming a packaging ratio of 5) would have a fibre length of 10,800 nm. 100 kb packaged into a 30 nm fibre would have a fibre length of 916 nm.

From these rough calculations it is possible that the ESI approach is not revealing all of the chromatin in the image. I therefore wonder if it is difficult to categorically say that stem cell chromatin has a decompacted 10-nm fibre structure.

Figure 6
The same comments for figure 4 apply for figure 6. It is very clear that in the images shown there is a difference in chromatin packaging but I wonder if it is difficult to make an assessment about the entire population of chromatin fibres as there just is not enough yellow marking to account for all the DNA that should be in a chromocentre. Is it possible that the ESI is revealing something slightly different or only showing a snap-shot of chromatin fibres?

B. As for figure 3B I am not sure how informative this analysis is as it will depend on where the linescan is drawn. Also as DAPI staining is showing a very different level of chromatin organisation to the ultrastructure of the chromocentres I think it is difficult to relate these two pieces of data to each other (in the text it states:"thus, DAPI line scan analysis supports the LM/ESI data")

Fig 7B and Figure 8B
As for previous figures I feel this linescan analysis might be difficult to accurately quantify.

The authors mention "alpha satellite" in a few places. I think the satellites in mouse are either called minor or major satellites.

I wonder if the title of the paper is too prescriptive and could be "Constitutive heterochromatin reorganisation during somatic cell reprogramming"

1st Revision - authors' response 24 December 2010

Specific Responses to referees' Comments:

Referee #1

1. Much is made of the apparent absence of 30 nm chromatin fibers and presence of 10 nm fibers. In fact, clearly defined 30 nm fibers are seen only in specialized transcriptionally inactive nuclei such as avian erythrocytes, and, despite the widespread expectation that some 'higher order' chromatin organization is present in compact chromatin (based largely on the structure of isolated material), there is very little evidence for this. In the absence of any obvious higher order structure, the authors interpret and discuss their TEM images in terms of 10 nm chromatin fibers. In fact, only in Fig 4C are structures resembling fibers actually seen, and even here, the distinction between randomly folded arrays of nucleosomes and 10 nm fibers is not clear, especially since no stereo views or 3D information is presented. The micrographs clearly support the absence of well-defined chromatin higher order structures, but do not justify the repeated use of the term 10-nm fiber unless they define it as a randomly folded chain of nucleosomes. For most of the comparisons, it is more correct to phrase the differences simply in terms of compaction. For example, in the 2nd sentence of the Discussion, the word 'fibre' should simply be omitted.

REPLY: We would like to thank the reviewer for this comment and we have addressed the concern that without three-dimensional information we could only comment on the absence of well-defined higher-order chromatin structures and could not define the nucleosomes observed in our micrographs as 10 nm chromatin fibres. We have since generated three-dimensional images containing H3K9me3-enriched regions in J1 ES cells and have measured the fibre width to be 10.8 nm both within and outside of the biochemically defined heterochromatin regions, using Fourier analysis. We have added these data to Figure 4, Supplemental Figure 5 and Supplemental Movies 3 and 4. We have also referred to domains as "open" and "closed" in many sections of the manuscript rather than in terms of 10 nm or higher-order chromatin fibre assemblies.

2. In some of the box plots (e.g. 4B), it is not clear which comparisons show significant differences.
REPLY: We have removed the ambiguity of the significant differences obtained and have clarified these differences in all relevant figures.

3. Scale bars are missing or unexplained in many micrographs.

REPLY: We apologize for the omission in our initial submission and have added scale bars and descriptions in all the Figure legends, with the exception of the Chimera model of the chromatin fibres visualized after tomographic reconstruction in Figure 4E as this is a perspective representation and the scale varies in the z-direction throughout this image. Scale bars of tomographic slices are, however, included in Supplemental Figure 4 for clarity.

4. Much is made of the DAPI distribution, and it is often referred to as a 'counterstain'. It should be explained that DAPI preferentially binds A-T rich sequences, such as found in the repetitive pericentromeric DNA, and is the reason for its focal distribution in mouse cells. It's relative distribution is not a measure of compaction per se.

REPLY: We agree that we had not clearly made this distinction in the text, in that without proper imaging controls, DAPI as a measure of density must be interpreted with caution. Reviewer 3 also noted our deficiency of an appropriate description. Therefore, we have added a section that

discusses this on page 9: "DAPI is not a good indicator of chromatin fibre density *per se* because it is also affected by AT-richness and nucleosome-repeat length. Therefore, to interpret differences in heterochromatin organization observed by DAPI counter stain we employed Electron Spectroscopic Imaging (ESI)."

5. It also needs to be clarified that mouse cells are unusual in that the pericentromeric chromatin from different chromosomes tends to self-associate resulting in DAPI-bright. Human cells, for example, do not share this property.

REPLY: Related to this comment, we have included the following on Page 4: "These chromocentres are easily identified in mouse nuclei by their DAPI-rich staining, and are specifically marked by H3K9me3 and H4K20me3 (Peters et al, 2001). This clustering makes mouse heterochromatin an attractive model system for studying chromatin domain organization. Human cells, on the other hand, contain repetitive sequences that are distributed more evenly across the genome and in most contexts do not cluster to the same degree as in mouse cells."

6. The image processing steps used in Photoshop and ImageJ should be spelled out

REPLY: We thank the reviewer for noticing this deficiency and have included a section in the Materials and Methods section on Page 19:
"*Image analysis*

DAPI line scan analyses were performed using ImageJ on optical sections where DAPI foci were at optimal focal planes. 50 pixel (approximately half the diameter of the average chromocentre analyzed) histograms were generated through the foci and the background (outside the nucleus) was subtracted from the nucleoplasmic and chromocentre signals. Variations in these data were calculated as a ratio of chromocentre peak height to nucleoplasmic signal.

Integrative phosphorus analyses of ESI images were preformed on unprocessed phosphorus jump ratio maps in ImageJ. The integrated average phosphorus intensities within H3K9me3-enriched regions were compared to the integrated average phosphorus intensity outside the H3K9me3-enriched region within the same image field. Phosphorus intensities were background subtracted using a small region within the field devoid of nucleic acid or phosphorus signal."

