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Replication-coupled passive DNA demethylation for the erasure of genome imprints in mice

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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 13 August 2012

Thank you very much for submitting your research paper for consideration to The EMBO Journal editorial office.I received extremely coherent input from two independent expert referees and am thus able to communicate a decision.

As you will recognize, both scientists appreciate timeliness and value of the presented results. Their minor critiques center around further integration of the DNA-methylation status with the cell cycle. I would thus urge you to attend particularly to the comments on Fig4 from ref#2 (bisulfite sequencing in PGCs at different cell cycle states) and as raised from both referees employ PCNA staining/short BrdU-pulse in Fig 5d and e.

Please also attend carefully to the other minor issues raised (adding a few controls and clarifications) and adjust the paper format to The EMBO Journal article guidelines before submitting an ultimate version for final assessment.

Please do note that The EMBO Journal considers only one round of major revisions and do not hesitate to get in touch in case of further questions (preferably via E-mail).

I am very much looking forward to your amended manuscript and remain with best regards.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

The authors present a well-validated in vivo system to assay expression and epigenetic changes in primordial germ cells (PGC), during the period in embryogenesis in which they undergo genome-wide demethylation. Their results show that during the critical period, PGCs lack mRNA and protein products for most of the known mammalian demethylation pathways, with the exception of TET1 that could be excluded as mice lacking this enzyme do not display PGC anomalies. Similarly PGCs for this period, also lack known DNA de novo methyltransferases and, although they continue to express the maintenance DNMT1 hemi-methyltransferase, it mostly does not translocate to replication foci of S phase cells, due to the lack of the UHRF1 cofactor. The authors then propose, in contrast to previous publications, that PGC demethylation occurs passively due to the unavailability of methylation enzymes during PGC differentiation. They test this hypothesis in two ways. First they show that differentiating PGCs have a higher proliferation rate than previously shown, which is sufficient to allow a passive loss of DNA methylation during the critical period. Next, they examined the exact timing of erasure of DNA methylation imprints on six differentially methylated regions (DMRs) and of methylation changes on one type of retrotransposon. The authors use bisulphite sequencing applied to F1 mice carrying SNPs that allow the two parental alleles to be distinguished. Interestingly, five DMRs already showed some 'imprint' erasure by 10.5dpc, earlier than shown by previous publications that did not use an allele-specific assay. All six DMRs substantially lose DNA methylation by 12 - 13.5 dpc in a manner fully consistent with the proposed model of replication-coupled passive demethylation. Lastly, the authors show in contrast to two previous publications, that major changes in Chromocenter appearance, Histone H1 linker and H3K27me3 abundance do not occur during the critical window for PGC demethyation. However, they confirm reports in the same publications that nuclear enlargement is seen in early differentiating PGC. The authors suggest that their data, which examined PGCs within the genital ridge, provides more accurate information than the two previous publications that analyzed dissociated single PGCs attached to slides.

Overall the authors present very convincing data to support their model that PGC genome-wide demethylation is explained by 'replication-coupled passive-demethylation'. This model is a challenge to the current understanding of this process, which is based on three previous publications (Hajkova et al, 2008; Hajkova et al, 2010, Popp et al., 2010) providing evidence for active demethyation using a DNA repair pathway. The strengths of the authors data is their analysis of PGC in vivo, their careful quantification and their ability to accurately identify PGC using the Stella-EGFP transgenic mouse. This is an important result that has clear implications for studies to identify demethylation pathways in development and in human disease. The paper is well presented in good written English and is suitable for publication as it is. However I do ask that the following minor points, including one additional immunofluorescence experiment, are attended to as they will improve accessibility of the manuscript for those outside the field.

Minor comments that should be attended to:

1) Figure 1 legend and relevant text: The explanation of plotted colors should be improved. The authors do not explain why they are assaying PGC from mixed sex 9.5dpc - 11.5 dpc embryos and from single sex 12-13.5 dpc embryos or what is the purpose of the in vitro data. It would help if the text/legend could categorize the assayed genes to help the reader follow the logic of the analysis in Figure 1 (e.g., the results section on Blimp1, Prdm14, Mvh does not say why the expression of these genes is relevant).

2) Figure 2: the image quality looks blurred in my merged PDF copy - perhaps the originals are sharper. 2A: should label the non-STELLA positive cells that show UHRF1 expression as hindgut endoderm/somatic cells of genital ridge.

