Stella preserves maternal chromosome integrity by inhibiting 5hmC-induced γH2AX accumulation

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Editor: Esther Schnapp

1st Editorial Decision 08 September 2014

Thank you for the submission of your research manuscript to EMBO reports. I apologize for the slight delay in getting back to you; we have only now received the full set of referee reports on your study that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also point out that no causal links between 5hmC, gH2AX and replication defects are established in Stella null (or wild type) embryos and that the current data are not sufficiently convincing or conclusive. The referees further indicate that the interpretation of the data needs to be done more carefully and accurately, and they have several suggestions for how the study could be improved and strengthened. Referees 1 and 3 mention that the colocalization of 5hmC and gH2AX should be quantified, and referee 3 adds that it should be investigated whether 5hmC colocalizes with H3K9me2. Importantly, referee 3 (and indirectly referee 2) also remark that it needs to be examined whether TET3 knockdown in the zygote reduces 5hmC and gH2AX levels, and whether TET3 overexpression in vivo phenocopies Stella null phenotypes. Referee 2 is further asking for stronger evidence to support an effect of Stella on replication timing, for controls and better data presentation, and for analysis of gH2AX foci in 2 cell embryos.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully
addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. mean +/- SEM, SD) and the statistical tests used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

The manuscript by Nakatani et al extends on the phenotypic characterisation of the Stella mutant embryos previously described by the same authors and the Surani group. The authors present evidence of delay in developmental progression and aberrant chromosome segregation mostly on maternal chromatin. The authors document precocious appearance of gH2A.X in the maternal pronucleus, which persists at the 2-cell stage.

The authors present at least three novel observations: a defect in the temporal pattern of gH2A.X, a defect in replication and a defect in chromosome segregation as a result of Stella loss.

While the first two observations are temporally correlated, there is no causal relationship, nor it is established that this could be the 'mechanism' why the developmental arrest is affected, and instead alternative interpretations are plausible, for example: Can it be that Stella/K9me2 are important in recruiting scaffolding proteins important for segregation? Also, it could be that the persistent H2A.X foci are related to deficient recruitment/function of BER proteins, and not to replication. With the presented data the authors cannot formally conclude either way and I therefore think they should carefully rewrite their statements/interpretations.

I find the phenotype on phospho H2A.X of interest and novel, together with the delayed replication. However, I find the manuscript slightly oversated in the mechanistic/causal conclusions since there is no causal relationship of any of the data provided. I suggest that the authors rewrite significantly their conclusions avoiding a 'mechanistic' link, and instead limiting to the description of the data and possible interpretations.

In this sense the manuscript is weak and could benefit of at least one experiment to try to link the phenotypic observations. For example, if the gH2A.X levels are indeed responsible for the phenotypic arrest, the authors could attempt inhibiting ATR/ATM activity in Stella null embryos with specific inhibitors and address whether increased gH2A.X is indeed the 'mechanism' of arrest in Stella null embryos. Culturing embryos and assessing developmental potential in the presence/absence of these inhibitors should provide some answers.

Along these lines, on page 7, the authors conclude 'These data support the notion that accumulation of gh2a.x foci correlates with the induction of 5hmc'. As mentioned above, this is only a temporal correlative analysis and is therefore quite weak considering all other changes in chromatin taking place at this early stages. Can the authors quantify colocalisation of 5hmc and gh2a.x in 3D nuclei?

Minor comments:
1. The reference of earlier work documenting asymmetric gH2A.X distribution in the zygote should be cited (Ziegler-Birling 2009)
2. First sentence on page 7 that H2A.X phosphorylation limits pre-implantation development is strictly speaking not correct, as the paper referenced documents defective development dependent on GABA stimulation, so this argument should be removed/rephrased.
3. Figure 2D, the panel gh2a.x is very difficult to appreciate. Can the authors provide a better contrast image? (or else a gray scale instead of the red, making the differences more visible?)

4. The quantification of Figure 2A should be improved: what does ‘weak’ mean? Is it a threshold comparison?

Referee #2:

The authors report on the role of Stella for early mouse development. In particular they aim to understand the consequences of altered maternal chromatin caused by the accumulation of 5hmC and its consequences for development. The main observation is that Stella null embryos have a strongly impaired preimplantation development. The authors claim that this is caused by impaired DNA replication and chromosome missegregation (mitotic catastrophe). This conclusion is based on observations concerning a shifted "timing of replication" (in the zygote, data reported but not shown), the accumulation of g-H2AX foci in the maternal chromosomes of Stella minus zygotes/embryos (known before), the co-appearance of gH2AX and chromosome „fragments“ in later stages of development (> 2 cell) and the appearance of microchromosomal fragments in stalled embryos.

The authors compile a number of very interesting observations. However the proposed links between altered replication timing and the molecular changes induced by the absence of stella remain somewhat obscure. The interpretation that maternal (enhanced) 5hmC causes the replication stress is one possibility - the connection to impaired developmental competence is another. For both the data are not convincing enough.

