De novo DNA methylation drives 5hmC accumulation in mouse zygotes

Rachel Amouroux1, Buhe Nashun1,7, Kenjiro Shirane2,7, Shoma Nakagawa3,7, Peter W. S. Hill1, Zelpha D’Souza1, Manabu Nakayama4, Masashi Matsuda5, Aleksandra Turp1, Elodie Ndjetehe1, Vesela Encheva1,6, Nobuaki R. Kudo3, Haruhiko Koseki5, Hiroyuki Sasaki2 and Petra Hajkova1,8

Zygotic epigenetic reprogramming entails genome-wide DNA demethylation that is accompanied by Tet methylcytosine dioxygenase 3 (Tet3)-driven oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC; refs 1–4). Here we demonstrate using detailed immunofluorescence analysis and ultrasensitive LC-MS-based quantitative measurements that the initial loss of paternal 5mC does not require 5hmC formation. Small-molecule inhibition of Tet3 activity, as well as genetic ablation, impeded 5hmC accumulation in zygotes without affecting the early loss of paternal 5mC. Instead, 5hmC accumulation is dependent on the activity of zygotic Dnmt3a and Dnmt1, documenting a role for Tet3-driven hydroxylation in targeting de novo methylation activities present in the early embryo. Our data thus provide further insights into the dynamics of zygotic reprogramming, revealing an intricate interplay between DNA demethylation, de novo methylation and Tet3-driven hydroxylation.

In the mouse zygote, the paternal genome undergoes genomewide loss of DNA methylation shortly after fertilization5,6. This has been mainly attributed to the activity of the Tet3 hydroxylase responsible for the accumulation of 5hmC on the paternal DNA (refs 1,3,4). To elucidate the exact involvement of 5hmC during this DNA demethylation, we examined the detailed kinetics of 5hmC appearance in connection with loss of 5mC signal (Fig. 1a,b). We confirmed accumulation of 5hmC in the paternal pronucleus at late zygotic stages1–4, consistent with the described localization and expression pattern of the Tet3 enzyme (Supplementary Fig. 1a,b). Detailed analysis of 5mC and 5hmC immunofluorescence data, however, revealed that whereas 5mC disappears from the paternal genome by early PN3 stage, 5hmC starts to accumulate only after the main drop in 5mC has occurred, and increases considerably from PN4 onwards7 (Fig. 1a–c). Consequently, paternal DNA of late PN2–early PN3 zygotes shows neither 5mC nor 5hmC signal (Fig. 1d). Moreover, the accumulation of 5-formylcytosine (5fC) and 5-carboxycytosine (5caC; refs 8,10) is detectable concomitantly to 5hmC appearance in the paternal pronucleus, a few hours after the loss of 5mC (Supplementary Fig. 1c; ref. 10). The delayed appearance of 5hmC is also not due to the low sensitivity of the 5hmC antibody, as 5hmC is detectable with considerably higher sensitivity than 5mC (Supplementary Fig. 1d,e).

Tet proteins belong to the family of 2-oxoglutarate (2OG) and iron (Fe2+) -dependent dioxygenases11. To assess the involvement of Tet3-driven 5mC hydroxylation in zygotic demethylation, we eliminated 5hmC by using dimethyloxallyl glycine (DMOG), a small-molecule inhibitor of 2OG-dependent oxygenases. DMOG effectively blocks the activity of Tet enzymes in vitro (Supplementary Fig. 2a); consequently, the presence of DMOG during in vitro fertilization (IVF) leads to an absence of 5hmC, 5fC and 5caC in the paternal pronucleus (Fig. 2a and Supplementary Fig. 2c,d). Of note, DMOG treatment does not affect the presence of maternal 5hmC (Fig. 2a), the normal asymmetry of histone modifications observed between pronuclei (Supplementary Fig. 3a), or the development of preimplantation embryos (Supplementary Fig. 2e,f). Strikingly, the lack of 5hmC formation following DMOG treatment did not impact on the extent of DNA demethylation in early PN3 mouse zygotes (Fig. 2a and Supplementary Fig. 2b), which reinforced our initial observations showing that loss of paternal 5mC signal and accumulation of 5hmC are temporally disconnected. We further confirmed our findings by inhibiting Tet3 activity in the zygote using a Fe2+/...
Figure 1 5hmC and 5mC kinetics during mouse zygotic development. (a,b) 5mC (a) and 5hmC (b) enrichment in mouse zygotes at different developmental stages (as in ref. 38) assessed by immunofluorescence using 5mC- and 5hmC-specific antibodies. DNA is stained using propidium iodide (PI). Representative images are shown and correspond to the 5mC and 5hmC signal quantification presented in c. (c) Quantification of 5mC (red, left axis) and 5hmC (green, right axis) staining as a ratio between signal from the paternal (pat.) pronucleus relative to the signal from the maternal (mat.) pronucleus. Values are plotted against the area of the mid-sections of the paternal pronuclei. Each data point represents a zygote. Experiment reproduced three times (n > 100). (d) Loss of paternal 5mC and accumulation of 5hmC are temporally separated. Early PN3 zygotes do not show any detectable 5mC or 5hmC in the paternal pronucleus. PN, pronuclei; p, female pronucleus; o, male pronucleus; pb, polar body. Scale bars, 5 μm.

