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Transcription and histone methylation changes correlate with imprint acquisition in male germ cells

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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 03 August 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below. As you will see from their comments they find the study to be interesting and require several issues are addressed, one of the most important being the concerns raised by referee #2 regarding the difference in the H3K4me3 marks, which clearly needs to be resolved. Should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

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):

In this manuscript, the authors examined epigenetic changes controlling the establishment of paternal genomic imprints in male germ cells. They showed that two paternally methylated mouse ICRs, the H19 DMR and the Ig-DMR, lose histone H3 lysine methylation marks (both active and inactive marks) before acquisition of DNA methylation imprints in male germ cells. By contrast, two maternally methylated ICRs showed methylation at H3K4 (an active mark) and served as active promoters in male germ cells. Interestingly, transcriptional read-through at the paternally methylated ICRs was found only in male germ cells and this occurred concomitantly with the acquisition of DNA methylation. These findings lead the authors to propose that transcription and loss of histone H3 lysine methylation pre-empt acquisition of the paternal imprints.

The study is on the whole very well performed and the findings are novel. The proposed model is fine and will be useful to guide future studies. The only thing that I want to point out is that, because they only showed simultaneous occurrence of transcriptional read-through and DNA methylation, the use of the word "pre-empt" in the title (Transcription ... pre-empt acquisition of ...) is not appropriate. The authors should use a more accurate wording in the title. Also, H3 should be "histone H3".

Other points

1. Page 7, 1st paragraph: The text says that "high transcript levels were detected ... in one of the first exons of Snrpn", but Figure 4A shows a result for exon 5. This is confusing and should be corrected appropriately.
2. Page 7, 2nd paragraph: Supplementary Figure 4A should be Figure 4A (no Supplementary).
3. Page 7, 3rd paragraph: Figure 4 should be Figure 4A.
4. Page 9, 2nd paragraph: Delete one of the duplicated "in post-meiotic".
5. Figure 5B: Primers for region C' is not listed in Supplementary Table 1. Also, the extent of region B-C does not look what is expected from the locations of regions B and C in Figure 5A. The second bar from the right should be marked "D-E".
6. In some graphs (eg. Figure 3A), bars of different colors indicate different cell origins (somatic, female germ, male germ) but in others (eg. Figure 4A), the same color pattern shows different developmental time points (13.5, 15.5, 17.5 dpc). The figures will become more comprehensible if the graphs are prepared according to one common rule.

Referee #2 (Remarks to the Author):

DNA methylation is thought to be the primary mark determinant for genomic imprinting acquisition during gametogenesis. Using a developmental study based on cell sorting during the fetal stages of gametogenesis, the authors analyzed here the variations in histone modifications and transcription events occurring before and concomitantly with the process of de novo methylation at imprinting control regions (ICRs) in male germ cells. By highlighting a specific enrichment in H3K4me3 methylation at maternal ICRs in male germ cells, their cchIP approach pointed towards a negative correlation between this mark and DNA methylation acquisition. On the other side, paternal ICRs lacked this mark but were rather traversed by transcripts originating from further promoters. The authors then concluded that DNA methylation at paternal ICRs is preceded by specific changes in chromatin states and read-through transcription occurring specifically in male germ cells.

This work provides an important and completely novel piece of information regarding the chromatin states of ICRs of both parental origins, in a physiological imprint-free situation (13.5dpc PGCs) and during the process of imprint acquisition (15.5-17.5dpc) in fetal male germ cells. An interesting part is notably the observation that erasure of DNA methylation patterns at 13.5dpc is also accompanied by erasure of H3K9 and H4K20me3 patterns, while methylation at H2A/H4R4 is persistent. This work also extends further the positive correlation between transcription and DNA methylation that

can be observed at non-promoter regions.