Major points:

* Fig 1C shows a normal timing in the appearance of 2-cell embryos while the BrdU based replication „timing“ (Fig 2A) suggests a delayed replication in the zygote. In this experiment the time of fertilisation and thus the development will be variable. „in vivo“ fertilized embryos are not synchronous. The fertilisation of embryos should occur around 14h posthCG - hence the time window of strong BrdU labelling at 19-21h is much too early (5-7 h postfertilisation)!!! The decrease of the signal around 20post hCG is in principle very interesting. Here real imaging intensity data should be provided complemented by images of embryos to confirm at which (pronuclear) stage control and stella +/- zygotes are. This is important since the argument of an impaired replication is the basis for all later interpretations. It remains unclear why this replication timing was not confirmed in in vitro fertilized embryos, i.e. in a much more controllable fashion. Moreover replication timing in two cell embryo should be analysed as well using BrDU labelling. Such a 2-cell experiment should reveal a more pronounced delay according to figure 1C.

* The experimental linking chromosomal abnormalities and the variable nomenclatures used to describe these chromosomal aberrations are more than confusing. The authors use the terms fragmentation, ACS and micronuclei formation. Fig 1 F essentially describes the formation of micronuclei as in Fig 2D only indicting that also maternal chromosomes are affected. The comparison in Fig 2G is misleading since the micronuclei formation in ICSI and Stella-null cannot be compared. The Stella null analysis was not performed with ICSI - there should at least a comparison to normal ICSI or normal Stelly+/+ be included. Moreover pictures should not be so heavily overcontrasted and stacked images should be provided. Besides micronuclei counting the authors should catch metaphases in early phases to convincingly show chromosomal missegregation.

The authors the finding of Stella induced „mitotic catastrophies“ to an enhanced yH2Ax appearance (particularly in the maternal chromosomes). It should be noted that yH2AX does not „inhibit DNA-replication“ - it is one major (but not the only one) indicator of replication stress (accumulates also in normal zygotes at PN4 !!). The counted foci in the supplementary table (Fig2c) are rather variable. At least 4/15 zygotes show a strong paternal dominance. Hence the interpretation should be done with more caution. The statement that foci are equally distributed in paternal and maternal pronuclei is only partially true - the numbers of embryos low. In fact both paternal and maternal PNs appear to be much more „stressed“ which would contrast the fact that micronuclei are predominantly
formed in the maternal chromosomes as suggested by figure 1G.

The rational of the TDG experiment and its contribution to the overall picture remains unclear. The authors show that the over-expression of TDG removes 5fC and 5caC but leaves 5hmC level unchanged (the statement is based on IF-pictures - not on real image analysis). They also claim that the number of γH2AX foci remains also unchanged - arguing that 5hmC and not 5caC and 5fC is triggering γH2AX foci formation. They do not take into account that the quantity of 5fC and caC formation is very unclear and that these modifications are formed rather late during zygotic development. Here an analysis of γH2AX foci in 2 cell embryos would have been a must! Minor comment: The numbers paternal foci in controls in 2C and 3E are surprisingly different and that the DG foci are (nonsignificantly) lower showing the interexperimental variation that needs to be accounted for when comparing results.

The final experiment aims to extend the link between 5hmC accumulation (by Tet3 overexpression!!) and replication stress in mouse 3T3 cells. The data are nice but the question is of which relevance the findings are for the early embryonic phenotypes particularly in the context of Stella. There are many parameters such as the different isoforms of Tet3 and non 5hmC effects that should be respected to allow this cross reference. Finally authors suggest that Tet3 and Tet2 do not process 5mC to 5hmC. This statement should be considered since others (Hu et al, Cell2013) have clearly show that at least Tet2 is capable of processing 5hmC further.

Referee #3:

This study by Nakatani and colleagues investigates the molecular mechanisms underlying the ablation of Stella causes early embryonic lethality. This work advances previous study on Stella-null mouse (Surani, 2003) by presenting the potential underlying mechanisms. However, the novelty is also perturbed by the previous studies. The major conclusions of this manuscript are: 1) Stella-null mouse has impaired DNA replication and abnormal chromosome segregation (ACS) in the maternal DNA. 2) γH2AX were aberrantly accumulated in the maternal chromatin of Stella-null zygotes. 3) Ectopic appearance of 5hmC of maternal chromatin induced abnormal accumulation of γH2AX and the subsequent growth retardation. Thus, the author concluded that Stella protects maternal chromosomes from aberrant epigenetic modifications, thus ensuring early embryogenesis. However, I have reservations on the interpretation of the data. Specifically, recent genome-wide allele-specific DNA methylation mapping in mouse early embryos (Wang et al. 2014) has been clearly shown that 5hmC and 5fC is present in both maternal and paternal genome in 2-cell embryos. However, the methods applied in the manuscript is mainly depend on immunostaining, which is less quantitative and can not unambiguously support the conclusions.