chelator, deferoxamin (DFX). This inhibitor also effectively blocks the formation of 5hmC, but has no impact on the loss of paternal 5mC signal in the early PN3 zygotes (Supplementary Fig. 3b,c), as also observed with DMOG. Collectively, these results suggest that the Tet3-driven accumulation of 5hmC observed in the late zygote is not required for the initial loss of paternal 5mC signal.

Line1 represents a class of non-LTR repetitive elements that has been shown to lose methylation during zygotic reprogramming. Although these elements have been previously suggested as targets for 5mC hydroxylation in zygotest, our results clearly show that Line1 elements (L1Md_Tf and L1Md_Gf subtypes) undergo DNA demethylation in the absence of 5hmC formation to a similar extent as that observed in the control zygotes and two-cell-stage embryos (Supplementary Fig. 3d,e).

Immunofluorescence analysis provides an indirect quantification of DNA modifications, relying on the specificity and the affinity of the antibodies used. Additionally, differences between staining protocols, signal acquisition and approaches used to normalize the resulting signal make direct comparison between studies problematic. Bisulphite analysis can provide sequence-specific information; however, the results are compromised by the inability to distinguish between 5mC and 5hmC and between C, 5fC and 5caC (refs 8,9,14). In addition, whole-genome bisulphite analysis is subject to amplification biases and the lack of information regarding the
Figure 2 Small-molecule inhibition of Tet protein activity abrogates 5mC formation but does not prevent DNA demethylation. (a) 5mC and 5hmC staining of control and DMOG-treated zygotes (IVF). Quantification of both DNA modifications is represented as a ratio between the pronuclear signals (pat./mat.). For 5mC staining, n=18 PN3 zygotes and n=40 PN4–5 zygotes; for 5hmC staining, n=17 PN3 zygotes and n=48 PN4–5 zygotes. This experiment was replicated four times independently. (b) Quantification of the 5mC/dG and 5hmC/dG ratios in sperm, MII oocytes and in zygotes without polar bodies (control or treated with DMOG) by LC-MS (n=3 independent experiments with two technical replicates each, except for DMOG-treated zygotes; replicate of this experiment shown in Supplementary Fig. 4e). Limits of quantification are summarized in Supplementary Fig. 4a. For peaks below the quantification limit, an overestimation of the 5mC/dG ratio is calculated based on the limit of detection of 5hmC. (c) Quantification of DNA modifications in two-cell embryos derived from DMOG-treated or control zygotes analysed by LC-MS (n=2 independent experiments and two technical replicates for each point). Statistical analysis was carried out using Student’s t-test (two-sided). Error bars indicate s.d. Scale bars, 5 μm.

Copy number of various repetitive elements in the genome precludes precise quantitative assessment of the dynamic changes in global DNA modifications. Considering these facts we further supported our findings by an independent quantitative approach using an ultrasensitive liquid chromatography–mass spectrometry (LC-MS) method (Supplementary Fig. 4a).

