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Dynamic link of DNA demethylation, DNA strand breaks and repair in mouse zygotes

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1st Editorial Decision 16 October 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal, and please excuse the delay in getting back to you with a decision. We have now received the reports and recommendations of three expert referees who had agreed to take a detailed look at your study. As you will see, all referees find your analysis on DNA demethylation and repair patterns and kinetics in mammalian zygotes interesting in principle and also potentially important for advancing our understanding of this process. At the same time, however, all three of them also clearly remain somewhat ambivalent in their judgment of the suitability of the study for publication in The EMBO Journal at this stage. A main concern in this respect is obviously the absence of clear support for a causal relationship of DNA demethylation and the appearance of DNA repair markers. Related to this, the referees question whether the current data can provide sufficiently strong evidence to rule out that pre-existing DNA damage or repair-independent processes contribute to the appearance of markers such as gamma-H2AX in the zygote.

Given the importance of the topic, as well as the overall interest still expressed by all referees, I am nevertheless inclined to give you the opportunity to respond to their criticisms through a revised version of the manuscript. However, please understand that given their rather cautious enthusiasm and substantive concerns, I do currently not see myself in the position to make strong commitments with regard to publication of a revised manuscript - in this case, acceptance or rejection of the manuscript will ultimately depend on whether you may have been able to strengthen the demethylation-repair connection through additional experimentation in the spirit of the reviewers' detailed comments and suggestions. Therefore, should you feel confident that you might be able to address the key criticisms and to convince the critical referees, we should be able to consider a revised manuscript further for publication. However, please bear in mind that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential that you

diligently answer to all the points raised at this stage if you wish the manuscript ultimately to be accepted. If required, we could in this case also discuss an extension of the revision duration (normally limited to three months). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding this decision or your revision.

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):

Review of EMBO Journal-2009-72486 by Wossidio et al., (Walter Lab)

The authors are investigating the dynamics of DNA demethylation in the mammalian embryo, an area of intense and general interest. The work addresses the possible use of a DNA repair/modification mechanism for 5mC removal during pronuclear stages, as opposed to passive modes of demethylation that may occur due to replication.

The strengths of the work are the quality of the approaches and the clarity of the data. They support a considerable quantity of active demethylation in pre-replicative phases, while noting a considerable disconnect between the quantity of demethylation observed in 5mC staining (very high) and that by bisulphite sequencing of line elements (moderate), which leaves open the possibility for 5hmC or other modes of 5mC alteration. There is extensive data on a correlation between gH2A.X, SSBs and PARP-1 association with the phase of active demethylation, which is interesting. The weakness of the work is the lack of evidence that these associations of repair factors are directly coupled to demethylation. Manuscripts in EMBO Journal typically come to more firm conclusions via more direct connections. However, the experiments needed to directly connect these are extremely difficult and will challenge the field for years to come. Instead, this paper adds in a significant way to the growing notion that at least a portion of the DNA demethylation in mammals is likely associated with a DNA repair process, with BER the most intuitive (though apparently not PolB).

Major comments:

1) The bisulphite sequencing of LINE1 elements does suggest a moderate replication-independent demethylation, followed by a replication-dependent phase. I think this is significant, as there is much debate about the number of bases that are subjected to demethylation/repair. Here, however, the authors test LINE1 elements in Figure 1, which is a fine element to test. However, they want to make fairly general conclusions about the status of DNA methylation genome-wide. It seems necessary to test other loci, such as IAP elements, ETNs, Oct4, and other loci to make clear statements. Also, the authors should verify the presence of paternal imprints and the lack of maternal imprints to verify the purity of sperm samples. It is also unfortunate that methods are lacking to understand 5hmC, which appears as 5mC in bisulphite sequencing formats.