Major points:

1. Concurrence of 5hmC and γH2AX in maternal genome need more vigorous checking in control and Stella-null mouse. Meanwhile, I also have reservations in the interpretation of data. As showed in Figure 2D and 2E, "both γH2AX foci and 5hmC were detectable only in the H3K9me2-negative paternal chromatin (Fig 2D, E and S1). In contrast, γH2AX foci and 5hmC were diffusely distributed in the nuclei of Stella-null 2-cell embryos (Fig 2D and E)." But the Figure 2D does not support that γH2AX foci were not diffusely distributed in the nuclei of Stella-null 2-cell embryos. Instead, the figure supports that γH2AX foci is detectable only in the H3K9me2-negative paternal chromatin in Stella-null mouse. Similarly, the results in Figure 2E, 5hmC in stella-null mouse also showed mutually exclusively localized in H3K9me2-negative paternal chromatin. Therefore, more experiments using more embryos should be done to confirm the distribution.

2. The amount of the 5hmC antibody used in the method should be titrated with positive control nucleotides. Those evidences will help the understanding of the recent sequencing data in 2-cell embryo of the control mouse (Wang et al. 2014), and elucidate whether 5hmC is also modified in maternal genome.

3. Previous studies support that Stella protects the DNA demethylation on H3K9me2 labeled regions through 5hmC (Wossidlo et al.2011, Nakamura et al.2007), it was intriguing to explore carefully
whether 5hmC is co-localized with H3K9me2 staining. Although from the Figure 2E, the 5hmC and H3K9me2 showed quite different distribution.

4. co-staining 5hmC and γH2AX will give better idea, whether 5hmC is response to γH2AX accumulation in maternal genome in Stella-null mouse.

5. siTET3 in zygote is required to elucidate whether the induction of 5hmC and γH2AX accumulation is impaired especially in maternal genome of wide-type mouse. This should reconstructed the phenotype of Stella-null mouse to support the major conclusion of this manuscript.

Minor comments
1) Figure 1D, it was not quite clear how to determine the micronucleus-like phenotype. The micronucleus showed in the third panel is quite different to the others in case of the distance to the nuclear.
2) The labels of Figure3 may contain typo. The author may want to change to AG-TET2 instead of AG-et2 et al.
3) The staining of γH2AX in Figure 2D is barely seen especially in control mouse.

1st Revision - authors' response  
19 December 2014

Referee#1:

The authors present at least three novel observations: a defect in the temporal pattern of gH2A.X, a defect in replication and a defect in chromosome segregation as a result of Stella loss.

While the first two observations are temporally correlated, there is no causal relationship, nor it is established that this could be the 'mechanism' why the developmental arrest is affected, and instead alternative interpretations are plausible, for example: Can it be that Stella/K9me2 are important in recruiting scaffolding proteins important for segregation? Also, it could be that the persistent H2A.X foci are related to deficient recruitment/function of BER proteins, and not to replication. With the presented data the authors cannot formally conclude either way and I therefore think they should carefully rewrite their statements/interpretations.

Thanks for the valuable comments. As the referee pointed out, we reported that Stella/K9me2 was important for the maintenance of DNA methylation (Nakamura et. al., Nature, 2012). However, as reported in the paper, Stella/K9me2 localized only in the maternal chromatin of zygotes. Therefore, even if some scaffolding proteins are recruited to Stella/K9me2, it is quite unlikely that such proteins play critical roles in chromosome segregation, which is the phenomenon of both maternal and paternal chromatin.

As for the relationship between γH2AX and BER proteins, we interpreted that the referee #1 asked whether the dysfunction of BER pathway caused the persistent γH2AX accumulation. If BER pathway was impaired in Stella-null zygotes and affected γH2AX foci, the number of γH2AX foci in paternal chromosome should also be affected. However, the number of γH2AX was changed only in the maternal chromatin (Fig. 2C). Therefore, we concluded that abnormal states in maternal chromatin, namely 5hmC and γH2AX, were well associated with DNA replication.

To avoid the overstatements on the causal relationship among defects of γH2AX, DNA replication, and chromosome segregation in the Stella-null condition, we deleted several sentences from the Results and Discussion of the original manuscript (original manuscript file, page 6, line 5-10; page 7, line 2-3, line 6-12) and, instead, added a sentence (page 7, line 19-20) and two references (reference no. 16 and 19). In addition, the running title was changed from “Effect of 5hmC on maternal chromosome integrity” to “Effect of Stella on maternal chromosome integrity”.

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I find the phenotype on phospho H2A.X of interest and novel, together with the delayed replication. However, I find the manuscript slightly oversated in the mechanistic/causal conclusions since there is no causal relationship of any of the data provided. I suggest that the authors rewrite significantly their conclusions avoiding a 'mechanistic' link, and instead limiting to the description of the data and possible interpretations.

Following the referee’s comment, we deleted several sentences from the Conclusion of the original manuscript (original manuscript file, page 9, line 1-12, line 18-19, line 21-22; page 10, line 5, line 6) and, instead, rewrote several sentences (page 8, line 11-12, line 17-19; page 8, line 24-page 9, line 5; page 9, line 9-13) to avoid the overstatement about the mechanistic/causal relationships.