However, there are 2 major caveats I would like to raise, to give the opportunity to the authors to discuss more accurately the reach of their results:

1- H3K4me3 is a typical mark for promoter regions. Its study in the context of maternal ICRs (KvDMR and Snrpn DMR) is then fully justified. However, as paternal ICRs map to intergenic regions, H3K4me3 is not expected in such regions, in somatic or germ cells, and this is indeed in agreement with what the authors found. It can be seen from Fig 3A that paternal ICRs do not harbor H3K4me3 in imprint-free situation (13.5dpc), during the course of imprint acquisition (15.5dpc) and in fully imprinted states (somatic cells of the fetal gonads). So, is it really legitimate to argue that maternal and paternal ICRs have different states of histone methylation at the time of imprint acquisition in male germ cells? They are actually rather similar in terms of H3K9, H4K20 and H2A/H4R3 methylation, the only difference at 15.5dpc is this H3K4me3 mark, which may at the end rather reflect genomic features than epigenetic differences. The mark that should have been studied is H3K4me2, which reflects a permissive chromatin state in promoter and non-promoter regions. This point is honestly raised page 9 by the authors, who recognized they faced a specificity issue with the antibodies they used. Unfortunately, I don t see how the conclusion that maternal and paternal ICRs are different at the time of imprint acquisition in male germ cells, and therefore that H3K4me3 may play a role in parental imprint distinction, can be maintained.

2- The deposition of methyl groups at paternal ICRs and the production of transcripts originating from upstream locations are completely coincident. De novo methylation is indeed initiated at 15.5dpc and further consolidated at 17.5dpc; transcript level at both the H19 and ig-DMR loci is also progressively increasing from 15.5dpc to 17.5dpc. According to the authors, this can be taken as evidence that the transcriptional read-through guides the acquisition of DNA methylation at H19 ICR and ig-DMR. However, equally possible is the hypothesis that DNA methylation favors the elongation of these transcripts initiating upstream from the ICRs, and this would actually be in agreement with the fact that gene body methylation is associated with active transcription. In this regard, the authors wrongly cite the Wu et al. 2010 paper. The original interpretation from that paper is that Dnmt3a-dependent DNA methylation in gene bodies is a pre-requisite for active transcription of these genes (based on the use of Dnmt3a-/- NPCs). The authors mention this work in their manuscript to emphasize the fact transcription is a pre-requisite to de novo methylation.

I would suggest the authors to either discuss equally the 2 possibilities (transcription elongation favors DNA methylaton or inversely, DNA methylation favors transcription elongation) and/or to include a later time point to assess the level of production of the traversing RNA, when imprints are fully established, in early post-natal stages. It may indeed be interesting to see if this transcript is still produced once methylation is fully acquired.

Then I have a few minor points:

- In the introduction page 3, it should be pointed that DNMT3A itself is also able to sense H3K4 methylation states, not only DNMT3L. See Li et al., 2011 and Otani et al., 2009.
- Page 7, beginning of 2nd paragraph; the figure call should be Figure 4A (and not Supplementary Figure 4A).
- Figure 5B: the D-E legend is missing from the drawing illustrating the amplification product mapping.
- It is not said why female germ cells at 15.5dpc were not studied in Figure 3A, while they were in Figure 4. Why?

Referee #3 (Remarks to the Author):

This paper addresses the question what signals paternal imprint acquistion in male germ cells. Previously DNA methylation was assayed in PGCs but no extensive histone methylation analysis has been reported. Here, the investigators assay DNA methylation, H3K9me3, H4K20me3, H2A/H4Rme2s and H3K4me3 in PGCs. From these experiments, it appears that DNA methylation precedes repressive histone modifications at paternally methylated regions in male PGCs.

Additionally the authors make the surprising observation that high transcriptional read-through is detected at the paternal ICRs H19-DMR and Ig-DMR during the time of imprint establishment. Together these results suggest a role for DNA methylation, transcription and histone modifications in acquisition of parental imprints. The data are clear and robust and the paper is well written. Although some important new information has been uncovered by this work, the mechanism by which parental imprints are placed on specific regions of the genome remains unclear.

Specific comments:

Abstract: "To address this question, we explored, for the first time, histone methylation at ICRs in mouse primordial germ cells (PGCs)." This concept of "for the first time" is written a few times in the manuscript and while it is true that PGC chromatin was assayed in detail at ICRs for the first time here, Szabo and colleagues reported some histone methylation at the H19 ICR in a 2010 PLoS Genetics publication. Thus, this claim is not technically accurate and must be modified or removed.