3) Figure 4: Bisulphite analysis of IAP retrotransposon - the authors should say if this is a mass analysis of all IAP retrotransposons, or if the primers used allowed analysis of one IAP locus.

4) Figure 4. As the authors note in the text, the rate of imprint erasure varied amongst the six studied DMRs, which the authors suggest can be explained by similarity to retrotransposons. Could the

authors also say if this correlate with the presence of tandem direct repeats that are a features of many gDMRs, or if it correlates with size of the DMR?

5) Figure 5: the authors should say how they quantified Chromocenter appearance and if H1 abundance was quantified as described for H3K27me3. The authors show that H3K27me3 does not display an abrupt loss in during PGC differentiation. However, they do detect a heterogeneity in all stages such that 60% of cells are strongly positive and 40% are weakly positive. The authors attribute this to the ~40% of S phase cells present in the population in Figure 5F. I would ask if the authors could not demonstrate this with their PCNA antibody. I ask this of the authors because much of this section argues against conclusions of two previous publications (Hajkova et al, 2008; Hajkova et al, 2010) and it would be better to be clear on this point.

6) Nomenclature issues: Naming of genes or DMRs - the authors looked at the changing methylation status of known DMRs (differentially methylated regions) that are referred to as 'genes' in the text. The term 'gene; is not correctly used here, as some of these regions (e.g., the H19 DMD) lie outside of a gene. Some do overlap the promoter CpG island of a gene, but none extend the full length of a gene. DMR is the optimal term to use here as these regions were previously identified as such. The genomic location of the DMRs assayed, and also if they are gametic or somatic DMRs, should be included somewhere in the Text, legend or Methods and the reference given that demonstrated these are DMRs. The official HUGO gene names must be mentioned once in the text (Lit1=Kcnq1ot1). Protein names should be written in all capitals e.g., STELLA, mouse gene names as Stella in italics.

7) Data issues: microarray data which is the basis of Figure 1, should be deposited in a publically available database such as GEO and details of the statistical analysis should be provided in the Methods.

8) Manuscript type: The manuscript is not laid out in the EMBO J style.

Referee #2

Figure 1: nice to see all expression data together. High quality
Figure 2a and 2B: good. But I would like to see a positive control for Uhrf1 in cycling somatic cells (can be e.g. ESCs). Maybe confirmation with a second antibody would be good.
Figure 2c and d: it is difficult to interpret the quality of Dnmt1 staining.

Figure 3: good quality and interesting results.
Figure 4: very important and key to the paper. I think that the authors can gain more weight by performing bisulfite in PGCs at different stages of the cell cycle (combine methods of Fig 3a and Fig 4 in a new experiment). If the level of DNA methylation is about 2-fold different in G1 vs G2 cells at the two consecutive days, this would be very strong!

Figure 5d and e: it would be good when these data could be related to the cell cycle (PCNA staining, short pulse of BrdU). Nap1 staining was a strong point in the Hajkova paper - should be repeated.

1st Revision - authors' response 04 November 2012

We would like to sincerely thank the reviewers for their encouraging and constructive comments, and have used these as the basis for revising the manuscript. Here we address the reviewers' specific comments.

Referees' Comments:

Referee #1:
The authors present a well-validated in vivo system to assay expression and epigenetic changes in primordial germ cells (PGC), during the period in embryogenesis in which they undergo genome-wide demethylation. Their results show that during the critical period, PGCs lack mRNA and