In this sense the manuscript is weak and could benefit of at least one experiment to try to link the phenotypic observations. For example, if the γH2A.X levels are indeed responsible for the phenotypic arrest, the authors could attempt inhibiting ATR/ATM activity in Stella null embryos with specific inhibitors and address whether increased γH2A.X is indeed the 'mechanism' of arrest in Stella null embryos. Culturing embryos and assessing developmental potential in the presence/absence of these inhibitors should provide some answers.

We deeply appreciate these truly constructive comments. We performed immunostaining analysis of zygotes which were treated with three kinase inhibitors of H2AX, to clarify which kinases were responsible for the phosphorylation of H2AX. We found that only ATR inhibitor suppressed the phosphorylation of H2AX at PN3-4 (Fig S3A and B). Treatment of ATR inhibitor, however, did not rescue the developmental arrest of Stella-null embryos. Meanwhile, developmental arrest was brought about even in the control embryos as well as the Stella-null embryos (Fig S3C). Therefore, we could not conclude that increased γH2A.X was the direct cause of developmental arrest in Stella-null embryos. Several sentences and three references were added in the Results and Discussion (page 6, line 22-page 7, line 2) and in the References (reference no. 22, 23, and 24), respectively. One sentence was added in the Supplemental Information (page 10, line 19-20). Figure S3 and its legend were added (supplemental information file, page 8, line 16-23).

Along these lines, on page 7, the authors conclude 'These data support the notion that accumulation of gh2a.x foci correlates with the induction of 5hmC'. As mentioned above, this is only a temporal correlative analysis and is therefore quite weak considering all other changes in chromatin taking place at this early stages. Can the authors quantify colocalisation of 5hmC and gh2a.x in 3D nuclei?

γH2AX accumulation and Tet3-induced 5hmC appears at the same time in zygotes (Iqbal et al., Proc. Natl. Acad. Sci. U. S. A., 2011, Wossidlo et al., Nat. Commun., 2011, and Gu et al., Nature, 2012). This concomitant appearance had been reported to be just a transient event around PN3-4. However, we found the concomitant localization of 5hmC and γH2AX at 2-cell stage embryos, in which Tet3 was no longer detected (Fig 2D, E, and S4). We also found that both 5hmC and γH2AX foci had increased in maternal chromatin of Stella-null zygotes until PN3-4 stage zygote (Fig 2B, C, and Nakamura et al., Nature, 2012). These data of high correlation implicate the potential interaction between 5hmC and γH2AX. Then we carried out in vitro experiments using NIH-3T3 cells, to examine the direct mechanism linking 5hmC with γH2AX. Enforced expression of Tet2 or Tet3 induced only 5hmC accumulation among three oxidative derivatives. Moreover, Tet3 induced γH2AX in a catalytic activity dependent manner. Therefore, we concluded that Tet enzymatic activity, namely oxidative product 5hmC, induced γH2AX accumulation in Figure 4. We believe that our conclusion is reasonable enough.

Although we tried to quantify the co-localization of 5hmC and γH2AX, their double immunostaining was not succeeded. The major technical reason is that HCl treatment necessary for the immunostaining of 5hmC degenerates γH2AX antigen. We sincerely appreciate if the referee kindly understands the situation.

Minor comments:
1. The reference of earlier work documenting asymmetric gH2A.X distribution in the zygote should be cited (Ziegler-Birling 2009)
We should have cited this paper. This paper was cited in the Results and Discussion (page 6, line 18, line 21) and corresponding sentences were added in the References (reference no. 21).

2. First sentence on page 7 that H2A.X phosphorylation limits pre-implantation development is strictly speaking not correct, as the paper referenced documents defective development dependent on GABA stimulation, so this argument should be removed/rephrased.

Following the referee’s comment, this part was deleted from the Abstract and the Results and Discussion of the original manuscript (original manuscript file, page 2, line 8 and page 7, line 1, respectively).

3. Figure 2D, the panel gh2a.x is very difficult to appreciate. Can the authors provide a better contrast image? (or else a gray scale instead of the red, making the differences more visible?)

Sorry for the photograph with low contrast. The photographs were replaced with the new gray ones with higher S/N ratio.

4. The quantification of Figure 2A should be improved: what does ‘weak’ mean? Is it a threshold comparison?

We analyzed the fluorescence signal intensity of BrdU in the pronuclei of zygotes by Image J, and then classified the zygotes into three categories (no, weak, and strong) by the threshold comparison following the previously reported method (Posfai et. al., Genes. Dev., 2012). Representative images of no, weak, and strong BrdU signals were added as examples in Figure S2. A sentence to explain the category of the intensity was added in the Materials and Methods (supplemental information file, page 3, line 4-6). A corresponding sentence was rewritten in the Results and Discussion (page 7, line 6-8) and sentences were added in the Supplementary legends (supplemental information file, page 8, line 7-14).