6. The ms needs to be carefully checked for typos - e.g. glutaraldehyde misspelled, 'plan' in Fig 8 legend should be 'plane'

REPLY: We have made every effort to correct for typos and thank the reviewers for noticing these.

Referee #2

1. The most important discovery of the paper seems to be that the authors found prevalence of 10 nm fibres within the compact heterochromatin domains of MEFs, where, according to their own words "only 30 nm or higher-level fibre organization might be expected". If indeed true, this seems to be a significant and important observation but that has little to do with pluripotent cells or iPS cells.

REPLY: We thank the reviewer for the expressed interest in this major finding of our paper. Our intention was not to imply that there were not any higher-order chromatin fibres in MEF heterochromatin domains, as we are unable to determine the precise higher-order chromatin fibre structures in regions where multiple fibres overlap within the entire EM section. We have clarified this on Page 15: "We were surprised to observe 10 nm chromatin fibres in the very densely packed chromocentres of MEFs and partial iPS cells although we cannot exclude the possibility that 30 nm fibres can also be found in these structures. However, prevalence of 10 nm fibres in both compact and disrupted heterochromatin domains indicates the transition between open and closed chromatin domains involves, at least in part, transitions between closely packed and highly folded 10 nm chromatin fibres. This challenges the absolute requirement for transitions between 10 and 30 nm chromatin fibres in defining heterochromatin domains." The major finding of the paper is that the typical meso-scale genome organization of compact and open domains of somatic cells is not

present in pluripotent ES cells and fully reprogrammed iPS cells. This point was not made clear in the previous version of the paper. We have thus changed several of the "10 nm mesh" descriptions throughout the text to reflect the striking difference in meso-scale organization that we have observed by describing domains in terms of open and closed chromatin domains.

2. The title is problematic because it implies in a way that the chromatin fiber width changes during the somatic reprogramming and that is not what is shown in this paper. It also implies that 10 nm fibres can only be detected at a late stage during reprogramming. However, as noted above, the authors find that heterochromatin in MEF cells is also comprised of 10 nm fibres.

REPLY: We would like to thank the reviewer for suggesting a change of the title. A title change was also suggested by reviewer 3 and we hope that reviewer 2 will agree with Reviewer 3's suggestion, "Constitutive heterochromatin reorganization during somatic cell reprogramming".

3. Abstract: Their opening statement is portrayed as common knowledge, while there's in fact very little evidence at the moment for the global epigenetic changes in the field. I believe that it should be written with more caution, especially when referring to heterochromatin changes.

REPLY: Our opening statement described the works of Jaenisch and others, which are described in the first sentence of the Introduction as well. The reprogramming process involves global epigenetic changes, chromosome X reactivation, and hypomethylation of major satellite sequences. To deal with the concern about referring to heterochromatin changes, we added the following revisions to the Abstract, "In contrast to loci-specific epigenetic changes, heterochromatin domains undergo epigenetic resetting during the reprogramming process but the effect on the heterochromatin ultrastructure is not known." We have also clarified this point in the Introduction on page 3: "As they complete reprogramming, full mouse iPS cells acquire epigenetic marks of pluripotency including X chromosome reactivation and genome-wide establishment of ES cell-like histone H3K27 and H3K4 trimethylation patterns (Maherali et al, 2007)."

4. The CREST staining of the partial iPS cells (Fig S1) should be referred to in the results section.

REPLY: We thank the reviewer for noting we had not clarified in which cell lines these domains could be identified as chromocentres and have consequently referred to the CREST staining (Figure S1) on page 8: "These domains were identified as chromocentres in the parental MEFs, ES cells and full and partial iPS cells, on the basis of H4K20me3 enrichment, DAPI density and proximity to centromeres, visualized with CREST antisera (Figure S1)."

5. Figure 1: The results for each panel are on a different set of cells. Perhaps not all 5 cell types are crucial for every experiment, but at least add J1 ES cells to panel C: pMX-LTR (bottom right).

REPLY: We have used J1 ES or MEF cells as our control cell lines where appropriate. However, we did not perform ChiP analysis on the J1 ES cells in this experiment since they were not infected with any of the pMX vectors. We feel that in this panel it is most appropriate to compare the epigenetic modifications of the reprogramming transgenes in a full (EOS3F-29) and partial (EOS3F-24) iPS cell line.

6. Page 2 (of the Results section - please add page numbers!): the authors compare the numbers of differentially expressed genes between the different cell types. According to the microarray analyses, it seems that the partial iPS cells are more similar to MEFs than the ES cells. If this is the case, then why do the authors conclude that reorganization of heterochromatin is a late event during reprogramming?

REPLY: We apologize for the omission of page numbers in our initial submission and have added them subsequently. We also thank the reviewer for pointing out that the table in Figure 2 and the description of these data were confusing. The partial iPS cells are actually more similar to ES cells than to MEFs. We have changed the label on the table itself to more accurately represent the

comparisons. In fact, the number of differentially expressed genes between EOS3F-24 and J1 ES cells is smaller than between EOS3F-24 and MEF cells.

Furthermore, EOS3F-24 partial iPS cells both grow similarly to ES cell colonies and can give rise to all three germ layers (demonstrated in Hotta, 2009 and referenced in the text), but do not robustly activate the pluripotency transcriptional network. Collectively, these data indicate that the changes in heterochromatin structure and retroviral silencing observed following the 2i treatment accurately represent late events in the reprogramming process.

7. Page 3: it is very important to show the correlation between DAPI and H3K9me3 enrichment that is stated in page 3 of the Results section (data not shown), especially since in Fig.3A the DAPI-H3K9me3 correlation is not very clear for pluripotent cells (ES and full iPS cells).