Methods: The methods in this paper are quite brief. More specifically, it would be useful to know how many times each of the experiments were performed on independently isolated populations of germ cells, including for the DNA methylation and RNA expression analysis.

Page 4: "At the Ig-DMR controlling the Dlk1-Dio3 domain (Lin et al, 2003), the timing of imprint acquisition was comparable to the H19 DMR, with no apparent difference between the parental chromosomes." How does one decide that there is "no apparent difference?" Given reports showing asymmetry between acquisition of DNA methylation on the H19 and Snprn parental alleles, a difference would be expected. Is there a statistical analysis to be used? As mentioned in the previous comment, how many times was the experiment performed?

Figure 2 legend: "Bound chromatin fractions were quantified by real-time PCR and corrected for background precipitation (percentile precipitation with standard deviation)." What is background precipitation? IgG?

Figure 3. Since the bar graphs for H19 DMR suggests essentially no H3K4me3, how can the depiction of the data in part 3 (bottom) have 50/50 for that chromatin mark? Also, there appear to be some parental biases for Snrpn DMR and KvDMR1 in the gels shown in B. The legend states that there is no parental bias for the H2A/H4Rme2s, but there seems to be significant bias in the gels for Snprn.

Page 7. "To assess whether transcription originated from within the KvDMR1, we also amplified the cDNA with a primer pair at the 5' extremity of the KvDMR1, upstream of the somatic transcription start site. As in somatic cells, no amplification was detected in the germ cells at this region, suggesting that the KvDMR1 itself acts as a promoter in PGCs as well."
To show that the same promoter is used, the investigators should use RACE or tiling arrays or a related technique. The use of only two primers does not fully address the question.

Discussion: "These data evoke, for the first time, a link between histone methylation, transcription, and imprint acquisition in male germ cells." Although highly suggestive, the link is only correlative and there is no data to support that these properties are mechanistically related or that one may be the initial trigger.

Revision 05 October 2011

Answers to the reviewers

We thank the reviewers for their interest in our study on imprinting control regions (ICRs) in mouse PGCs, and for their pertinent comments and suggestions. Our answers to their points are given below. The corresponding textual changes are marked in red in the revised manuscript.

Referee 1:

Suggestion to change the title:

We agree that our study shows a correlation between non-coding RNA transcription and changes in histone H3 lysine methylation and imprint acquisition. To more carefully convey the conclusions and limitations of our study, we changed the manuscript title as follows (see also answer to point 1 of referee 2):

'Transcription and histone methylation changes correlate with imprint acquisition in male germ cells'

Other points:

1. Page 7: We corrected this error, and changed the sentence as follows:

'At the Snrpn DMR, transcripts are readily amplified (from both the parental alleles) between Snrpn exons 1 and 3 (Supplementary Figure S4); similarly high transcript levels were detected with primers at exon 5 (Figure 4A).'

2. Page 7: We corrected this mistake: 'Next, we explored transcription at the *H19* DMR and the Ig-DMR (Figure 4A).

3. Page 7: We now put 'Figure 4A'.

4. Page 9: We deleted 'in post-meiotic', which by mistake was put twice in the original text.

5. We corrected the omission of these primer sequences. They are now provided in Supplementary Table 1. Figure 5A has been modified as suggested by the reviewer.

6. Colour codes in the figures. These have now been homogenised throughout.

Referee 2:

This reviewer asks us to discuss more accurately the reach of our findings, and provides helpful suggestions on how this can be done.

Main point 1:

We agree with the reviewer that the difference in H3K4me3 between maternal and paternal ICRs is not specific to the time of imprint acquisition. We observed that during PGC development, this mark becomes enriched on both the parental alleles of "maternal" ICRs, while it is hardly detected on "paternal" ICRs. Our claim is that H3K4me3 and the associated promoter activity could play a role

in the specificity of imprint acquisition, by protecting maternal ICRs against acquisition of DNA methylation in the developing male germ cells.