protein products for most of the known mammalian demethylation pathways, with the exception of TET1 that could be excluded as mice lacking this enzyme do not display PGC anomalies. Similarly PGCs for this period, also lack known DNA de novo methyltransferases and, although they continue to express the maintenance DNMT1 hemi-methyltransferase, it mostly does not translocate to replication foci of S phase cells, due to the lack of the UHRF1 cofactor. The authors then propose, in contrast to previous publications, that PGC demethylation occurs passively due to the unavailability of methylation enzymes during PGC differentiation. They test this hypothesis in two ways. First they show that differentiating PGCs have a higher proliferation rate than previously shown, which is sufficient to allow a passive loss of DNA methylation during the critical period. Next, they examined the exact timing of erasure of DNA methylation imprints on six differentially methylated regions (DMRs) and of methylation changes on one type of retrotransposon. The authors use bisulphite sequencing applied to F1 mice carrying SNPs that allow the two parental alleles to be distinguished. Interestingly, five DMRs already showed some 'imprint' erasure by 10.5dpc, earlier than shown by previous publications that did not use an allele-specific assay. All six DMRs substantially lose DNA methylation by 12 - 13.5 dpc in a manner fully consistent with the proposed model of replication-coupled passive demethylation. Lastly, the authors show in contrast to two previous publications, that major changes in Chromocenter appearance, Histone H1 linker and H3K27me3 abundance do not occur during the critical window for PGC demethyation. However, they confirm reports in the same publications that nuclear enlargement is seen in early differentiating PGC. The authors suggest that their data, which examined PGCs within the genital ridge, provides more accurate information than the two previous publications that analysed dissociated single PGCs attached to slides.

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Minor comments that should be attended to:
1) Figure 1 legend and relevant text: The explanation of plotted colours should be improved. The authors do not explain why they are assaying PGC from mixed sex 9.5dpc - 11.5 dpc embryos and from single sex 12-13.5 dpc embryos or what is the purpose of the in vitro data. It would help if the text/legend could categorize the assayed genes to help the reader follow the logic of the analysis in Figure 1 (e.g., the results section on Blimp1, Prdm14, Mvh does not say why the expression of these genes is relevant).

Response 1. We first analysed the expression of genes such as *Blimp1*, *Prdm14*, *Stella*, and *Mvh* in order to validate the results of our microarray analysis.

We analysed the gene expression of PGCs at E12.5 and E13.5 in both sexes (males and females). The reason why we analysed the PGCs from E9.5 to E11.5 from mixed sexes is that it is known that the gene expression of PGCs is indistinguishable until E11.5 in both sexes (Jameson et al, 2012). According to the suggestion by the referee, we categorized the genes analysed and explained the logic of the analysis in Figure 1 more clearly in the revised manuscript (p 5, and the legend to Figure 1 in the revised manuscript).

2) Figure 2: the image quality looks blurred in my merged PDF copy - perhaps the originals are sharper. 2A: should label the non-STELLA positive cells that show UHRF1 expression as hindgut endoderm/somatic cells of genital ridge.

Response 2. As the referee suggested, one of the reasons why the image in the original Figure 2A looked blurred was that the merged PDF file was compressed to a size that was suitable for online submission. To improve the image, we chose a different method for the image compression, so that the images look sharper even in the merged PDF file in the revised manuscript.

According to the referee's suggestion, we labelled the *Stella-EGFP*-negative somatic cells in the revised manuscript (Figure 2A in the revised manuscript).

3) Figure 4: Bisulphite analysis of IAP retrotransposon - the authors should say if this is a mass analysis of all IAP retrotransposons, or if the primers used allowed analysis of one IAP locus.

Response 3. The primers we used for the bisulfite analysis of IAP retrotransposon amplify a mass population of essentially all IAPs. We clarified this point in the legend to Figure 4B and in the bisulfite sequence analysis section of the Materials and Methods in the revised manuscript.

4) Figure 4. As the authors note in the text, the rate of imprint erasure varied amongst the six studied DMRs, which the authors suggest can be explained by similarity to retrotransposons. Could the authors also say if this correlate with the presence of tandem direct repeats that are a features of many gDMRs, or if it correlates with size of the DMR?

Response 4. According to the referee's suggestion, we examined the correlation between the rate of imprint erasure and the presence of tandem direct repeats in the DMRs or the size of the DMRs. However, we did not find a clear correlation. We have added a mention of this analysis in the Discussion section of the revised manuscript (p 13, paragraph 2 in the revised manuscript).

5) Figure 5: the authors should say how they quantified Chromocenter appearance and if H1 abundance was quantified as described for H3K27me3. The authors show that H3K27me3 does not display an abrupt loss in during PGC differentiation. However, they do detect a heterogeneity in all stages such that 60% of cells are strongly positive and 40% are weakly positive. The authors attribute this to the ~40% of S phase cells present in the population in Figure 5F. I would ask if the authors could not demonstrate this with their PCNA antibody. I ask this of the authors because much of this section argues against conclusions of two previous publications (Hajkova et al, 2008; Hajkova et al, 2010) and it would be better to be clear on this point.