REPLY: We have revised the text and the figures to better represent this result. We have added two color line scans showing the enrichment of the H3K9me3 is co-incident with the DAPI. We have also generated supplemental movies of J1 and MEFs (Supplemental Movies 1 and 2) to demonstrate that the two signals are coincident through the optical sections. We have also included the following revision in the text to emphasize these data on Page 8 "Analysis of optical z-stack images and two-channel line scans confirmed that DAPI enrichment is always associated with H3K9me3 enrichment in these cells (Figure 3A and Supplemental movies 1 and 2)."

8. Page 3 (of the Results section): Please add additional references regarding chromocenters in pluripotent and differentiated cells in addition to Ahmed et al., 2009.

REPLY: We have added two additional references (Gaspar-Maia et al, 2009; Martin et al, 2006) and would welcome additional suggestions if the reviewer feels we have omitted any critical studies.

9. In order to demark the constitutive heterochromatin domains and to correlate them with ESI, H3K9me3 immunostaining is used (Fig. 4). In this case the analysis of the chromocenters by scan plot and analysis of the bright regions in relationship to the nuclear background should be made on the H3K9me3 stained nuclei and not on the DAPI signal (Fig. 3). Alternatively, one can correlate the ESI images to the DAPI signal. This is important to get coherent and strong evidences for these correlations.

REPLY: Related to the response to Reviewer 2 comment 7 we have subsequently provided data showing the relationship between DAPI and H3K9me3 immunostaining. Unfortunately it is not possible to use DAPI for correlative LM/ESI studies. We hope that our discussion and inclusion of these new data will satisfy the reviewers concerns.

10. Figure 3B: The figure is labeled incorrectly. I believe 'MEF' should be at the right?
REPLY: We thank the reviewer for noticing this error. The reviewer's assumptions were entirely correct and we have relabeled Figure 3B.

11. In the Materials and Methods part, please provide a description of the scan plot analysis and the calculation of the background to chromocenter ratio shown in Fig.3B for example.

REPLY: We apologize for the omission of this description in our original submission and have included a comprehensive description of both the scan plot analysis and of the integrative phosphorus density analysis in the Materials and Methods. (Details sighted above in relation to Reviewer 1, comment 5.)

12. Page 4 of Results: the authors discuss their findings of 10 nm fibres in constitutive heterochromatin of MEFs. Since this is a crucial part of the paper it is important to strengthen this point. Do other somatic cells show the same 10 nm fibres? Is the 30 nm fibre never detectable? Do all iPS cells show prevalence of 10 nm fibres? Some additional cell measurements and statistics would be appropriate.

REPLY: We feel that these are very interesting and critical questions, though some are beyond the scope of this paper. This study is the first study that we have undertaken to examine the structure of constitutive heterochromatin regions using correlative LM/ESI analysis. We are just embarking on evaluating whether a transition between 10 and 30 nm chromatin assemblies is involved in other

reprogramming experiments. The 30 nm chromatin fibre is readily detected by ESI. For example, starfish sperm chromatin appears entirely as 30 nm fibres when observed by ESI, which we have now referenced in the revised manuscript (Bazett-Jones, 1992), and discussed on page 11: "Although we detect no 30 nm fibres in these dispersed chromatin regions, 30 nm fibres can be detected by ESI. For example, 30 nm chromatin fibres have been imaged by ESI in starfish sperm (Figure S4) (Bazett-Jones, 1992)." We have also prepared a supplemental Figure for the reviewers' consideration demonstrating these 30 nm chromatin fibres *in situ* in these cells. Since similar images have been published, we do not think that this figure should be in the final manuscript, but will defer to the reviewer and the editor on this point.

We also cannot state that all fully reprogrammed iPS cells show a prevalence of 10 nm fibres in chromatin domains marked with heterochromatin-specific histone modification. We thought it important, however, to examine additional fully and partial reprogrammed cell lines, which we obtained from Dr. Yamanaka. These lines were generated in a completely independent manner than those in our lab. We have confirmed that disruption of constitutive heterochromatin domains of this fully reprogrammed iPS cell also occurs and well-defined chromocentres are maintained in the partially reprogrammed iPS cell line. The difference between these two cell lines based on both DAPI line scan analysis and by integrative ESI phosphorus analysis is extremely statistically significant. We have included these data in Supplemental Figure 3.

We have also added what we believe to be very exciting new data in Figure 4, three-dimensional tomographic information of the H3K9me3-enriched regions and the surrounding unmarked chromatin fibres in the pluripotent J1 cells. The dispersed chromatin fibres in these domains are entirely 10 nm chromatin fibres. The visual impression of this is confirmed by Fourier transform analysis.

13. In addition, as mentioned above for the title of the paper, the last sentence of this same section (page 5 of Results section): 'acquisition of the dispersed 10 nm fibre meshwork correlates with the fully reprogrammed state' does not reflect the data and should be rephrased.

REPLY: Related to our experiments described above we thank the reviewers for encouraging us to make these supporting data stronger by including the three-dimensional data of the J1 ES cell. Together with our 2i experiments we hope the reviewer will no longer take exception to this conclusion.

14. Figure 7A: the DAPI panel is too small to discern. Please show the DAPI panel at the same size as the Nanog panel. Figure 7B: how many nuclei were analyzed? Please indicate in figure legend. Figure 7C: the figure is not very convincing. Please provide the merged images and quantify Nanog positive / GFP-positive cells. (if the merged images do not provide more clarity, please remove it and the associated conclusions).

REPLY: We have enlarged the DAPI panel in Figure 7A to match the size of Nanog immunocytochemistry image. We wish to clarify in response to this comment that these were not the DAPI images used to generate Figure 7B or the analysis. Figure 7A is simply used to demonstrate a variegated Nanog expressing ES cell colony. We analyzed 30 high and 30 low nanog-expressing nuclei and have included this information in the figure legend. We have removed Figure 7C as this was a qualitative observation. Given other supporting data from previous studies we feel this does not change our conclusions. We have added to the text on the bottom of Page 13 "This is consistent with other reports showing that the retroviral reprogramming factors are specifically silenced in Nanog-GFP positive iPS cell colonies (Nakagawa et al, 2008)."