As stated by the reviewer, analysing H3K4me2, should have been more appropriate to reveal possible "true" epigenetic differences between maternal and paternal ICRs, the latter not having promoter activity. Unfortunately, we were unsuccessful in the study of this mark due to the insufficient quality of the control experiments with the antisera tested.

We agree with the reviewer that our carrier ChIP data on H3K4me3 needed to be interpreted in a more restricted manner. Consequently, we toned down our interpretation of these data at different places in the revised manuscript:
-In the title we no longer use the term 'loss of H3 methylation'. The new title uses the term 'histone methylation changes', to account not only for the loss of H3K9me3 and H4K20me3, but also for the observed gain of H4R3me2s:
'Transcription and histone methylation changes correlate with imprint acquisition in male germ cells'

-In the Abstract, we now comment only on a possible role for H3K4me3 at maternal ICRs:
'Furthermore, during male PGC development, H3 lysine-4 trimethylation becomes biallelically enriched at "maternal" ICRs, which are protected against DNA methylation, and whose promoters are active in the male germ cells'.

-In the first paragraph of the Discussion, we changed the last sentence as follows:
'These data evoke the possibility that histone methylation and transcriptional events are linked to the specificity of imprint acquisition in male germ cells'.

Main point 2:
The reviewer raises the interesting possibility that 'DNA methylation could favour the elongation of these transcripts initiating upstream from the ICRs, and that this would be in agreement with the fact that gene body methylation is associated with active transcription'

We agree that this alternative hypothesis should be mentioned in the Discussion as well. In fact, we detected high transcription through these paternal ICRs at 17.5 dpc, when the acquisition of DNA methylation at the Ig-DMR and *H19* DMR was virtually complete (all molecules have substantial DNA methylation). This strongly suggests that transcription across these regions continues after imprint acquisition and may therefore have been influenced by the presence of the newly acquired CpG methylation. In an earlier study (K. Delaval et al., 2007) on the organisation of ICRs in post-meiotic male germ cells (which have full DNA methylation at paternal ICRs), we did not obtain evidence for transcription at the Ig-DMR and *H19*-DMR in spermatocytes, round spermatids or elongating spermatids.

The reviewer is right that the Wu et al. (2010) paper was not quoted appropriately in our original discussion. In our revised manuscript, we now quote this study to indicate the possibility that the acquired CpG methylation could have augmented transcript levels across these two paternal ICRs.

Our initial hypothesis to explain the transcription data remains unaltered in the Discussion. It proposes that it is the transcription across these ICRs that facilitates imprint establishment, a mechanism which has been shown to act on one ICR in the female germ line (by Gavin Kelsey, Cambridge).

We now discuss both these non-exclusive hypotheses, and changed this part of the Discussion as follows: *'In the male germ cells, we detected high levels of transcription through the H19 DMR and Ig-DMR at 15.5 dpc, at the beginning of imprint establishment. Transcription levels were still high at 17.5 dpc, when methylation acquisition was almost complete. One hypothesis, therefore, could be that the acquired CpG methylation had led to enhanced transcription through these ICRs. A recent study on neurogenic gene loci provides evidence for such a mechanism in which DNA methylation enhances transcription (Wu et al. 2010). In an earlier study on post-meiotic male germ cells (which have full DNA methylation at paternal ICRs), we did not detect transcription across the Ig-DMR and H19-DMR (Delaval et al., 2007). An alternative, non-exclusive, hypothesis is that the transcription across the paternal ICRs facilitates imprint acquisition in the male PGCs. Insights into such a mechanism have recently been obtained relative to imprint acquisition in the female germline. At the Gnas locus on mouse distal chromosome 2, DNA methylation acquisition in growing oocytes requires transcription across the ICR (Chotalia et al, 2009). How, precisely, transcription could contribute to acquisition of de novo methylation in germ cells is unknown, but insights have emerged from studies on somatic cells. X-linked genes, for instance, have higher levels of DNA methylation on the transcriptionally active- than on the inactive X chromosome (Hellman and Chess, 2007). The observed strand-preference of transcription across both H19 DMR and Ig-DMR could be relevant in relation to recent work on rDNA genes, showing RNA-dependent acquisition of DNA methylation is linked to the formation of RNA-DNA triplexes (Schmitz et al, 2010). Together with insights from other model systems, our combined data make us propose a working model (Figure 6) in which a specific histone modification state and transcriptional read-through, producing long non-coding RNAs, guide the acquisition of DNA methylation at paternal ICRs.'*