Response 5. According to the referee's suggestion, we defined the criteria for the quantification of the chromocenters in the revised manuscript (major axis > 0.4 mm, relative staining intensity more than twice that of the nucleoplasm (p 10, paragraph 1, and the legend to Figure 5B in the revised manuscript).

In our hands, nearly all the *Stella-EGFP*-positive PGCs showed essentially equivalent staining for H1. We therefore simply indicated the number of *Stella-EGFP*-positive PGCs we analysed and explained this point in the revised manuscript (p 10, paragraph 2 in the revised manuscript).

To respond to the referee's comment, we investigated the relationship between the intensity of the H3K27me3 level in PGCs and the cell-cycle state of PGCs. Although we performed a number of trials, the staining of *Stella-EGFP*-positive PGCs for PCNA and H3K27me3 (triple labelling of the samples for Stella-EGFP, PCNA, and H3K27me3) did not yield a convincing outcome, which was partly because the pattern of PCNA staining does not distinguish the early S phase from the G1 phase. We therefore tried a different approach: As we have shown in Figure 3A, we labelled E11.5 embryos by BrdU and immunostained *Stella-EGFP*-positive PGCs for BrdU and H3K27me3 (triple labelling of the samples for EGFP, BrdU, and H3K27me3). As we discussed in the original manuscript, this experiment revealed that *Stella-EGFP*-positive PGCs that incorporated BrdU, i.e., PGCs in the S phase of the cell cycle, exhibited a higher H3K27me3 level (Figure 6E, F). Based on this experiment, we conclude that the heterogeneity of H3K27me3 levels in PGCs reflects their cell cycle state: PGCs at the S phase generally have a higher H3K27me3 level, whereas those outside the S phase have a lower H3K27me3 level. We provided the relevant data and statements in the revised manuscript (p 10-11 in the revised manuscript).

6) Nomenclature issues: Naming of genes or DMRs - the authors looked at the changing methylation status of known DMRs (differentially methylated regions) that are referred to as 'genes' in the text. The term 'gene; is not correctly used here, as some of these regions (e.g., the H19 DMD) lie outside of a gene. Some do overlap the promoter CpG island of a gene, but none extend the full length of a gene. DMR is the optimal term to use here as these regions were previously identified as such. The genomic location of the DMRs assayed, and also if they are gametic or somatic DMRs, should be included somewhere in the Text, legend or Methods and the reference given that demonstrated these

are DMRs. The official HUGO gene names must be mentioned once in the text (Lit1=Kcnq1ot1). Protein names should be written in all capitals e.g., STELLA, mouse gene names as Stella in italics.

Response 6. As suggested, we have used the term DMR throughout the text in the revised manuscript. In addition, we have shown the genomic locations of the DMRs analysed (Figure 4A in the revised manuscript), stated their origin and provided appropriate references in the revised manuscript (p 15, Materials and Methods in the revised manuscript). We also stated the official HUGO gene names at their first appearance and wrote the protein and gene names as suggested by the reviewer.

7) Data issues: microarray data which is the basis of Figure 1, should be deposited in a publically available database such as GEO and details of the statistical analysis should be provided in the Methods.

Response 7. We have presented our microarray data in the NCBI Gene Expression Omnibus (GEO) database. The accession number is GSE40412, which is included in the Microarray analysis section of the Materials and Methods. We also provided the details of the statistical analysis we employed in the Microarray analysis section of the Materials and Methods.

8) Manuscript type: The manuscript is not laid out in the EMBO J style.

Response 8. We revised the manuscript to conform to the EMBO Journal style.

Referee #2

Figure 1: nice to see all expression data together. High quality.

Response 1. According to the suggestion by the referee, we combined Figure 1 and Supplementary Figure S1 in the original manuscript to create the new Figure 1 in the revised manuscript.

Figure 2a and 2B: good. But I would like to see a positive control for Uhrf1 in cycling somatic cells (can be e.g. ESCs). Maybe confirmation with a second antibody would be good.

Response 2. We provided immunofluorescence and Western blot analysis of UHRF1 expression in ESCs in the revised manuscript (Figure 2A, p 7 in the revised manuscript).

Figure 2c and d: it is difficult to interpret the quality of Dnmt1 staining.