15. Figure 8B, the six low and high Nanog panels are bleary (out of focus??) in comparison with the Cdx2 and the GATA6 images. This is important as it may affect the measurements shown. Please provide other representative pictures (and corresponding measurements), which will be clearer.

REPLY: We apologize for the quality of the images used in the original submission. We replaced this panel with images of an embryo that contained cells where the measured chromocentres and the edge of the nucleus are in the same focal plane, the latter assessed by DAPI. We also applied deconvolution to aid in distinguishing the various fluorescence signals in the whole embryo. We hope the reviewer finds these new images to be more satisfactory.

16. Third sentence "... we show here that heterochromatin specifically enriched in H3K9me3 is composed of 10 nm fibers in ES and full iPS cells." Once again, more cautious is warranted, since 10 nm fibers are also reported in the partial iPS and MEF cells, the statement may cause confusion.

REPLY: We thank the reviewer for highlighting the logic problem, which we had inadvertently established in our first submission. This issue was also raised by Reviewer 1, comment 1. We have clarified this on Page 15, "We were surprised to observe 10 nm chromatin fibres in the very densely packed chromocentres of MEFs and partial iPS cells although we cannot exclude the possibility that 30 nm fibres can also be found in these structures. However, prevalence of 10 nm fibres in both compact and disrupted heterochromatin domains indicates the transition between open and closed chromatin domains involves, at least in part, transitions between closely packed and highly folded 10 nm chromatin fibres. This challenges the absolute requirement for transitions between 10 and 30 nm chromatin fibres in defining heterochromatin domains."

We have also changed several of the "10 nm mesh" descriptions to reflect the striking difference in meso-scale organization that we have observed by describing domains in terms of open and closed chromatin domains throughout the text. We have also included the following argument on Page 14: "Although generally dispersed chromatin was previously observed in ES cells (Efroni et al, 2008), we show here that heterochromatin specifically enriched in H3K9me3 is composed entirely of 10 nm fibres in ES and full iPS cells. This is compatible with the general concept that pluripotent stem cells have more open chromatin structure to make the cells more responsive to differentiation cues that they receive."

Reviewer #3

1. For me the authors need to further address what the ESI approach is showing and to further develop the DAPI line scan assay so that it is more quantifiable.

REPLY: As noted by all reviewers, our description of the DAPI line scan analysis was not sufficient and has been remedied in the Materials and Methods section. The line scan analysis terminology was misleading since the width of the "line" corresponded to several pixels (~1/2 the width of the average chromocentre). Hence these scans are an integrated intensity band across the nucleus, normalized to both the background outside of the cell and relative to the nucleoplasmic signal. Normalization allows for comparisons between data sets acquired both on different days, microscope settings and camera parameters. We also analyzed a subset of these data using a variance analysis as recommended by Reviewer 3 (referred to on page 9 "These line scan analyses are supported by whole nucleus variance analyses which show a 9-fold increase in the signal variance between MEF feeder and J1 ES cells within the same image field." and the caption for Figure 3). We found the variance analysis to be entirely consistent with our DAPI line scan analysis, but could only be used to compare data acquired with consistent microscope parameters and specimen illumination.

We will address what ESI is detecting under comment 13.

2. The authors use of the term heterochromatin is quite loose. In the abstract they both refer to the silencing of genes as heterochromatinisation (line 3) and then talk about studying heterochromatin domains (line 4) although these are both quite different. At the end of the abstract they imply retroviral silencing is heterochromatinisation and then talk about constitutive heterochromatin structures. In the first line of the introduction they talk about heterochromatinisation of tissue specific genes. Later in the introduction (start of paragraph 3) the authors talk about changes in heterochromatin marks (presumably at genes) but then talk about heterochromatin domains (presumably constitutive heterochromatin) but these structures are entirely different in cells.

REPLY: We agree with the reviewer that we need to distinguish between gene loci heterochromatinization associated with gene-silencing vs. constitutive heterochromatin domains which are the focus of this study. We have now clarified this distinction throughout the manuscript. For example, in the first example in the Abstract "In contrast to loci-specific epigenetic changes, heterochromatin domains undergo epigenetic resetting during the reprogramming process but the effect on the heterochromatin ultrastructure is not known."

To further clarify the distinction of silencing epigenetic marks and heterochromatin, we

added on Page 4, "ES cells are known to have unique heterochromatin domain organization with hyperdynamic binding of histone and associated heterochromatin structure proteins (Meshorer et al, 2006). In contrast to loci-specific epigenetic changes compatible with altered gene expression, changes to the physical structure of heterochromatin domain organization during reprogramming remain unexplored."

3. As this study focuses on constitutive heterochromatin domains I think the authors need to be more clear about this and make the distinction that constitutive heterochromatin is very different from the "heterochromatinisation" that occurs during stable gene silencing. It could also be misleading to relate what is found at constitutive heterochromatin to the silencing of genes - as the processes and chromatin structures involved will be entirely different.

REPLY: We agree with the reviewer's point. We think that this is very important in our study, which focuses on constitutive heterochromatin. We hope we have sufficiently clarified this distinction with the changes to the manuscript described above in response to comment 2.

4. *In the introduction the authors say evidence for 30-nm fibres in vivo is weak. This is true but there are two key pieces of data (i) low angle x-ray scattering clearly indicates that chromatin in cells is packaged into 30-nm fibres and (ii) chromatin isolated from cells adopts a 30-nm fibre structure under physiological conditions.*

REPLY: We appreciate the reviewer highlighting the omission of these data and have included a description of these experiments in the manuscript on Page 10 "Previous low angle x-ray scattering (Langmore & Paulson, 1983) and EM with conventional heavy atom contrast agents or of isolated chromatin experiments have indicated that the genome is comprised entirely of 30 nm and higher-order chromatin fibres (Gilbert et al, 2004; Sinclair, 2010)." It should be noted that Langmore's study was performed on sperm, chicken erythrocytes, isolated nuclei, and lymphocytes. We agree that 30 nm fibres are prevalent in sperm (see Reviewer 2, comment 12), and possibly in avian erythrocytes as well. Conditions of isolation and buffer conditions of purified nuclei can affect chromatin fibre transitions, and thus such results must be interpreted with caution. We hope in future to explore the chromatin fibre morphology of the very compact chromatin domains found in some cell types such as lymphocytes, but such findings are outside of the topic of this paper.