2) The initial gH2A.X experiments are somewhat informative, providing a qualitative survey of DNA damage profiles. However, it is hard to make the argument that the few foci in the parental nucleus in P2/3 are truly signs of demethylation; the authors simply have a moderate correlation. More interesting are the results with aphidicolin. Here, it is apparent that foci increase, which bolsters the notion that repair is taking place during that phase. The limitation is that there is no evidence that this repair is truly related to 5meC removal. The Cpt is a nice control for the affects of replication vs. repair, but there is no additional information content as the authors already established the timing of demethylation relative to replication in earlier data.

3) I found the section with the application of SCNT a very interesting approach. However, it is not clear to this reviewer whether the authors have a pre-replicative phase of demethylation (by anti-5-mC), as there is considerable decondensation of the chromatin and a larger area. Here, it seems important to quantify the 5mC signal relative to bulk DNA. Also, it would be very informative to perform bisulphite sequencing on the regions suggested in my first comment above (on Figure 1) to verify this, and to discriminate between complete repair/replacement and possible retention of 5hmC. Finally, the data do show the same correlation as in embryos, with Aph increasing gH2A.X foci. That helps make the connection to demethylation, but is far from definitive.

4) The PARP-1 data is likewise consistent with a mode of repair occurring at the phases described, and the expected overlap of foci are observed. I especially liked the experiments detecting nicks in the DNA using the modified nick translation protocol. I think the authors have a fairly convincing case for nicks. The limitation is that nicks are expected in a genome that has witnessed such a massive remodeling of its structure, and there is no direct quantitation or connection to demethylation.

Minor point: In the introduction, the authors appear fairly dismissive of the demethylation systems that involve GADD45. I agree that the knockout data for the mouse argues that Gadd45a is not the sole player. However, the DNA repair field is rife with examples where other players (paralogs and other systems) take over when a component is lacking. Also, there are several papers that do support a role for Gadd45.

Referee #2 (Remarks to the Author):

The rapid loss of DNA cytosine methylation in the paternal pronuclei of mouse zygotes has been interpreted as evidence for active DNA demethylation. The underlying molecular mechanism, however, has not been identified. In this manuscript, Mark Wossidlo and colleagues present experimental work addressing the dynamics of this demethylation process. Making use of Na-bisulfite DNA sequencing, in vivo DNA labeling and immunofluorescence (IF) technology, they show that 5-meC loss in the paternal pronucleus occurs in waves before and (presumably) during DNA replication, suggesting an involvement of active and passive modes of demethylation. At both stages, demethylation coincides with an appearance of γ-X2A.X and PARP foci, indicating an occurrence of DNA strand-breaks. The dynamics of the process is similar upon in vitro and natural fertilization, as well as in cloned embryos. On the basis of this evidence the authors conclude that active DNA demethylation in the paternal pronucleus involves DNA excision repair.

The study is well-designed, carried out and presented. It clearly provides an advanced temporal resolution of the DNA demethylation - replication processes in mouse zygotes, thereby clarifying a number of critical points including a general uncertainty associated with IF-based analyses of 5-meC levels. The observation that the dynamics of demethylation measured by IF and bisulfite-sequencing is rather different seems important to me, and so does the separation of early demethylation from DNA replication. The advancement in temporal resolution here is significant and, in my opinion, the strongest point of the manuscript. I am less convinced, however, that the γ-X2A.X and PARP data are compelling enough to support the rather strong conclusion that DNA excision repair drives active DNA demethylation. Addressing the following points may help strengthen the manuscript as a whole and the DNA repair part in particular.

General point:

Title: I don't think linking demethylation with DNA repair in the Title of the manuscript is appropriate, repair was not directly addressed. "DNA demethylation coincides with DNA strand-breaks in mouse zygotes" would seem more to the point.