Referee #2:

Major points:

* Fig 1C shows a normal timing in the appearance of 2-cell embryos while the BrdU based replication „timing“ (Fig 2A) suggests a delayed replication in the zygote. In this experiment the time of fertilisation and thus the development will be variable. „in vivo“ fertilized embryos are not synchronous. The fertilisation of embryos should occur around 14h posthCG - hence the time window of strong BrdU labelling at 19-21h is much too early (5-7 h postfertilisation)!!! The decrease of the signal around 20post hCG is in principle very interesting. Here real imaging intensity data should be provided complemented by images of embryos to confirm at which (pronuclear) stage control and stella -/- zygotes are. This is important since the argument of an impaired replication is the basis for all later interpretations. It remains unclear why this replication timing was not confirmed in in vitro fertilized embryos, i.e. in a much more controllable fashion. Moreover replication timing in two cell embryo should be analysed as well using BrDU labelling. Such a 2-cell experiment should reveal a more pronounced delay according to figure 1C.

We repeated the BrdU incorporation experiments using in vitro fertilized embryos, in which the timing of fertilization was synchronized. The result of the in vitro fertilized embryos was same as that of natural mating of the control and the Stella-null conditions. We considered that the data of the in vitro fertilization was more accurate and changed the Figure 2A to the novel one. We analyzed the fluorescence intensities of BrdU by Image J and classified them into three categories (no, weak, and strong) following the previously reported method (Posfai et. al., Genes. Dev., 2012), and the representative real images for each category were shown in Figure S2. As for BrdU intensity, we confirmed that the strong BrdU intensity was already observed at 6-8 hpf in the repeated experiment as well.

Thanks for the comment on the DNA replication timing in 2-cell embryos. We re-examined the delay of the Stella-null embryos in more detail following the referee’s suggestion (Fig S2). Figure 2A was replaced with the new data of the more detailed experiments and the data of the BrdU
incorporation at 2-cell stage was added in the Figure S2. Corresponding sentences were rewritten in the Results and Discussion (page 6, line 10-14; page 7, line 6-8) and in the Figure Legends (page 16, line 25, page 17, line 3; page 17, line 5-6) and added in the Results and Discussion (page 6, line 14-15), in the supplemental information (page 10, line 18), in the Figure Legends (page 17, line 3-5), and in the Supplementary legends (supplement information file, page 8, line 7-14).

* The experimental linking chromosomal abnormalities and the variable nomenclatures used to describe these chromosomal aberrations are more than confusing. The authors use the terms fragmentation, ACS and micronuclei formation. Fig 1 F essentially describes the formation of micronuclei as in Fig 2D only indicting that also maternal chromosomes are affected. The comparison in Fig 2G is misleading since the micronuclei formation in ICSI and Stella-null cannot be compared. The Stella null analysis was not performed with ICSI - there should at least a comparison to normal ICSI or normal Stelly/#/ be included. Moreover pictures should not be so heavily overcontrasted and stacked images should be provided. Besides micronuclei counting the authors should catch metaphases in early phases to convincingly show chromosomal missegregation.

Reading the comments, we assume that the referee would mention about Figure 1D and G, not Figure 2D and G. Anyway we agree that micronuclei formation is more suitable than fragmentation and changed “fragmentation” to “micronuclei formation” in the Figure Legends (page 16, line 14, line 15), following the referee’s comment. We leave the word “ACS”, because abnormal chromosome segregation (ACS) can be used as a process to cause micronuclei formation.

As for the comparison in Figure 1F and G, we used ICSI embryos derived from the crossing of Stella## female and BDF1 male mouse as a reference of the H3K9me2-negative micronuclei. ICSI embryos have been reported to give rise to ectopic micronuclei frequently and those micronuclei are derived from paternal chromosomes. The embryos were suitable for the analysis of paternal chromosome derived micronuclei. Then we decided to use the ICSI embryos as a reference to discriminate whether the origin of micronuclei was paternal chromosome or maternal chromosome. Thus, the data of the ICSI embryos are not used as the control but as a kind of reference. Embryos derived from Stella## female mice were developed to blastocyst stage similarly to the embryos derived from Stella## female mice, because Stella is a maternal factor. Therefore, we used the embryos derived from female Stella## as a source. Micronuclei formed in the ICSI experiments were H3K9me2 negative, which is consistent with the previous result from ICSI-embryos (Yamagata et al., Hum. Reprod., 2009).

The images of Figure 1F were replaced by not over-contrasted ones and 3D-stacked images of micronucleus in Stella-null 2-cell embryo were added in Figure S1 (page 5, line 23; page 10, line 17). Corresponding sentences were added in the Materials and Methods (supplemental information file, page 5, line 22-page 6, line 1, page 6, line 7-8) and in the Supplementary legends (supplemental information file, page 8, line 2-5).

ACS could be observed at anaphase in the Stella-null embryos as shown in the Movie S2. However, unfortunately, ACS could not be traced in the metaphase, because all chromosomes were aligned at the metaphase plate.