5. In the introduction (paragraph 4) the authors refer to one of their previous studies (Efroni et al., 2008) showing that "ES cells display a meshwork of 10 nm chromatin fibres through out the nucleus, and a paucity of the blocks of condensed chromatin observed in somatic cells". This was originally published as supplementary data and although there is a clear difference between stem cells and differentiated cells by ESI I feel describing it as a 10-nm chromatin fibre mesh is too prescriptive. At the end of paragraph 4 the authors also suggest that it is accepted that ES cell chromatin is organised as a 10-nm fibre structure, which I feel might be overstated.

REPLY: We have revised this section in the Introduction as follows on page 5: "When visualized by electron spectroscopic imaging (ESI) (Ahmed et al, 2009), the only technique that provides high contrast of unstained chromatin at high-molecular resolution (Bazett-Jones and Ottensmeyer 1981; Dehghani et al., 2005), the predominant chromatin configuration in ES cells is a mesh of "open" dispersed chromatin fibres, and displays a paucity of the blocks of condensed "closed" chromatin observed in somatic cells (Efroni et al, 2008)." Also, please see our response to Reviewer 1, comment 1.

6. Figure 2C. The authors show considerable differences in expression by qRT-PCR (4 orders of magnitude). Can I just confirm the scale is correct. The authors also do not mention what the error bars are.

REPLY: The scale in this Figure is correct. The error bars represent the standard error of the mean. This piece of information is now included in the Figure legend.

7. Figure 2D. Would a Western blot show the differences in expression more quantitatively than IF?

REPLY: Since western blot analysis is typically more quantitative than IF, we have now included

both approaches in Figure 2.

8. Figure 3.
The authors use DAPI and antibody staining to show that chromocentres are different between stem cells and differentiated cells. This analysis is very dependent on where the line is drawn through the nucleus. Rather than quantifying this as background:chromocentre DAPI ratio can the authors measure signal variance across the entire nucleus? From the data you would expect increased variance in differentiated cells.

REPLY: We have confirmed that our DAPI line scan analysis is consistent with a variance analysis, and have added the results of variance analysis in comparing chromocentre DAPI signals in one of the reprogrammed lines, compared to chromocentres of feeder cells (page 9). Unfortunately, our original description of the line scan analysis was inadequate to evaluate this analysis, and have clarified the description of the analyses in the Materials and Methods section.

9. I feel the authors need to emphasise that DAPI staining is both affected by chromatin packaging (e.g. can see a Barr body using DAPI in female cells) and by DNA sequence (preferentially binds AT DNA). This therefore makes it difficult to interpret what DAPI staining is actually showing us about chromatin compaction.

REPLY: We thank the reviewer for highlighting this important point. We agree that interpreting DAPI as a measure of chromatin density must be approached with caution, because of its preference for AT-rich DNA, and other reasons. Because DAPI and other fluorescence microscopy approaches are not necessarily linear relative to chromatin density, we have resorted to ESI, which is linear and hence quantitative. We have now addressed this concern in the manuscript in the Results on Page 9: "DAPI is not a good indicator of chromatin fibre density *per se* because it is also affected by AT-richness and nucleosome-repeat length. Therefore, to interpret differences in heterochromatin organization observed by DAPI counter stain we employed Electron Spectroscopic Imaging (ESI)."

10. If the DAPI linescan assay is reflective of a change in chromocentre structure then as the chromocentre structure changes with differentiation so will the area the chromocentres occupy in the nucleus. As DAPI staining is both reflective of chromatin compaction and AT-binding maybe another technique could be used. It would be possible to stain chromocentres using FISH for major satellite and quantifying the chromocentre area in the nucleus. Alternatively the cells could be transfected with GFP-H3 (or equivalent) to mark the "compact" heterochromatin and then quantify the area they occupy in the nucleus.

REPLY: Given that we have analyzed these chromatin domains with ESI, which produces very high contrast and spatial resolution, superior to fluorescence microscopy methods, and because it is a quantitative technique, we feel that using a lower resolution technique such as FISH, which also causes disruptions to local meso-scale chromatin organization, would not add to the findings of this study. We also argue that a GFP-H3 experiment would not provide any additional insight not revealed by DAPI (equivalent resolution and lacks linearity) or ESI analysis (resolution at the molecular level and is fully quantitative).

11. The authors need to label the y axis of the graph in B and the size of the scale bar in A is not mentioned in the figure.

REPLY: We apologize for missing this critical detail. The Y-axis label has been added and the scale bar is now mentioned in the Figure legend.

12. Figure 4
For me this is really the crux of the paper. The authors use LM/ESI to visualise the chromocentres by H3K9me3 staining and analyse the phosphorus/nitrogen content of the chromocentres as a measure of chromatin compaction. The results clearly show there is a difference in chromocentre architecture by ESI and the authors interpret this to mean chromatin structure has changed and that stem cells have a dispersed 10-nm fibre structure.

In fig 4A the authors show considerable phosphate/nitrogen enrichment (yellow) in the

chromocentre for MEFs which they interpret to indicate they have a high density of chromatin (i.e. compact chromatin). In contrast they show that stem cell like chromocentres (e.g. EOS3F-28) have the same chromatin compaction as euchromatin. Previously GFP-H3 (or equivalent) has been visualised in a number of stem cells and differentiated cells. By fluorescence microscopy the chromocentres in ES cells clearly have more GFP-H3 associated with them. This would argue that chromocentres do have a more compact structure than euchromatin. If this is not seen by ESI could this mean that ESI is visualising something different to chromatin structure. These figures need scale bars. Fig 4B. I am not sure this analysis is very informative as it depends on where the analysis line is drawn through the nucleus.