Quantitative assessment of 5mC levels in normal fertilized zygotes (PN4–5) by LC-MS showed a clear 5mC loss in comparison with 5mC values measured in either of the gametes (Fig. 2b and Supplementary Fig. 4c) providing additional evidence for zygotic DNA demethylation. To assess the relative contribution of parental genomes to the measured 5mC levels, we quantified 5mC in parthenogenetically activated oocytes that lack the contribution of a sperm-derived genome. Our LC-MS measurements show that parthenotes contain a quantitatively similar amount of 5mC to MII oocytes (P=0.075, t-test; Supplementary Fig. 4c). The global level of DNA methylation is also not significantly affected by DNA replication as shown by 5mC levels in parthenotes treated with aphidicolin (P=0.078, t-test; Supplementary Fig. 4bc). As the maternal methylation remains globally stable (Supplementary Fig. 4c), the measured 5mC quantity in zygotes implies that the paternal genome at PN4–5 (10 h post-fertilization) has lost ~70% of 5mC compared with the sperm (Supplementary Fig. 4d). We confirmed that this measured loss of global 5mC occurs independently of the completion of S phase (P=0.058 between control and aphidicolin-treated zygotes, t-test; Supplementary Fig. 4f–h). Although we cannot preclude that
demethylation of some loci requires S-phase progression\textsuperscript{15,16}, our quantitative measurements demonstrate that on a genome-wide level the zygotic DNA demethylation is predominantly an active, replication-independent process\textsuperscript{5,17,18} (Supplementary Fig. 4f–h).

Our LC-MS data further show that the absolute 5hmC level in zygotes is very low compared with the amount of paternal 5mC lost during this wave of DNA demethylation (Fig. 2b and Supplementary Fig. 4e). Importantly and confirming our immunofluorescence data (Fig. 2a and Supplementary Fig. 2b), the measured global loss of 5mC is not affected by the absence of 5hmC in the DMOG-treated zygotes. These results thus demonstrate that 5hmC is not required for removal of most 5mC in the early PN3 zygotes (Fig. 2a,b and Supplementary Fig. 4e).

To provide further genetic validation of our results, we generated a conditional oocyte-specific Tet3 knockout (KO) mouse model (Fig. 3a). Gdf9-Cre-driven Tet3 deletion in early oocytes resulted in a complete absence of Tet3 messenger RNA in Tet\textsuperscript{3\textsubscript{mat}}/MII oocytes (\(P < 0.001\), t-test, Fig. 3b) and Tet3 protein in zygotes (Fig. 3c). This results in a lack of 5hmC formation in the paternal pronucleus following fertilization as assessed by immunofluorescence and by LC-MS of zygotes\textsuperscript{8} (Fig. 3d,e). In agreement with our data using dioxygenase inhibitors (Fig. 2a,b and Supplementary Fig. 3c), ablation of maternal Tet3 did not affect early loss of paternal 5mC observed in PN3 zygotes by immunofluorescence (Fig. 3d and Supplementary Fig. 4i) nor global 5mC levels in Tet\textsuperscript{3\textsubscript{mat}/pat\textsuperscript{−\textsubscript{−}}} zygotes as measured by LC-MS (Fig. 3e). Combined, these results thus do not corroborate the accepted model whereby 5hmC is an intermediate for global DNA demethylation that occurs in early pre-replicative (PN3) mouse zygotes\textsuperscript{1–4}.

Further assessment of DMOG-treated and Tet3\textsuperscript{3\textsubscript{mat}/pat\textsuperscript{−\textsubscript{−}}} zygotes revealed that whereas the initial DNA demethylation proceeded normally in the absence of 5hmC formation, a slight accumulation of 5mC was detectable by immunofluorescence in the paternal pronucleus of later (late PN4–PN5) stage zygotes (Figs 2a and 3d and Supplementary Figs 2b and 4i). Although our LC-MS measurements did not detect any significant difference in 5mC in either Tet3\textsuperscript{3\textsubscript{mat}/pat\textsuperscript{−\textsubscript{−}}} or DMOG-treated zygotes, most likely owing to pooling of slightly different stages of zygotes (Figs 2b and 3e and Supplementary Fig. 4e), loss of 5hmC in the zygote leads to a detectable, albeit small, increase in 5mC in the two-cell-stage embryos derived from DMOG-treated (\(P < 0.001\), t-test, Fig. 2c) and Tet3\textsuperscript{3\textsubscript{mat}/pat\textsuperscript{−\textsubscript{−}}} zygotes (\(P < 0.05\), t-test, Fig. 3f). The observed limited accumulation of 5mC in late-stage Tet3-KO and DMOG-treated zygotes is in agreement with the previously described contribution of Tet3 to zygotic DNA demethylation\textsuperscript{1,4}. However, and contrary to previous interpretation, our data document that the accumulation of 5mC occurs only once the initial wave of DNA demethylation has been completed (Figs 1c, 2a and 3d and Supplementary Figs 2b and 4i).