Major points:

1.) Statistical assessment of observations: It appears that most of the findings presented in the manuscript base on the observations of just a few zygotes. I understand that, for biological and

technical reasons, such analyses can't be done on large populations. Nevertheless, I think there should be some indication as to the variance associated with the key measurement (as done for γ-X2A.X foci in Figure 4). What, for instance, is the variance in time of replication onset in these experiments? How frequently would replication be expected to start already in PN2 or early PN3, and how synchronous is this really (also considering variance) between maternal and paternal nuclei? Also, what is the frequency and variance of pronuclear PARP-1 foci at the different zygotic stages?

2.) DNA bisulfite sequencing: I reckon the methylation data shown in Figure 1 reflect the methylation status of one DNA strand only. Is it possible that the pre-replicative wave of active demethylation is directed to one DNA strand only, i.e. generates hemi-methylated DNA, and full demethylation is achieved by replication (active-passive). The authors should provide some assessment on this and discuss this issue in the context of their model. They should consider (which I am sure they did) that symmetrical excision repair at mCpGs would generate DNA double-strand breaks, most likely even gaps, that would be rather complex to repair.

3.) γ-X2A.X foci: While there are very few γ-X2A.X foci in the paternal pronuclei (even less in nuclei of the cloned embryos) before DNA replication, it seems to me that the number of γ-X2A.X foci increases rather massively in both nuclei once they start replicating the DNA, i.e. in late PN3. Regarding these observations I feel there are a couple of points that should be clarified. (A) Is it possible that the few pre-replicative foci in the paternal pronuclei indicate sites of sperm-DNA damage, which is recognized and repaired only after fertilization but before the onset of DNA replication. Accumulation of damage in sperm DNA due to inefficient or event absent repair is well-documented. (B) How do the authors explain the strong increase of γ-X2A.X foci in the maternal pronucleus upon replication onset. If DNA repair mediated demethylation indeed occurs in the paternal pronucleus, an accumulation of repair intermediates could explain the increase there, but what is going on in the maternal pronucleus?

4.) Aphidicolin: The effect of Aph on γ-X2A.X in the paternal pronuclei could again be explained by repair of pre-existing DNA damage of any kind. Can this be addressed and excluded? Also, Aph does not affect the DNA polymerase (Polb) most likely involved in base excision repair mediated cytosine deamination. So, either Aph inhibition interferes with the repair of other types of lesions (NER, DSBR), or indeed base excision repair in zygotes does not engage Polb. To clarify this point, the data on Polb exclusion from pronuclei referred to in the discussion would be helpful and should be shown.

5.) PARP-1 and Nick-translation: Without statistical analysis, I can't judge the significance of these data. For instance, in the absence of Aph, there seems to be only a minority of PARP foci localizing to the paternal pronucleus and co-localizing with γ-X2A.X? In the presence of Aph, there is again the ambiguity of potential pre-existing damage in the sperm DNA.

Minor point:
I suggest combining Figures 3 and 4 as well as the staining of the un-phosphorylated X2A into one Figure 3 (panels A-C). Also, I recommend showing the two-cell stage embryo control in the main manuscript, in my opinion this is an important control.

Referee #3 (Remarks to the Author):

Cytological assays with 5meC antibodies have revealed a rapid loss of DNA methylation at the paternal pronucleus during mouse development. This observation has been regarded as an evidence for the existence of active DNA methylation in mammals. The function and the mechanism behind this demethylation is unclear. The paternal-specific demethylation is not conserved in all the mammalian species. Certain classes of retrotransposons have been confirmed to undergo demethylation at this stage by bisulfite sequencing, but this assay does not give the parental specificity and may suffer from biases inherent to PCR-based approaches to the study of highly repetitive interspersed sequences. Finally, enzymes with biochemical properties of active demethylases are not identified in mammals. Repair coupled mechanisms involving enzymes of the

glycosylase family are nonetheless known to be operant in promoting active demethylation in Plants.
The authors provide here a detailed analysis of the DNA methylation dynamics within the few hours following fertilization in mouse, in conjunction with replication timing and DNA repair mechanisms related to DNA breaks. They conclude that there is indeed a significant level of replication-independent demethylation occurring in the zygote and that this event is coincident with increased phosphorylation of histone variants H2A.X in the paternal pronucleus. Gamma-H2AX is associated with DNA breaks and accumulates for a few hours around sites of DNA damage