REPLY: We appreciate this concern that the reviewer has raised. If the GFP-H3 signal corresponding to a chromocentre is above the background, then the nucleosome density must indeed be greater than the surrounding background region. If a higher chromatin density is not seen with ESI, then the reviewer is justified in asking what ESI is really detecting. Foci of DAPI and GFP-H3 have been used to argue that normal chromocentres exist in ES cells or epiblast cells in early mouse embryo. With contrast enhancement, even small increases in fluorescence intensity in these domains above the surrounding nucleoplasm or other surrounding chromatin domains can create the impression that chromocentres consist of densely packed chromatin. Adjusting contrast settings of fluorescence micrographs leads to nonlinearity and can create false impressions of chromatin density. Unfortunately, we may have created the impression that the chromatin density in the dispersed chromocentres is absolutely equal to that of the surrounding chromatin when visualized by ESI. That is not necessarily the case. Sometimes the H3K9-labelled chromatin domain does show a higher chromatin density compared to surrounding chromatin when the region is examined by ESI. We have clarified this point in the text, including on page 10, "At higher resolution, we observed an abundance of dispersed chromatin within chromocentre domains of ES and full EOS-28 iPS cells that is difficult and sometimes impossible to distinguish from the surrounding chromatin (Figure 4C)." We agree that DAPI and GFP-H3 could detect local changes in chromatin density, indeed we used DAPI extensively for this purpose, but ESI is a superior method for detecting and quantifying either large or small differences in chromatin organization and density. The major point that we have re-stated for clarity throughout the text is that chromocentres consist of densely packed chromatin in differentiated and partial iPS cells, but consist of chromatin fibres that are significantly more dispersed in ES cells (as demonstrated in Efroni et al., 2008) and full iPS cells. This dispersal of chromocentre chromatin fibres is a hallmark of pluripotency.

In the context of this concern, we state that we have added scale bars to these figures. We have also clarified both in the Materials and Methods section and the figure legends that line scans were not used to analyze the phosphorus density of heterochromatin relative to the surrounding chromatin within the field, instead an integrated density analysis was used to compare these structures to the surrounding regions. (Also, see response to Reviewer 1, comment 6.)

13. The Reviewer's concern about what ESI can detect is further indicated by:

Fig 4C. This panel is highly significant. The authors say they are visualising individual chromatin fibres, including 10-nm fibres, within the chromocentres. If these are individual chromatin fibres it should be possible to calculate how much chromatin is in the chromocentres. As a quick calculation 8% of the mouse genome… I therefore wonder if it is difficult to categorically say that stem cell chromatin has a decompacted 10-nm fibre structure… . . Is it possible that the ESI is revealing something slightly different or only showing a snap-shot of chromatin fibres?

REPLY: We wish to emphasize that ESI is a fully quantitative analytical method. The elemental signals obey Beer's Law, and the detection sensitivity surpasses that required to visualize pure B-form DNA (many references for ESI (also known as Electron Energy Loss Spectroscopy) from the materials sciences, and Bazett-Jones et al., 1999; Bazett-Jones, 1999). We have shown that linker DNA between reconstituted nucleosomes can predict both the mass and phosphorus content of the nucleosomes themselves (e.g. Bazett-Jones, D.P., Côté, J. Landel, C.C., Peterson, C.L., Workman, J.L. (1999) SWI/SNF complex creates loop domains in DNA and polynucleosome arrays and can disrupt DNA histone contacts within these domains. Mol. Cell. Biol.19: 1470-1478.). Hence, the sensitivity and linearity of the method for DNA detection are well documented. However, to demonstrate this further in this revised manuscript, we used measured phosphorus density of individual nucleosomes in images of the 70 nm sections to estimate the total DNA content within

the physical section, and by extrapolation, the total DNA content of the entire mouse nucleus. This analysis has been added to the Supplemental Materials and Methods, even though similar stoichiometric and quantitative demonstrations have been previously published (e.g. Bazett-Jones et al., 1999). We think that this analysis is superior to attempts to calculate the DNA content of chromocentres. First, there is no *a priori* way of knowing how much DNA is in a chromocentre since the number of chromosomes that contribute to a single chromocentre is variable, and there is more than just major satellite DNA in the structure. Hence, we argue that the analysis shown in the Supplemental Materials and Methods demonstrates that ESI can detect and display all of the DNA in a field. Further, whether ESI can detect 30 nm chromatin fibres in sectioned material, we demonstrated this previously (Bazett-Jones, 1992). We have also included images of 30 nm chromatin fibres imaged in starfish sperm nuclei (Supplemental Figure S4). This demonstration is superior to an *in vitro* reconstitution experiment since it shows "real" 30 nm fibres in a physiological context.

14. B. As for figure 3B I am not sure how informative this analysis is as it will depend on where the linescan is drawn. Also as DAPI staining is showing a very different level of chromatin organisation to the ultrastructure of the chromocentres I think it is difficult to relate these two pieces of data to each other (in the text it states:"thus, DAPI line scan analysis supports the LM/ESI data"). Fig 7B and Figure 8B. As for previous figures I feel this linescan analysis might be difficult to accurately quantify.

REPLY: We have clarified the DAPI line scan analysis and have confirmed that it is consistent with a variance analysis (see comment 1, above). DAPI analysis shows significant differences in the organization of chromocentres in these cell lines, as does the ESI analysis. Hence, we think that it is fair to state the DAPI analysis supports the ESI analysis.

15. The authors mention "alpha satellite" in a few places. I think the satellites in mouse are either called minor or major satellites.

REPLY: We agree with the reviewer and have replaced these occurrences with "major" satellite.

16. I wonder if the title of the paper is too prescriptive and could be "Constitutive heterochromatin reorganisation during somatic cell reprogramming"

REPLY: We thank the reviewer for suggesting this alternative title, which we have adopted.

2nd Editorial Decision 25 January 2011

Your revised manuscript has been reviewed once more by the original referees. There are a few issues still remaining that should be addressed prior to publication.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.
When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have responded to reviewers' comments with substantive and effective changes to the ms. that have greatly strengthened it. It now appears suitable for publication.