For the transcript traversing the *H19* DMR, we include additional data to show that transcript levels are not linked to the activity of the *H19* gene itself. Although we find that this long ncRNA starts close to the *H19* gene promoter (Figure 5), but is transcribed in the opposite orientation, comparing male and female 15.5 dpc PGCs we find no correlation with the level of expression of the *H19* gene. In female germ cells, *H19* is more highly expressed than in male primordial germ cells, whereas the antisense transcript crossing the *H19* DMR is expressed at the highest levels in male PGCs. In the somatic control cells, *H19* was expressed about twenty fold higher than in male PGCs, and yet, the upstream ncRNA was not detected.

This is depicted in a new Supplementary, Figure S8, and strongly suggests that the upstream ncRNA is regulated independently from the *H19* gene transcript. We added a few sentences to this effect in the Results section (Page 8): *'Combined, these data suggest that the transcription across the H19 DMR predominantly initiates upstream of the H19 gene, which itself is highly transcribed in the male PGC cells as well. However, expression of the RNAs crossing the H19 DMR seems not linked to the expression of the H19 gene. In female PGCs, H19 is more highly expressed than in male PGCs, whereas the antisense transcript crossing the H19 DMR is expressed at highest levels in male PGCs. In somatic control cells, H19 is expressed twenty-five fold higher than in the male PGCs, and yet, the upstream ncRNA is not detected (Supplementary Figure S8)'*

Minor points:

-Page 3: DNMT3A itself also senses the H3 lysine-4 methylation state: We now included a sentence into the Introduction to indicate this important point: *'Recent studies show that DNMT3A itself is also sensitive to the H3 lysine-4 methylation status. Its 'ATRX-DNMT3-DNMTL' (ADD) domain binds to the H3 tail most efficiently when lysine-4 is unmethylated (Li et al, 2011; Otani et al, 2009; Zhang et al, 2010).*'

-We corrected this mistake: '(Supplementary Figure 4A)' was changed into '(Figure 4A)'.

-Figure 5B: We now show the D-E legend in this figure.

-Figure 3A: For our project on imprint acquisition in male germ cells, we performed carrier ChIP on both male and female PGCs, at 13.5dpc, but we limited this demanding approach to male cells at 15.5 dpc. Indeed, both DNA methylation and expression studies (which required low numbers of cells) indicate a dynamic process from 13.5 dpc onwards in male germ cells only. We therefore focused on the male germ cells in our chromatin studies at 15.5 dpc.

Referee 3:

Abstract: We agree with the reviewer that the earlier study by Piroska Szabo and her colleagues already reported chromatin studies in PGCs, at one ICR. Therefore, we now removed 'for the first time' from the abstract, the last sentence of the Introduction, and also from the Discussion.

Methods: We agree that the Materials and Methods section was rather concise and added further information, particularly to explain how many times experiments were performed. The following texts were added to Materials and Methods (marked in red, in the revised manuscript):

'cChIP data were obtained from at least three (and up to five) assays, performed on independent chromatin preparations, except for the data on male germ cells at 15.5dpc (Figure 3A), which derive from two independent experiments'

'DNA extraction and bisulphite sequencing were conducted on batches of ~15.000 cells, as described (Henckel et al, 2009). For each region analysed, CpG methylation profiles were obtained from at least three independent bisulphite treatments, with analysis of two or more independent PCR products per treatment. For each amplicon, methylation patterns were also assessed by digestion with "diagnostic" restriction endonucleases (the "Cobra" approach) followed by direct sequencing (data not shown). The two different approaches gave concordant results.'

Page 4, DNA methylation acquisition at the Ig-DMR:

In the representative methylation data presented in Figure 1 (results obtained from two independent PCR products from one treatment), we observed that 27% of CpG were methylated on the maternal allele and 32% on the paternal allele. Similar results were obtained from independent other bisulphite treatments. This is now explained in the legend to the Figure.