The authors the finding of Stella induced „mitotic catastrophies” to an enhanced γH2Ax appearance (particularly in the maternal chromosomes). It should be noted that γH2AX does not „inhibit DNA-replication” - it is one major (but not the only one) indicator of replication stress (accumulates also in normal zygotes at PN4 !!). The counted foci in the supplementary table (Fig2c) are rather variable. At least 4/15 zygotes show a strong paternal dominance. Hence the interpretation should be done with more caution. The statement that foci are equally distributed in paternal and maternal pronuclei is only partially true - the numbers of embryos low. In fact both paternal and maternal PNs appear to be much more „stressed” which would contrast the fact that micronuclei are predominantly formed in the maternal chromosomes as suggested by figure 1G.

We agree with referee’s comment that γH2AX doesn’t inhibit DNA replication but are just an indicator of replication stress. However, previous reports showed that DNA replication was affected by γH2AX directly or indirectly in the DNA damage response-independent manner (Andang et. al., Nature, 2008 and Fernando et. al., Proc. Natl. Acad. Sci. U. S. A., 2011). As one of the comments to
the referee #1, to avoid the overstatements on the causal relationship between γH2AX accumulation and a defect in replication, we deleted several sentences from the Results and Discussion of the original manuscript (original manuscript file, page 6, line 5-10; page 7, line 2-3, line 6-12) and, instead, added a reference (reference no. 19).

Sorry for the confusion of the second part. Following the referee’s comments, we changed the “γH2AX foci were equally distributed in paternal and maternal” to “γH2AX foci were increased in maternal pronuclei” (page 6, line 21-22). We carried out additional experiments, in which we increased the number of embryos for the analysis. We confirmed that the number of maternal γH2AX foci, but not paternal γH2AX foci, were significantly increased in Stella-null embryos (Fig 2C and Table S1). Statistical analysis of the new data was carried out and sentence was rewritten in the Figure Legends (page 17, line 13).

The rational of the TDG experiment and its contribution to the overall picture remains unclear. The authors show that the over-expression of TDG removes 5fC and 5caC but leaves 5hmC level unchanged (the statement is based on IF-pictures - not on real image analysis). They also claim that the number of γH2AX foci remains also unchanged - arguing that 5hmC and not 5caC and 5fC is triggering γH2AX foci formation. They do not take into account that the quantity of 5fC and caC formation is very unclear and that these modifications are formed rather late during zygotic development. Here an analysis of γH2AX foci in 2 cell embryos would have been a must! Minor comment: The numbers paternal foci in controls in 2C and 3E are surprisingly different and that the DG foci are (nonsignificantly) lower showing the interexperimental variation that needs to be accounted for when comparing results.

To capture the overall distribution and signal intensity of 5fC and 5caC in the TDG-expressing zygotes, we performed 3D reconstruction of confocal image stacks using the identical exposure time for the immunofluorescence. The data revealed that the signals of 5fC and 5caC, but not 5hmC, were completely removed at PN3-4 (Fig S5, page 7, line 17). Several sentences were added in the Supplemental Information (page 10, line 22), in the Materials and Methods (supplemental information file, page 5, line 22-page 6, line 1), and in the Supplementary legends (supplemental information file, page 9, line 8-12).

Following the referee’s comment, we analyzed γH2AX foci in the control and TDG-expressing 2-cell embryos. γH2AX foci were localized at H3K9me2-negative region in the control 2-cell embryos. On the other hand, only less than half of TDG-expressing zygotes proceeded to 2-cell stage and γH2AX foci were dispersed in the whole chromosome of surviving TDG-expressing 2-cell embryos. This γH2AX pattern in TDG-expressing 2-cell embryos would not be solely due to 5hmC. Because dying cells have been reported to accumulate γH2AX in response to the apoptotic signals (Lu et al., FEBS Lett., 2008). It is quite likely that TDG overexpression-induced cell death would be the major cause of the γH2AX accumulation, since survived 2-cell embryos stopped the development before the blastocyst stage (data not shown). Considering that this is basically a negative data, we did not mention about this experiment in the revised manuscript.

The results of Figure 2C and 3E could not be compared directly, because these two data were obtained by using different antibodies against γH2AX. The usage of different antibodies is due to the technical reason of double staining. Staining of Stella and Myc-tagged TDG required rabbit and mouse antibodies in Figure 2C and 3E, respectively. Therefore, mouse monoclonal and rabbit polyclonal anti-γH2AX antibodies should have been used in the experiments of Figure 2C and 3E, respectively.

The final experiment aims to extend the link between 5hmC accumulation (by Tet3 overexpression!!) and replication stress in mouse 3T3 cells. The data are nice but the question is of which relevance the findings are for the early embryonic phenotypes particularly in the context of Stella. There are many parameters such as the different isoforms of Tet3 and non 5hmC effects that should be respected to allow this cross reference. Finally authors suggest that Tet3 and Tet2 do not process 5mC to 5hmC. This statement should be considered since others (Hu et al, Cell2013) have clearly show that at least Tet2 is capable of processing 5hmC further.