Referee #2 (Remarks to the Author):

Comments on 'Constitutive heterochromatin reorganization during somatic cell reprogramming' by Fussner et al.

In the revised manuscript the authors address some of the concerns which were raised in the previous version and improved a good deal of the data. However, the paper still suffers from some important issues that must be addressed before publication is recommended.

1. The authors refer to the changes that they observe in heterochromatin organization as "a late event". This statement is not supported by any data in this manuscript and should be omitted from the paper. Early and late can only be referred to when actual time course experiments are being conducted. The authors rely on partial iPS cells, which are somewhere between MEF cells and iPS cells but there is no reason to believe that they are similar to ES cells / iPS cells. The authors' microarray analysis and PCA analysis supports the idea that the partial iPS cells are as dissimilar from iPS cells (~5000 differentially expressed genes) as they are from MEFs (~5600 differentially expressed genes). Moreover, partial iPS cells merely represent a stable state somewhere along the axis of reprogramming. These cells are much more subjective than iPS cells, which can be characterized based on similarities to ES cells. Since there is no gold standard for characterizing partial iPS cells, they should simply be regarded as a stable state between MEFs and iPSCs. The additional experiment that the authors present using 2i was also done on a period of 1-2 weeks and lacks the time course dimension that is required to reach this conclusion.

2. In regard to the comments made by all three reviewers concerning the dependency on DAPI staining throughout the manuscript, the authors added line scans for H3K9me3 staining in Figure 3, but performed the analyses on DAPI (Figure 3B). Similar DAPI-based analyses are also provided in Figures 6B, 7B and 8B. I still feel, as in the previous version, that these analyses would be more informative and accurate if they were performed on H3K9me3 rather than DAPI.

3. Figures 7-8 remain unconvincing. The DAPI staining in Figure 7A and 8A (even when blown up to reach the maximum resolution) looks identical between all the different cells whether Nanog high or low. Figures 7B and 8B do not add much confidence in this assay. Once again, it might be useful to analyze H3K9me3 instead or omit these figures from the manuscript, perhaps as supplementary figures. I do not feel that they are as strong and convincing as the rest of the paper.

Referee #3 (Remarks to the Author):

I am satisfied with the authors additional revisions and I feel this is an interesting manuscript that discusses a number of controversial issues. I still have some concerns over some of the conclusions (i) ES cell heterochromatin is entirely composed of 10-nm fibres and (ii) ES cells having poorly defined chromocenters indicating they have an open structure. Although I accept these are possible interpretations of the data.

However, my opinions should not detract from this manuscript and if anything shows there is significant scope for more studies on constitutive heterochromatin and ES cell chromatin structure using different approaches.

My only comment is that the last line of the abstract is not so clear. If I understand correctly could it be better worded as "... heterochromatin reorganizes into 10-nm chromatin fibres in the very late stage of iPS cell reprogramming"

2nd Revision - authors' response 09 February 2011

Referee #1 (Remarks to the Author):

The authors have responded to reviewers' comments with substantive and effective changes to the ms. that have greatly strengthened it. It now appears suitable for publication.

REPLY: We thank the reviewer assistance in strengthening the manuscript.

Referee #2 (Remarks to the Author):

Comments on 'Constitutive heterochromatin reorganization during somatic cell reprogramming' by Fussner et al.

In the revised manuscript the authors address some of the concerns which were raised in the previous version and improved a good deal of the data. However, the paper still suffers from some important issues that must be addressed before publication is recommended.

1. The authors refer to the changes that they observe in heterochromatin organization as "a late event". This statement is not supported by any data in this manuscript and should be omitted from the paper. Early and late can only be referred to when actual time course experiments are being conducted. The authors rely on partial iPS cells, which are somewhere between MEF cells and iPS cells but there is no reason to believe that they are similar to ES cells / iPS cells. The authors' microarray analysis and PCA analysis supports the idea that the partial iPS cells are as dissimilar from iPS cells (~5000 differentially expressed genes) as they are from MEFs (~5600 differentially expressed genes). Moreover, partial iPS cells merely represent a stable state somewhere along the axis of reprogramming. These cells are much more subjective than iPS cells, which can be characterized based on similarities to ES cells. Since there is no gold standard for characterizing partial iPS cells, they should simply be regarded as a stable state between MEFs and iPSCs. The additional experiment that the authors present using 2i was also done on a period of 1-2 weeks and lacks the time course dimension that is required to reach this conclusion.

REPLY: We have removed the "late-stage" conclusion from the abstract and text. We would like to emphasize that our use of late-stage refers to the acquisition of hallmark characteristics of pluripotency rather than a strict temporal order as used by the referee. In this regard, the partial iPS cells that we study (including those from the Yamanaka group) are already pluripotent, in that they form cells of the three germ layers *in vivo* in teratomas and *in vitro*. While we cannot conclude that the reorganization is temporally a very late event in the conversion process, our data shows that heterochromatin reorganization occurs after this level of pluripotency is attained in partial iPS cells but before the endogenous pluripotency network is activated in full iPS cells. Therefore we now state: "We speculate that this transition of heterochromatin domain reorganization occurs at a late stage in reprogramming as only the cells which have silenced the transgenes, a previously described late-stage event in iPS reprogramming, have disrupted heterochromatin."

2. In regard to the comments made by all three reviewers concerning the dependency on DAPI staining throughout the manuscript, the authors added line scans for H3K9me3 staining in Figure 3, but performed the analyses on DAPI (Figure 3B). Similar DAPI-based analyses are also provided in Figures 6B, 7B and 8B. I still feel, as in the previous version, that these analyses would be more informative and accurate if they were performed on H3K9me3 rather than DAPI.

REPLY: We apologize for not clearing this issue up in our last submission, we were under the impression that the issue taken by all three reviewers was whether or not the DAPI staining and H3K9me3 labeling was co-incident.