Although I agree that there is an interesting overlapping window of rapid DNA demethylation and intense H2AX phosphorylation of the paternal pronucleus, I do not see how these 2 events are causally related. The manuscript may give interesting clues about the kinetics of gamma-H2AX accumulation, the occurrence of DNA breaks during this key developmental period and how it can be affected by various drugs, but the data just do not support a functional link between DNA repair and DNA methylation. Also, while the authors conclude that gamma-H2AX foci in the early embryo are reflective of DNA breaks, a former study suggested that the accumulation of gamma-H2AX occurs in a DNA repair-independent manner during this period (Ziegler-Birling et al, 2009). The authors never discuss this former work. Here are my detailed comments on the manuscript that I unfortunately do not think suitable for a publication in EMBO in its actual form.
- It would be useful to have some background about PN staging from the beginning. Is the classification related to hours post-fertilization and/or to pronucleus aspects ?
- The replication timing study should logically come first, in relation to PN maturation.
Also, Fig2 includes a gammaH2AX staining experiment that is not reported in the text. This figure part is completely redundant with the results in Fig3A that give moreover a greater level of kinetic details.
- Bisulfite experiment : the authors conclude that there is a drop in Lines methylation preceding replication (before early PN3). How significant is this drop (p value please) ? There is a decrease from 68 to 54% from PN1 to PN2 but standard deviation are overlapping. To me the most significant decrease occurs in relation to replication, from early replication PN3 to PN4-PN5. Which would be suggestive of a passive mechanism of demethylation
- The authors exclude this hypothesis by arguing that the mosaic methylation pattern of bisulfite treated alleles can not be the result of the absence of maintenance methylation during replication. The dispersed nature of Lines1 make them likely to be regulated by multiple replication origins. Moreover, repeated sequences are prone to formation of chimeric molecules during PCR amplification. I don t think therefore that passive demethylation can be excluded based on this mosaic pattern argument.
- The authors never assess the direct relationship between gamma-H2AX and DNA methylation. By using DNA polymerase inhibitors (Aphidicolin), increased gamma-H2AX is observed and potentially reflects an accumulation of unrepaired DNA breaks. An obvious question would be to look at how it affects DNA methylation patterns?
- The discussion could be shortened.
In conclusion, the authors need to soften their conclusion about a relationship with DNA methylation and rather just focus on the simple description of gamma-H2AX kinetics and implication for repair mechanisms, by investigating other components of various repair pathways and how they can affect gamma-H2AX recruitment on the paternal pronucleus.

1st Revision - authors' response 27 February 2010

We are thankful to all reviewers for their highly valuable critical comments. All suggestions concerning the MS and additional experiments were taken into consideration. We also thank the editor for extending the deadline for resubmission. This became necessary since we performed a fair amount of additional experimental work (particular on cloned embryos) which certainly helped to substantiate the main conclusions of the paper. Before commenting on the individual points of criticism we would like to summarize the additional experiments performed for the revised MS:

1) As suggested by reviewers #1 we included ETn as a second class of repetitive element in our staged bisulfite analysis in zygotes. These data strongly support the notion of active pre-replicative demethylation. Interestingly the DNA methylation dynamics of ETn elements follows those of Line1 elements during pre-replicative stages but differs during post-replicative stages.