Although the measured percentages are not much different between the parental chromosomes, the reviewer is right that these data do not prove that the parental chromosomes have identical methylation levels. To provide a more accurately description, we changed the corresponding text in the Results section as follows: '*At the Ig-DMR controlling the Dlk1-Dio3 domain (Lin et al, 2003), the timing of imprint acquisition was comparable to the H19 DMR, with partial acquisition at 15.5 dpc and almost full DNA methylation at 17.5 dpc. The measured levels of DNA methylation were not much different on the parental chromosomes at 15.5 dpc'.*

Figure 2 legend, background precipitation levels:

This important issue is now further explained in the Materials and Methods:

'Input and antibody-bound fractions were quantified by real-time PCR amplification with a SYBR Green mixture (Qiagen) using an MX3000 apparatus (Stratagene). Background precipitation levels were determined by performing mock precipitations with a non-specific IgG antiserum (Sigma C-2288), and were only a fraction of that observed in the precipitations with specific antisera. Bound/Input ratios were calculated and were normalized, according to the antiserum used, against precipitation at IAP elements or Rpl30.'

Figure 3:

-The referee asks us how to reconcile the quantitative data shown to the left (Figure 3A), with the allele-specificity results shown to the right (Figure 3B). As concerns the *H19* DMR, we show in the left part of the figure that little H3K4me3 is precipitated (through analysis by real-time PCR). The allelic ratio determination shown in Figure 3 was performed following saturated PCR amplification (This is mentioned in the Figure legend). This qualitative assay determines the allelic ratio of the precipitated chromatin. Thus, at the *H19* DMR, little H3K4me3 was precipitated, but precipitation was similar on maternal and paternal chromosomes.

-Figure legend: It was misleading to write that there is no parental bias for the H2A/H4R3me2s. What we meant to say is that precipitation had occurred similarly on both the parental chromosomes and that no allelic enrichment greater than 1.5-fold was observed (based on relative band intensity

measurements, using ImageQuant software). We modified this sentence in the legend to this figure: '*At all four ICRs analysed, the histone methylations studied were similarly precipitated from the maternal (M) and the paternal (P) alleles (the ratio between the parental alleles were in all cases smaller than 1.5).'*

Page 7, RACE or tiling arrays:

The reviewer mentions that for the KvDMR1, we did not determine their precise transcription initiation site by RACE, or tiling arrays, in the male PGCs. In fact, we have tried to perform RACE experiments on batches of purified PGCs, but these experiments were non-conclusive, possibly as a consequence of the limited amounts of available RNA and/or structural features of the RNA. We agree with the reviewer that a tiling array-based approach would be powerful for transcriptome mapping. Data mining of available online databases indicates that this kind of analysis has not been performed yet at the PGC stage. Based on our expertise in isolation of pure population of germ cells, this will be one of our future objectives. However, such an analysis is a complete project on its own, and extends far beyond the determination of the KvDMR1 (*Kcnq1ot1*) trancription start site.
For the KvDMR1, our classical PCR amplifications of different regions indicate that there is initiation of transcription at the KvDMR1 region in the male PGCs, but it does not indicate the precise TSS. To more carefully discuss this result, we modified the corresponding sentence in he Results section as follows: '*Although we did not determine the precise transcription start sites, these results suggest that the KvDMR1 and Snrpn DMR act as promoters in PGCs as well.'*

Discussion:

In the revised manuscript, we took out the term 'for the first time', and rewrote this sentence of the Discussion as follows: '*These data evoke the possibility that histone methylation and transcriptional events are linked to the specificity of imprint acquisition in male germ cells.'*

2nd Editorial Decision 21 October 2011

Thank you for submitting your revised manuscript to The EMBO Journal. It has been reviewed once more by two of the original referees who find that you have addressed all the initial concerns raised. I am therefore happy to accept the manuscript for publication in The EMBO Journal. You will receive the official acceptance letter likely early next week.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

My former comments and concerns were properly addressed in this modified version. I am in favor of publication.

Referee #3

The authors have addressed all of my concerns. This is a very nice addition to the imprinting literature.