Response 3. To respond to the referee's comment, we provided immunofluorescence analysis of DNMT1 and PCNA expression/localization in ESCs at the S phase, which shows clearly that DNMT1 localizes at PCNA-positive replication foci in ESCs, validating the quality of our DNMT1 immunostaining (Figure 2C, p 7 in the revised manuscript).

We assume that the reason why it was difficult for the referee to interpret the quality of DNMT1 staining in PGCs was that DNMT1 was diffusively localized in the nucleus in most of the PGCs. However, please note that the DNMT1 staining in PGCs is highly specific to the nucleus and is excluded from the cytoplasm (please compare the DNMT1 staining panels with those of DAPI and Stella-EGFP in the revised manuscript), demonstrating the quality of the DNMT1 staining. In addition, for the revised manuscript we employed a better image-compression method when uploading the figures (Figure 2C in the revised manuscript).

Figure 3: good quality and interesting results.

Figure 4: very important and key to the paper. I think that the authors can gain more weight by performing bisulfite in PGCs at different stages of the cell cycle (combine methods of Fig 3a and Fig 4 in a new experiment). If the level of DNA methylation is about 2-fold different in G1 vs G2 cells at the two consecutive days, this would be very strong!

Response 4. The method we employed for the measurement of the cell-cycle state of PGCs shown in Figure 3 involves a treatment of the FACS-sorted cells with DNase for the detection of BrdU. This

treatment essentially digests genome DNA. It is therefore very difficult/impossible to obtain reliable/quantitative bisulfite sequence data using cells treated for the procedure of cell-cycle measurement used in Figure 3.

It is also important to note that PGCs are developmentally heterogeneous: For example, they initiate migration and undergo epigenetic reprogramming in a progressive, cell-by-cell manner (Molyneaux et al, 2001; Seki et al, 2007), and later, they also enter into meiosis in a progressive, cell-by-cell manner (Menke et al, 2003). Therefore the PGCs at the G1 and the G2 phase of the same embryos/embryos isolated at the same stage would be mixtures of developmentally more advanced and more retarded cells, making the suggested analysis rather redundant.

The key message of the present manuscript is to convey to the relevant research community that, contrary to the prevailing model emphasizing the involvement of active mechanisms, genome-wide DNA demethylation can essentially be explained by a replication-coupled passive mechanism. We consider that we have provided sufficient evidence to support our message in the revised manuscript. We also believe that, for a stronger demonstration of the involvement of the passive mechanism in the genome-wide DNA demethylation in PGCs, some functional experiment would be necessary, such as to evaluate the effect of over-expression of key repressed genes (e.g., *Uhrf1*) in PGCs. Therefore, such an experiment is currently underway in our laboratory. We have provided a relevant discussion of this matter in the revised manuscript (p 14, last paragraph in the revised manuscript).

Figure 5d and e: it would be good when these data could be related to the cell cycle (PCNA staining, short pulse of BrdU). Nap1 staining was a strong point in the Hajkova paper - should be repeated.

Response 5. To respond to the referee's comment, we labelled E11.5 embryos by BrdU and immunostained *Stella-EGFP*-positive PGCs for BrdU and H3K27me3 (triple labelling of the samples for Stella-EGFP, BrdU, and H3K27me3). As we assumed in the original manuscript, this experiment revealed that *Stella-EGFP*-positive PGCs that incorporated BrdU, i.e., PGCs in the S phase of the cell cycle, exhibited a higher H3K27me3 level (Figure 6E, F). Based on this experiment, we conclude that the heterogeneity of H3K27me3 levels in PGCs reflects their cell cycle state: PGCs at the S phase generally have a higher H3K27me3 level, whereas those outside the S phase have a lower H3K27me3 level. We provided the relevant data and statements in the revised manuscript (Figure 6E, F, p 10-11 in the revised manuscript).

In response to the referee's comment, we examined the localization of NAP1/NAP1l1 in PGCs by immunostaining. In contrast to the findings of Hajkova et al., we did not detect predominant localization of NAP1/NAP1l1 in the nucleus of PGCs around E11.5, which is consistent with our finding that H1 is consistently detected in the nuclei of PGCs throughout the period we examined. We provided the relevant data and statements in the revised manuscript (Figure 6B, p 10, paragraph 2 in the revised manuscript).