The accumulation of 5mC in the paternal pronucleus of late-stage DMOG-treated or Tet3-depleted zygotes indicates the presence of de novo methylation activity. However, the current models of zygotic epigenetic reprogramming assume the lack of 5mC maintenance during replication in the mouse preimplantation embryos\textsuperscript{13,15}. Additionally, given the general loss of DNA methylation during zygotic and early preimplantation development\textsuperscript{5,13,17}, the role or even existence of de novo DNA methylation in the early embryos has not been previously considered. To revise this assumption, we investigated the presence of maintenance and de novo DNA methylation activities in the zygotes using isotope labelling. The addition of isotope-labelled methionine during the IVF procedure resulted in a clear incorporation of labelled 5mC (5mC\textsuperscript{5}) detectable in late-stage (PN4–5) zygotes (Fig. 4a). This signal is reduced, but still clearly detectable, following treatment with aphidicolin (Fig. 4a), unambiguously demonstrating that both the maintenance (replication coupled) and de novo (replication independent) methylation activities are present in the mouse zygote.

This prompted us to consider whether the late appearance of 5hmC in paternal pronuclei could be mechanistically linked to the zygotic DNA methylation activity rather than to the sperm-derived 5mC as proposed in the current models\textsuperscript{13,4}. We carried out IVF in the presence of 5-azadeoxycytidine (azadC), an inhibitor of DNA methyltransferases that requires incorporation into DNA during replication. Zygotes incubated with azadC show a significant decrease in 5hmC level in the paternal pronucleus (Fig. 4b), demonstrating that 5hmC accumulation is linked to the generation of new zygotic 5mC. Notably, no significant difference in paternal 5mC is observed in azadC-treated zygotes, either by immunofluorescence or by bisulphite sequencing of Line1 elements (Supplementary Fig. 4j). This suggests that following the zygotic S phase the observed de novo DNA methylation is limited and most newly deposited 5mC is converted to 5hmC on the paternal genome. This conclusion is in agreement with the low absolute levels of 5hmC measured by LC-MS (Fig. 2b).

Previous reports have demonstrated the role of Dnmt3a and Dnmt3L in de novo DNA methylation during oocyte growth\textsuperscript{20–24}. In early mouse zygotes both Dnmt3a and Dnmt3L show pronuclear localization with progressive enrichment until PN5 (Supplementary Fig. 5a; refs 25,26). To address whether Dnmt3a/3L-driven de novo DNA methylation could provide a template for 5hmC formation during zygotic development, we assessed 5hmC levels in zygotes maternally depleted for Dnmt3a (Dnmt3a\textsuperscript{Zp3-Cre}), Dnmt3L (Dnmt3L\textsuperscript{KO}) and Dnmt3L (Dnmt3L\textsuperscript{−/−}). Consistent with previous reports, the lack of maternally inherited Dnmt3a and Dnmt3L results in loss of DNA methylation in the maternal genome\textsuperscript{20,22,27,28} (Fig. 4c and Supplementary Fig. 5b). Additionally, the loss of maternal Dnmt3a, but not Dnmt3L (Fig. 4c and Supplementary Fig. 5b), leads to significantly reduced paternal 5hmC in PN4 zygotes. We thus conclude that Dnmt3a-driven de novo DNA methylation is required for 5hmC formation in late zygotes.