2) We performed IF and bisulphite sequencing on cloned (SCNT derived) one cell embryos to follow the DNA methylation dynamics of LINE1 and ETn elements at pre-replicative and post-replicative stages in comparison to zygotes. The IF data strongly support the correlation between strand breaks and active DNA demethylation (pre-replicative presence of gH2A.X and reduction of the 5mC signal to about 50%). The additional bisulphite sequencing reveals a less pronounced molecular effect on CpG methylation at repetitive elements before S-phase in cloned embryos. Together these additional data support the correlative link between DNA demethylation and DNA strand breaks/DNA repair events and point towards minor but suggestive differences between zygotes and SCNT embryos in the extend and specificity of DNA-demethylation.
3) We included a detailed staged analysis of DNA single strand breaks during early PN staging. Several reviewers were pointing to the possibility of delayed remodeling associated repair in pre-existing sperm damage before the start of replication as an alternative explanation of pre-replicative DNA breaks and repair markers in the paternal pronucleus (as shown by gH2AX, PARP-1 staining and Nick translation assays). Using our modified Nick translation assay at earlier pronuclear stages we now present the full dynamics of DNA break signals at all relevant stages. From this analysis we conclude that we can clearly separate two distinct waves of repair: one early in both pronuclei and a second wave of newly appearing strand breaks at PN2/3 and following stages.

In summary all our data are in line with the interpretation that DNA demethylation and DNA strand breaks/ DNA repair events are dynamically correlated in the zygote. We have slightly changed the title to underpin the comparative developmental focus of our paper. We hope that particularly reviewer #3 will reconsider his/her critical view on our data and interpretations when studying the revised version. We are well aware of the fact that our study does not provide a direct mechanistic link between both molecular events, but the spatial and temporal overlap is more than suggestive.
Finally we would like to emphasize that our staged bisulphite sequencing analysis for the first time provides a **direct** molecular proof for active demethylation at **pre-replicative** stages and reveals interesting novel insights into the extend and dynamics of DNA-demethylation in the zygote.

We extensively revised the MS to create a more stringent and concise text and we followed the suggestion by reviewer #3 to shorten parts of the previous rather detailed discussion. We have corrected all minor mistakes outlined. We added a fair amount of new data and changed the figures accordingly. Some relevant supportive control data was therefore shifted into supplementary data (see below).

In the following we would like to give point-by-point answers to the reviewer comments:

Reviewer #1

Major points:

1. The reviewer points out that it would be helpful to analyze other elements by bisulfite sequencing of mouse zygotes to make more global statements of DNA demethylation changes. Therefore we also analyzed the proposed ETn element. Single copy sequences like Oct4 escape our attention because we want to make general statements about the global DNA demethylation. The purity of sperm sample was guaranteed by the extreme harsh purification of sperm DNA (after first ProteinaseK treatment only sperm heads are left to be processed further).

2. The reviewer questions that the few number of γH2A.X foci in the paternal pronucleus at PN2/early PN3 are true signs of DNA demethylation processes and points out that on the other hand the increased γH2A.X foci by inhibition with aphidicolin "bolsters" the notion that DNA repair is taking place. The appearance of only few γH2A.X foci without aphidicolin reflects a very fast and efficient repair process while the inhibition of DNA polymerases by aphidicolin partially "freezes" and hence visualizes these events. The strict reduction of such γH2A.X foci to only the paternal pronucleus along the late phase of DNA demethylation lets us assume, that the DNA demethylation is accompanied by the transient generation of DNA stand breaks and quick subsequent DNA repair (most likely single nucleotide BER). Unfortunately it remains an open question which modification induces the DNA repair mechanisms. We have examined the presence of TDG and AID as candidates which in our IF analysis are all negative

and hence are excluded from the MS. Still particularly the aphidicolin data provide a strong hint for a spatial (paternal pronucleus) and temporal link between repair and DNA methylation changes.
We disagree with the notion that "the authors already established the timing of demethylation relative to replication in earlier data". The present staged bisulphite analysis along with thorough replication timing analysis provides the first direct evidence for the extend of active demethylation in this time window (in the revised version also extended to cloned embryos). The work described in previous publications was focused on the analysis of later stages.