We agree with the referee’s comment that embryos and NIH-3T3 cells have different contexts. For example, there was no detectable 5hmC in NIH-3T3 cells and different Tet isoforms are expressed.
On the other hand, overexpression of Tet induced aberrant 5hmC formation in NIH-3T3 cells. Moreover, in the Tet3 overexpressing NIH-3T3 cells, aberrant γH2AX accumulation, defects in DNA replication, and delayed cell proliferation were induced in an enzymatic activity-dependent manner. Taken together, we consider it quite reasonable that artificially overexpressed Tet3 would be the cause of these phenotype. Therefore, it is quite likely that basic molecular mechanisms can be examined in NIH-3T3 cells for this purpose.

We are afraid that there is some misunderstanding. We agree with the referee #2’s comment that Tet2 is capable of processing 5mC to 5fC and 5caC further (Hu et al., Cell, 2013). However we did not mention that Tet3 and Tet2 did not process 5mC to 5hmC. Immunostaining in our hands showed that overexpression of Tet in NIH-3T3 cells gave rise to only 5hmC, but neither 5fC nor 5caC. This indicated that 5fC and 5caC might be removed by endogenous TDG shortly once produced, as shown previously (Nabel et al., Nat. Chem. Biol., 2012).

Referee #3:

Major points:

1. Concurrence of 5hmC and γH2AX in maternal genome need more vigorous checking in control and Stella-null mouse. Meanwhile, I also have reservations in the interpretation of data. As showed in Figure 2D and 2E, “both γH2AX foci and 5hmC were detectable only in the H3K9me2-negative paternal chromatin (Fig 2D, E and S1). In contrast, γH2AX foci and 5hmC were diffusely distributed in the nuclei of Stella-null 2-cell embryos (Fig 2D and E).” But the Figure 2D does not support that γH2AX foci were not diffusely distributed in the nuclei of Stella-null 2-cell embryos. Instead, the figure supports that γH2AX foci is detectable only in the H3K9me2-negative paternal chromatin in Stella-null mouse. Similarly, the results in Figure 2E, 5hmC in stella-null mouse also showed mutually exclusively localized in H3K9me2-negative paternal chromatin. Therefore, more experiments using more embryos should be done to confirm the distribution.

Sorry for the insufficient description of the result. We changed the “γH2AX foci and 5hmC were diffusely distributed in the nuclei” to “γH2AX foci and 5hmC were distributed in the H3K9me2-positive chromatin” (page 7, line 10-11). The images in Figure 2D were replaced to make the localization of H3K9me2 and γH2AX clearer as the referee #1 suggested in the minor comment.

As for the localization of H3K9me2 and γH2AX, their co-localization was quantified in 2-cell embryos and confirmed about three times more frequent in Stella-null (25.7%) than control (8.7%). Co-localization of H3K9me2 and 5hmC was also analyzed by Image J and confirmed that their overlap coefficient was significantly higher in the Stella-null (0.35) than the control (0.23). Therefore, we concluded that γH2AX foci and 5hmC were distributed in the H3K9me2-positive chromatin in Stella-null embryos.

2. The amount of the 5hmC antibody used in the method should be titrated with positive control nucleotides. Those evidences will help the understanding of the recent sequencing data in 2-cell embryo of the control mouse (Wang et al. 2014), and elucidate whether 5hmC is also modified in maternal genome.

To examine the specificity of anti-5hmC antibody, we performed immunostaining using the anti-5hmC antibody which had been pre-treated with 5hmC nucleotide. Fluorescence signal was disappeared by the treatment of 5hmC but not by that of 5mC nucleotide, which clearly demonstrated that fluorescence signal reflects 5hmC (data not shown).

3. Previous studies support that Stella protects the DNA demethylation on H3K9me2 labeled regions through 5hmC (Wossidlo et al. 2011, Nakamura et al. 2007), it was intriguing to explore carefully whether 5hmC is co-localized with H3K9me2 staining. Although from the Figure 2E, the 5hmC and H3K9me2 showed quite different distribution.

We are afraid that the referee might be a little confused. The previous paper only showed that Stella protects the DNA methylation through H3K9me2 to inhibit 5hmC formation (Wossidlo et al., Nat.

4. co-staining 5hmC and γH2AX will give better idea, whether 5hmC is response to γH2AX accumulation in maternal genome in Stella-null mouse.

Similar concern was raised as one of the major point of the referee #1. We tried double immunostaining of 5hmC and γH2AX, however did not succeed. The major technical reason is that HCl treatment necessary for the immunostaining of 5hmC degenerates γH2AX antigen. We sincerely appreciate if the referee kindly understands the situation.

5. siTET3 in zygote is required to elucidate whether the induction of 5hmC and γH2AX accumulation is impaired especially in maternal genome of wide-type mouse. This should reconstructed the phenotype of Stella-null mouse to support the major conclusion of this manuscript.

Thanks for the the valuable comments. We performed the knockdown analysis of the Tet3 in zygotes to clarify the direct effect of Tet3 on γH2AX accumulation. Knockdown of Tet3 exhibited significant decrease of the accumulation of γH2AX (Fig 3F). The data was added in Figure 3F. Corresponding sentences were rewritten in the Results and Discussion (page 7, line 17-20) and in the Conclusions (page 8, line 17-19). Sentences were added in the Figure Legends (page 18, line 10-12) and in the Materials and Methods (supplemental information file, page 4, line 7-12).