The remaining issue, which we did not clarify in the last submission, is that although the DAPI and H3K9me3 are co-incident they do not detect the same underlying biochemical components, and thus, are not equivalent. We observed qualitatively similar distributions of H3K9me3 fluorescence signals in differentiated vs. pluripotent cell types, whereas significant

differences were observed in the DAPI signals, which were confirmed and strengthened by the striking differences measured by ESI. We have added a sentence in the Results section and a paragraph in the Supplementary Information on the advantages and caveats of optical methods to measure chromatin density. The added sentences in the Results on pages 8-9 reads, "Although H3K9me3 enrichment is useful for identifying chromocenters in all analyzed cell types, it cannot be used to measure chromatin fibre density. On the other hand, though DAPI binds AT-rich DNA sequences preferentially, its signal intensity is a more accurate measure of chromatin compaction than detection of specific histone modifications. Indeed, close inspection of DAPI stained nuclei revealed significant differences between differentiated and pluripotent cell types. A description of the advantages and caveats of these optical methods to measure chromatin density is provided in Supplementary Information." The new section in Supplementary Information on measuring chromatin density reads,

"*Optical Methods to Measure Chromatin Density*

Constitutive heterochromatin was identified throughout this study on the basis of the biochemical enrichment of H3K9me3, a classic modification associated with constitutive heterochromatin domains and enriched in chromocentres in MEF cells. In fact, H3K9me3 is a common feature of chromocentres in all analyzed cell types. However, H3K9me3 immunofluorescence microscopy could be problematic for making conclusions about differences in chromatin density between differentiated and pluripotent cell types. The potential problem exists if primary antibody access in compact versus dispersed chromatin differs by even a small factor. Moreover, we do not know whether chromatin modifications (such as H3K9me3) in constitutive heterochromatin are absolutely conserved upon reprogramming. It is possible that changes in the distribution of histone modifications between repetitive and non-repetitive chromatin sequences accompany the acquisition of the pluripotent state. Reorganization of the H3K9me3 mark would preclude it from being used to make conclusions about changes in chromatin density. On the other hand, even though DAPI binds AT-rich DNA sequences preferentially, its signal intensity is a better reflection of chromatin density than immunofluorescence signals of specific histone modifications. ESI, however, is superior to both immunofluorescence microscopy of histone modifications and DAPI imaging for measuring chromatin fibre density. Not only does ESI provide much higher spatial resolution, but it also measures chromatin directly, without the caveats introduced by stains or contrast agents."

3. Figures 7-8 remain unconvincing. The DAPI staining in Figure 7A and 8A (even when blown up to reach the maximum resolution) looks identical between all the different cells whether Nanog high or low. Figures 7B and 8B do not add much confidence in this assay. Once again, it might be useful to analyze H3K9me3 instead or omit these figures from the manuscript, perhaps as supplementary figures. I do not feel that they are as strong and convincing as the rest of the paper.

REPLY: Again we apologize for not clarifying this point in our last submission. The DAPI staining shown in Figure 7A was taken at a very low magnification on a cell culture microscope and was meant only to indicate the distribution of individual cells, so that in very low Nanog expressing nuclei readers are able to identify these particular cells by DAPI counterstain. We have clarified this point in the figure legend by distinguishing the low versus high magnification (resolution) images.

"**A** Low magnification immunofluorescence of variegated Nanog expression in a J1 ES cell colony with DAPI shown to the right, scale bar 20 μm. **B** High-magnification and resolution representative image of a J1 ES cell field with high and low Nanog expressing cells (top left panel) and DAPI counterstain of a 0.3 μm z-stack series of these same nuclei."

Figure 8A is only showing the distribution of transcription factors, which were used to identify the different cell types. There is no DAPI image in Figure 8A. We have digitally enhanced the contrast slightly in Figures 7B and 8B. This has effectively reduced the background haze that arises from out-of-focus light of thick specimens (such as embryos). We hope that the reviewer finds the contrast enhanced images easier to interpret. In addition, we have added double-headed arrows to the line scans shown in Figure 8B to clarify the DAPI signal in chromocentres vs the nucleoplasmic background. We feel that both Figures 7 and 8 add significantly to this study, by supporting a role for Nanog in the early events of chromatin reorganization and reprogramming and

in generating insights into the timing of chromatin reorganization in reprogramming.

Referee #3 (Remarks to the Author):

I am satisfied with the authors additional revisions and I feel this is an interesting manuscript that discusses a number of controversial issues. I still have some concerns over some of the conclusions (i) ES cell heterochromatin is entirely composed of 10-nm fibres and (ii) ES cells having poorly defined chromocenters indicating they have an open structure. Although I accept these are possible interpretations of the data.

However, my opinions should not detract from this manuscript and if anything shows there is significant scope for more studies on constitutive heterochromatin and ES cell chromatin structure using different approaches.

My only comment is that the last line of the abstract is not so clear. If I understand correctly could it be better worded as "... heterochromatin reorganizes into 10-nm chromatin fibres in the very late stage of iPS cell reprogramming"

REPLY: We thank the reviewer for the suggestion to improve the Abstract. Because of the concerns raised by reviewer 2 on the timing and temporal nature of the reorganization events, we have removed the "very late stage" reference from the Abstract, but have adopted the essence of this reviewer's suggestion for the concluding statement. The final statement in our abstract now reads: "Thus, constitutive heterochromatin is compacted in partial iPS cells but reorganizes into dispersed 10 nm chromatin fibres as the fully reprogrammed iPS cell state is acquired."

3rd Editorial Decision 02 March 2011

Your revised manuscript has been re-evaluated once more by referee #2. S/he remains unconvinced by the quantitation of the heterochromatin foci, the other referees feel that this issue has been addressed and this remaining concern will be on the record in the Review Process File. I accept your reasoning for selecting the DAPI signal for quantitation and accept the manuscript for publication in The EMBO Journal. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
The EMBO Journal

Referee #2

The authors addressed the first point, but not the second and third. In essence, DAPI does not give the exact same pattern as a bona fide heterochromatin marker, say H3K9me3. Therefore, quantification of all the heterochromatin foci should not be done just by DAPI staining.