3. The reviewer suggests to quantify the 5mC immunosignal and to perform additional bisulfite sequencing experiments on cloned one cell embryos to verify pre-replicative DNA demethylation changes. In the revised version we add quantification data on the reduction of 5mC signals normalized to DNA signal and observe a reduction of approximately 50% of 5mC signal before the start of S-phase. We also add extensive bisulphite data on cloned one cell embryos which for Line1 elements suggests a minor but detectable demethylation at pre-replicative stages.

4. The reviewer points out that the observed nicks in the paternal pronucleus at early PN3 stage are expected in a genome "that has witnessed such a massive remodelling of its structure". The additional nick translation experiments on very early *in vitro* fertilized zygotes show that shortly after the protamine-histone exchange at late PN0/early PN1 no nicks remain on the parental pronuclei. We therefore conclude that a maintenance of "pre-existing" damages from sperm decondensation stage at PN0 up to early PN3 is more than unlikely. Hence the early PN3 γH2A.X foci mark newly generated nicks in the DNA.

Minor point: The Reviewer asks to put more emphasis on the putative role of GADD45 in our context. So far the published data on GADD45 is still very controversial and hence we are not so much in favour of GADD45 being the putative DNA demethylase in mouse zygotes. Recent data also do not support the role of GADD45 in the studied processes/time windows (Okada et al., 2010).

Reviewer #2

General point: The reviewer asks to change the title because he/she thinks that DNA repair is not directly addressed. We believe that the presence of overlapping γH2A.X and PARP-1signals points towards this direction. To put more emphasis on the correlative aspect of all processes we decided to introduce the change to the title "Dynamic link between DNA demethylation, DNA strand breaks and repair in mouse zygotes".

Major points:

1. The reviewer points out that it is difficult to statistically evaluate the IF data but asks for a clarification of key measurements of γH2A.X foci and their statistical assessment. For example he/she wants to know if the γH2A.X foci could be incorrectly assigned to already replicative stages. We carefully staged the embryos and performed at least three independent experiments for each stage with n>60 per stage for PN2-PN5 stages. We never observed incorporation of BrdU or EdU earlier than late PN3. Among the late PN3 stage embryos analysed we also **never observed** an example with asynchronously replicating (EdU/BrdU labeled) pronuclei. EdU or BrdU staining was either consistently positive or negative for both pronuclei. Furthermore we find all γH2A.X foci to co-localize with PARP-1 foci in pre-replicative stages. From late PN3 onwards this picture changes and during replication γH2A.X foci do not always overlap with PARP-1.

2. The reviewer suggests the problem of a possible strand bias in the DNA bisulfite sequencing analysis and asks to discuss the problem of DSB avoidance. Our comment to these very valid points: Both analyzed repetitive elements are scattered across the

genome (2200 LINEs and 160 ETns). Because of this "random" localization both types of elements have an equal relative orientation to replication origins. Hence the analyzed strands are theoretically in half of the cases on the lagging or leading strand, respectively and therefore "random". Since our analysis focuses indeed only on one of the DNA strands the possibility cannot be excluded that more strong effects of demethylation may exist on the other DNA strand and e.g. affected by transcription. We would like to note in this respect that a hairpin analysis performed on the same Line1 elements in (replicating) ES cells (unpublished results, data not shown) does not indicate any asymmetry between both DNA strands.
We agree that a symmetrical excision repair of CpG context indeed might lead to DSB formation. However glycosylase driven demethylation in plants is apparently performed by bifunctional enzymes which avoid such a risk of DSBs by processing one strand after the other, i.e. by blocking the excision of the opposite CpG (ROS1 in Arabidopsis, (Ponferrada-Marin et al., 2009).