Minor comments
1) Figure 1D, it was not quite clear how to determine the micronucleus-like phenotype. The micronucleus showed in the third panel is quite different to the others in case of the distance to the nuclear.

The micronucleus-like phenotype was determined from the time-lapse imaging of 3D image stacks. Chromosome dynamics was visualized by expressing the H2B-mRFP. Therefore, the moment of forming micronuclei could be caught at the segregation period, and divided H2B-mRFP positive fragments were shown as micronuclei in Figure 1D. The photograph was replaced to another one to show the micronuclei clearly.

2) The labels of Figure3 may contain typo. The author may want to change to AG-TET2 instead of AG-et2 et al.

We could not find the typographical error. It is presumably due to the some process to view the file.

3) The staining of γH2AX in Figure 2D is barely seen especially in control mouse.

Similar concern was raised as one of the minor points of the referee #1. Sorry for the photograph with low contrast. The photographs were replaced with the new gray ones with higher S/N ratio.

2nd Editorial Decision 13 January 2015

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it, and both support publication of the manuscript now. Referee 1 only has minor suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

Regarding statistics, it is good that you have added the number of embryos used for your analyses. However, it is not clear to me how many experiments were performed. Were all mentioned embryos treated at the same time, or are they a collection from independent experiments? It is important to add this information. In order to calculate statistics, 3 or more independent experiments would need to be performed, and the average values with error bars should be shown. It is not clear what the statistical test refers to in Fig 1B. If all embryos were treated in parallel in a single experiment only, no statistics can be calculated. The number of embryos is also missing for Fig 1C and E and Fig 4F.
I look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have any comments or questions.

REFEREE REPORTS:

Referee #1:

The manuscript has been improved and the authors have made a considerable effort to address the comments raised by the referees. It is unfortunate that the ATR inhibitor experiment did not produce exploitable results and I wonder whether a short treatment of ATR inhibitor (only in zygotes) would rescue at least the delayed replication phenotype, but this experiment is not necessary for the publication of this manuscript, I believe, which I now support based on the revisions by the authors. In this sense, re-interpretation of Fig.4 is welcome, and figure 3F is a strong addition, together with all the more rigorous quantifications and data analysis that was added to the revised manuscript.

I have one major and one minor point that I believe must be corrected before the paper can be published, but these are only text changes.
1. Minor: On page 9, line 4, English is not correct, please rephrase
2. MAJOR: On page 9, line10, 'We demonstrate is again overstated'. I suggest the following phrase instead, immediately after reference 3: "BASED ON OUR NIH3T3 DATA, WE SUGGEST THAT 5HMC WOULD PERTURB THE INTEGRITY OF......"

Referee #3:

Due to the technical reason, the authors cannot get results of 5hmC and H2AX co-staining which will give better mechanistic explanation of the observations. The manuscript has been extensively revised, and the authors have done new experiments to further address the mechanistic relationship (Fig3F). Therefore, I will suggest to publish the manuscript.

2nd Revision - authors' response 22 January 2015

Referee#1

The manuscript has been improved and the authors have made a considerable effort to address the comments raised by the referees. It is unfortunate that the ATR inhibitor experiment did not produce exploitable results and I wonder whether a short treatment of ATR inhibitor (only in zygotes) would rescue at least the delayed replication phenotype, but this experiment is not necessary for the publication of this manuscript, I believe, which I now support based on the revisions by the authors. In this sense, re-interpretation of Fig.4 is welcome, and figure 3F is a strong addition, together with all the more rigorous quantifications and data analysis that was added to the revised manuscript.

We consider that a long treatment with ATR inhibitor is required to inhibit the 5hmC-induced γH2AX accumulation. Because 5hmC is stably maintained from zygotes to 2-cell embryos, thus, we have not examined the effect of a short treatment on the DNA replication. We would appreciate the positive comments from referee #1 on our data of Fig 4 and Fig 3F.

I have one major and one minor point that I believe must be corrected before the paper can be published, but these are only text changes.
1. Minor: On page 9, line 4, English is not correct, please rephrase
Sorry for the incorrect English. Following the referee’s comment, we rewrote the sentence (page 9, line 3-5).

2. MAJOR: On page 9, line 10, 'We demonstrate is again overstated'. I suggest the following phrase instead, immediately after reference 3: "BASED ON OUR NIH3T3 DATA, WE SUGGEST THAT 5HMC WOULD PERTURB THE INTEGRITY OF......"

Following the referee’s comment, we rewrote the manuscript (page 9, line 9-10).

Referee #3

Due to the technical reason, the authors cannot get results of 5hmC and H2AX co-staining which will give better mechanistic explanation of the observations. The manuscript has been extensively revised, and the authors have done new experiments to further address the mechanistic relationship (Fig3F. Therefore, I will suggest to publish the manuscript.

Thanks for the understanding of the technical issue of double staining. We would like to express our gratitude for all the valuable comments from referee #3.

3rd Editorial Decision 26 January 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.