Paternal accumulation of 5hmC is severely affected but not completely abolished in zygotes lacking Dnmt3a, indicating a potential activity of another DNA methyltransferase. As Dnmt1 has been previously demonstrated to be present in both fully grown oocytes\textsuperscript{20,29} as well as in early mouse embryos\textsuperscript{30,31}, we have assessed the potential contribution of maternal Dnmt1 for the accumulation of 5hmC using the oocyte-specific Dnmt1-KO (Dnmt1\textsuperscript{Zp3-Cre}) (ref. 32). Interestingly, the lack of maternal Dnmt1 also leads to significantly lower levels of paternal 5hmC following fertilization (Fig. 4d). The reduced paternal 5hmC in Dnmt3a and Dnmt1 KO zygotes is observed despite the normal presence and localization of Tet3 protein in these zygotes (Fig. 4e,f). Cumulatively our results demonstrate that in the zygote, both Dnmt3a and Dnmt1 generate new 5mC that is targeted for hydroxylation.
Figure 3 Tet3 is not required for loss of 5mC in the early zygote. (a) A schematic illustration of the targeting strategy used to generate Tet3 conditional KO mice. (b) RT-qPCR analysis of Tet3 mRNA (exons 3 and 11) in control (Tet3mat+) and Tet3-depleted oocytes (Tet3mat−). Results are normalized to endogenous H3F3a and to control (Tet3mat+). Bars represent the mean of three technical replicates. (c) Tet3WT (n = 10) and Tet3mat−/pat+ (n = 8) zygotes were stained for Tet3 protein. Quantification is represented as the mean of intensity on the paternal pronuclei after background subtraction. (d) Tet3WT and Tet3mat−/pat+ zygotes were co-stained for 5mC and 5hmC at different time points post-fertilization. Quantification of both DNA modifications is presented as a ratio of paternal over maternal signal intensity. Each data point represents an independent zygote (n = 5 PN3, n = 6 PN3L and n = 13 Tet3WT zygotes; n = 5 PN3, n = 6 PN3L and n = 19 Tet3mat−/pat+ zygotes; two independent experiments). (e) Quantification of 5mC/dG and 5hmC/dG in Tet3WT and Tet3mat−/pat+ zygotes (with polar bodies) by LC-MS. Each point represents the mean of two technical replicates of a pool of about 100 oocytes or embryos. (f) Quantification of DNA modifications in two-cell embryos derived from Tet3WT or Tet3mat−/pat+ zygotes analysed by LC-MS. Each point represents the mean of two technical replicates of a pool of about 50 embryos. Statistical analysis was carried out using Student’s t-test (two-sided). Error bars indicate s.d. PN3L, late PN3; ♀, female pronucleus; ♂, male pronucleus. n.d., non-detectable; *P < 0.05; **P < 0.01; ****P < 0.0001. Scale bars, 5 μm.
**Figure 4** Tet3 targets newly deposited 5mC generated by Dnmt3a and Dnmt1. (a) Isotope-labelled 5mC (5mC*) quantified by LC-MS after incubation of zygotes with heavy methionine (\(^{15}C\), d<sub>5</sub>-methyl) in the presence or absence of aphidicolin (IVF). Each point represents a biological replicate \((n=2)\). An example of the 5mC* peak detected by LC-MS is depicted for isotope-labelled 5mC (5mC* / dG (%)). Inhibition of new zygotic 5mC generated by Dnmt3a and Dnmt1. (b) Inhibition of new zygotic DNA methylation by 5-azadeoxycytidine (azadC) (IVF) affects accumulation of paternal 5hmC as assessed by staining using 5mC and 5hmC specific antibodies. Only zygotes with a paternal mid-section area >200\(\mu m^2\) (PN4–5 zygotes) were considered to avoid developmental staging bias. Quantification of 5mC and 5hmC is represented as signal intensity in paternal and maternal pronuclei (left axis) or as a ratio between the pronuclei signal (pat./mat.) (right axis). \((n=6\) control and \(n=18\) treated zygotes; experiment replicated twice independently.) (c,d) 5mC and 5hmC staining in PN4–5 zygotes (paternal mid-section area >200\(\mu m^2\)) with maternal Dnmt3a \((\mu Dnmt3a^{lox/2lox}, Zp3-Cre \times c'WT)\) \((n=12\) WT and \(n=10\) KO zygotes; experiment replicated twice independently.) (c) or Dnmt1 \((\mu Dnmt1^{lox/2lox}, Zp3-Cre \times c'WT)\) \((n=13\) WT and \(n=11\) KO zygotes; experiments replicated twice independently) \((d)\) deletion. Note that only total signal intensity is plotted in (c) as Dnmt3a deletion affects 5mC and 5hmC level in maternal PN. (e,f) Tet3 localization and signal intensity is identical between WT \((n=4)\) and Dnmt3a KO \((n=3)\) or WT \((n=5)\) and Dnmt1 KO \((n=3)\) zygotes. Quantification is represented as the mean of intensity on the paternal pronuclei after background subtraction. Statistical analysis was carried out using Student’s t-test (two-sided). Error bars indicate s.d. *\(P<0.05\); **\(P<0.01\); ***\(P<0.001\). γ, female pronucleus; c’, male pronucleus.
To further validate the presence of de novo activity in the ooplasm and the role of 5hmC in targeting newly deposited 5mC, we performed somatic cell nuclear transfer (SCNT) using wild-type (WT) and triple KO Dnmt3a−/−, Dnmt3b−/−, Dnmt1−/− (TKO) embryonic stem cells (ESCs) as donor cells (Fig. 5a). As expected, the nuclei of TKO-ESCs are devoid of both 5mC and 5hmC (Supplementary Fig. 5c). At 5 h post-activation, 5mC and 5hmC are detected in the WT-ESC pseudopronucleus whereas the fully decondensed TKO-ESC pseudopronucleus does not show an appreciable amount of DNA modifications (Fig. 5a). However, at 14 h post-activation both the WT-ESC and the TKO-ESC pseudopronuclei exhibit comparable levels of 5mC and 5hmC (Fig. 5a). The presence of both DNA modifications on the originally 5mC/5hmC-free DNA template further demonstrates the presence of previously unappreciated de novo DNA methylation activities in the ooplasm and that Tet3 targets the newly formed 5mC. Interestingly, pericentromeric heterochromatin in WT-ESC- and TKO-ESC-derived pseudopronuclei is enriched for 5mC but not for 5hmC (Supplementary Fig. 5d), replicating the pattern observed on the paternal genome in PN4 zygotes.