3. The reviewer raises a couple of questions concerning the developmental dynamics of γH2A.X foci: A) Our extended analysis on early zygotic stages excludes the possibility that the γH2A.X foci are remains of pre-existing sperm damage as DNA breaks disappear at PN1 (see comment above to reviewer #1). B) The strong increase of γH2A.X foci upon replication onset in maternal and paternal pronuclei reflects stalled replication forks which occur frequently during S-phase (see S-phase in 2cell embryos for comparison, Sup_5). Still we would like to note that the paternal pronucleus shows more pronounced γH2A.X signal at the replicative stages (late PN3 and PN4). This may suggest that in the paternal pronucleus a mix of strictly replication induced and repair coupled foci might be present.

4. Aphidicolin: We excluded pre-existing sperm damage (see comment to reviewer #1 above) and we added DNA polymerase b immunostaings (Sup_9) showing the apparent absence of DNA Polymerase b in both pronuclei (note that the antibody is tested "positive" for Polb in MMS treated cultured cell lines, data not shown).

5. γH2A.X always co-localizes with PARP-1 foci before S-phase (see comment to reviewer #1). The absence of "nicks" at PN1 argues against the Aph induced enhancement of pre-existing "remaining" sperm damage up to PN2/earlyPN3 stage.

Minor points: We agree that pictures could be combined, but we would like to leave this decision open up to a potential editing process because some pictures will become too bulky and would require a huge size to be legible.

Reviewer #3

1. We would like to thank the reviewer for pointing out the missing reference (Ziegler-Birling et al., 2009) which we now refer to in the text. This study was published at the time of the first submission and we unfortunately missed the nice piece of work. The study includes an analysis of γH2A.X foci *in vivo* derived zygotes shown as PN2 and PN4 respectively. In our staging of *in vitro* fertilized zygotes the presented PN2 zygote in fig_1A of Ziegler et al. would refer to late PN3 stage – at least according to the morphology (size, decondensation) and close proximity of both pronuclei. In all our staging we refer to definitions set by ((Adenot et al., 1997; Santos et al., 2002). This staging includes both morphologic aspects and also the relation to hour post fertilization (possible with IVF).

2. The reviewer furthermore points to the fact that Ziegler et al. show the absence of 53BP1 as "an important factor of DNA double strand break repair". The authors interpret the absence of 53BP1 as a sign that the γH2A.X signal at the zygotic stages is not repair associated. However, it has been suggested, that γH2A.X marked DNA may be linked to repair processes or pathways other than DSB repair. E.g. Matsumoto et al. show the association of γH2A.X with NER (Matsumoto et al., 2007). Moreover our modified nick translation assays clearly reveals the presence of SSBs only. TUNEL assays (data not shown) are negative at PN2 and early PN3 (i.e. not DSBs). Irrespective of some staging discrepancies we highly appreciate the nice work by Ziegler et al. which also corroborates our

interpretation of a replication dependent enhanced γH2A.X signal in the paternal pronucleus at replicative stages.

3. The reviewer asks to first describe the replication timing. We would rather like to keep the order and show the major emphasis of our work on the DNA demethylation dynamics first to set the basis for other correlations such as DNA strand breaks, replication and repair markers. The discussed fig_2 shows the analysis of replication timing according to EdU incorporation (BrdU was also used – not shown in detail (Sup_1) and no difference) and co-staining also shows the associated γH2A.X signal in the same embryos. This allows to associate directly the appearance of γH2A.X according to the cell cycle. We refer to this in the chapter about the dynamics of γH2A.X during zygotic development.

4. The reviewer questions whether the drop of Line1 methylation before early PN3 is significant. We have put asterisks in fig_1 to relate to p-values. We are well aware that the statistical significances of these differences are not very strong. Still the data shown are the sum of several (up to 6) independent experiments (PN stages) which together confirm the tendency to decrease.

5. The reviewer points out that the passive demethylation cannot be excluded. We do not exclude this possibility but we find an increased mosaic pattern for Line1 elements in later PN stages, which disfavours the idea of a passive replication associated processes. Moreover the new data on ETn elements, even show an increase in DNA methylation after completion of the S-phase. This finding (still being not significant but strongly suggestive) does not go along with a picture of passive demethylation only – it rather shows that even *de novo* methylation is possible at particular zygotic stages and in particular elements such as active transposable elements.