References

Jameson SA, Natarajan A, Cool J, DeFalco T, Maatouk DM, Mork L, Munger SC, Capel B (2012) Temporal transcriptional profiling of somatic and germ cells reveals biased lineage priming of sexual fate in the fetal mouse gonad. *PLoS Genet* 8(3): e1002575

Menke DB, Koubova J, Page DC (2003) Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave. *Dev Biol* 262(2): 303-312

Molyneaux KA, Stallock J, Schaible K, Wylie C (2001) Time-lapse analysis of living mouse germ cell migration. *Dev Biol* 240(2): 488-498

Seki Y, Yamaji M, Yabuta Y, Sano M, Shigeta M, Matsui Y, Saga Y, Tachibana M, Shinkai Y, Saitou M (2007) Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. *Development* 134(14): 2627-2638

2nd Editorial Decision 15 November 2012

Your revised manuscript has now been re-assessed by one of the original referees. As you will see from the enclosed comment, this scientists recommends to be more specific with regard to the peri-centromeric heterochromatin localization and general conclusions that might arise from these results. I kindly ask you to amend the manuscript accordingly to enable efficient acceptance of such an ultimate version.

Yours sincerely,

Editor

The EMBO Journal

REFEREE REPORT:

Referee #1:

The authors have appropriately addressed the concerns of both reviewers. In principle, I recommend publication in EMBO Journal .

I have only one remaining concern that I recommend the authors to address in their final version of the manuscript. In the experiments describing the localization of Dnmt1 at replication foci marked by PCNA, the authors introduce the assay by focusing on the localization of both proteins at DAPI-intensely stained peri-centromeric heterochromatin regions in cell at mid to late S-phase. Subsequently, they describe the co-localization data in PGCs in more general terms, not referring to peri-centromeric heterochromatin regions anymore. In the figures, however, most examples of co-localization concern peri-centromeric heterochromatin. My question is how the authors interpret their data: does it mostly concern major satellites at peri-centromeric heterochromatin? If this is the case, the authors should discuss their data in that context. Of course, the authors can further speculate that a similar mechanism may apply to other regions of the genome as well.

2nd Revision - authors' response 16 November 2012

We would like to sincerely thank the reviewer for their constructive comments. Here we address the reviewer's comments.

Referees' Comments:

Referee #1:

The authors have appropriately addressed the concerns of both reviewers. In principle, I recommend publication in EMBO J.

I have only one remaining concern that I recommend the authors to address in their final version of the manuscript. In the experiments describing the localization of Dnmt1 at replication foci marked by PCNA, the authors introduce the assay by focusing on the localization of both proteins at DAPI-intensely stained peri-centromeric heterochromatin regions in cell at mid to late S-phase. Subsequently, they describe the co-localization data in PGCs in more general terms, not referring to peri-centromeric heterochromatin regions anymore. In the figures, however, most examples of co-localization concern peri-centromeric heterochromatin. My question is how the authors interpret their data: does it mostly concern major satellites at peri-centromeric heterochromatin? If this is the case, the authors should discuss their data in that context. Of course, the authors can further speculate that a similar mechanism may apply to other regions of the genome as well.

Response 1. We would like to thank the reviewer for this suggestion. We revised the relevant paragraph accordingly in the revised manuscript (p 7). We here paste the revised sentences.

As shown in Figure 2C and 2D, ~90% of the gonadal somatic cells at the mid-late S phase from E10.5 to E13.5 exhibited localization of DNMT1 at the PCNA-positive replication foci, indicating that, in these cells, maintenance DNA methylation occurs in a replication-coupled manner, at least at late replicating heterochromatic foci (including DAPI-positive peri-centromeric heterochromatin) and most likely in other regions of the genome. In sharp contrast, only ~10-20% and ~30% of the PGCs at the mid-late S phase showed localization of DNMT1 at replication foci at E10.5-E11.5 and at E12.5-E13.5, respectively (Figure 2C and 2D). These findings suggest the possibility that a majority of PGCs fail to undergo/are inefficient for replication-coupled maintenance DNA methylation, at least at late replicating heterochromatic foci and presumably also in other regions of the genome, especially at E10.5 and E11.5, and hence erase their DNA methylation via replication-coupled passive DNA demethylation.