6. Reviewer #3 strongly argues that we cannot build a direct mechanistic link between DNA breaks (particularly questioning γH2A.X dynamics), DNA repair and DNA demethylation. We agree that our work does not provide direct evidence for a mechanistic link. Still we would like to point out that the developmental correlation between DNA strand breaks (shown by different approaches), BER repair marker localisation and DNA methylation changes, i.e. the spatial, temporal and partially molecular link (bisulphite) between all three is indeed strongly suggestive. This correlation is now further substantiated since we document similar effects in **both** experimental systems: the zygote and the cloned embryos. We have considered the strong criticism more than carefully and tried to be cautious with our conclusions to avoid a potential dispute on "interpretations" only.

7. The reviewer asks to shorten the discussion part – we followed this advice.

References referred to in the letter:

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Okada, Y., Yamagata, K., Hong, K., Wakayama, T. and Zhang, Y. (2010) A role for the elongator complex in zygotic paternal genome demethylation. *Nature*.

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Santos, F., Hendrich, B., Reik, W. and Dean, W. (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev Biol*, **241**, 172-182.

Ziegler-Birling, C., Helmrich, A., Tora, L. and Torres-Padilla, M.E. (2009) Distribution of p53 binding protein 1 (53BP1) and phosphorylated H2A.X during mouse preimplantation development in the absence of DNA damage. *Int J Dev Biol*, **53**, 1003-1011.

2nd Editorial Decision 01 April 2010

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee 1 (comments to authors):

Review of revised version of EMBO Journal 2009-72486 "Dynamic link of DNA demethylation, DNA strand breaks and repair in the mouse zygote".

The revised version is improved in a substantial manner. I was pleased to see the addition of ETn elements, and agree this has strengthened the main conclusions, while also pointing to some possible differences in the rate at which these elements acquire DNA methylation later. There is also better quantification of the demethylation of Line1 elements, which I considered important. The differences between SCNT embryos and zygotes is also interesting, though still descriptive. Third, the authors were able to show that a substantial fraction of the marks does not result from residual damage in the sperm (repaired very early), but rather from a subsequent stage.

This was a good study in its original form, and I think these additions strengthen the manuscript considerably. Although the manuscript still lacks direct evidence for a mechanistic link between DNA demethylation and BER, this is a strong study that has sufficient breadth and fairly convincing correlative evidence. I also agree that the bisulphite sequencing in the manuscript does provide the best evidence to date for active DNA demethylation in the zygote prior to replication. For these reasons, I support publication of the revised version in the EMBO Journal.

Referee 2 (comments to authors):

The authors have addressed/rebutted my concerns, which is appreciated. Clearly, the strength of the manuscript is the direct molecular proof of pre-replicative DNA demethylation in the pronculei of in vitro fertilized eggs. This is an important achievement. The spatiotemporal association of DNA demethylaiton with DNA strand-break formation and repair is interesting but largely correlative.

Referee 3 (comments to authors):

The revised version of the manuscript of Wissidlo is greatly improved compared to the original submission. This is as a whole a nice and carefully executed piece of work that will raise important discussions in the field. The analysis of an other family of repeats certainly added confidence in the data and p values also render

the data more convincing.
I appreciate the fact that the authors "smoothened" the too strong emphasis on the fucntional link between DNA repair and DNA demethylation. I still regret that 5meC antibody staining was not performed on Aphidicolin treated PN3 stages, in the hypothesis that increased accumulation of g-H2AX foci in the maternal pronucleus could reveal a loss of 5meC staining... But in conclusion, in regards of the amount of work that was provided, the high quality of the data and the general interest of the subject, I recommend a publication of this manuscript in its present form.