Our detailed investigation of DNA modification dynamics in the zygote has revealed that 5hmC accumulation follows, but is not concomitant with, the loss of 5mC in the early (PN2–3) paternal pronucleus (Fig. 1). Furthermore, we have shown that the initial loss of paternal 5mC can be mechanistically uncoupled from 5hmC formation using a genetic loss-of-function model and small-molecule inhibitors of dioxygenases, providing mechanistic proof that these processes are independent (Figs 2 and 3 and Supplementary Figs 2b–d, 3c and 4d–i). In this context it should be noted that the methylation analysis of embryos depleted for Tet3 has been previously conducted on late-stage (PN5) zygotes, which has left the dynamics of the DNA demethylation process underappreciated.

Figure 5 De novo DNA methylation activity is present in the oocyte and generates a target for hydroxylation in the SCNT experiment. (a) Schematic of the somatic cell nuclear transfer (SCNT) experiment using wild-type (WT) or triple Dnmt-KO (Dnmt1−/− Dnmt3a−/− Dnmt3b−/−) (TKO) ESCs. 5mC and 5hmC staining following SCNT into enucleated oocytes using WT (left panel) or TKO (right panel) ESCs. Staining was carried out 5 and 14 h post-activation of the reconstituted embryos. Control: staining of the maternal genome (indicated by an arrow) in embryos following SCNT into non-enucleated oocyte (lower panel). The increase of 5mC and 5hmC intensity on the TKO nuclei 14 h post-activation reflects de novo DNA methylation activity in the oocyte, targeted by Tet3 hydroxylation. Representative images are shown (n = 7 and n = 6 WT-ESC and TKO pseudopronuclei, respectively). Scale bars, 5 μm. (b) Following genome-wide loss of sperm-derived 5mC in the male pronucleus of mouse zygote, newly deposited 5mC produced by zygotic Dnmt1 and Dnmt3a is hydroxylated by Tet3 (model). BER, base excision DNA repair.
The exact mechanism by which DNA demethylation proceeds in the zygote remains the subject of an intense scientific debate, with both active and passive models proposed\(^1\). Our study shows that, quantitatively, most observed global DNA demethylation proceeds independently of replication (Supplementary Fig. 4c,g,h) confirming previous bisulphite sequencing data\(^14,15,33,35\). Although we cannot rule out that a small proportion of loci lose their methylation through dilution\(^15,16\), this pathway provides only a limited contribution to the global methylation changes in the zygote. Combined with our findings regarding the lack of a major role for Tet3-driven oxidation, our data advocate the existence of an alternative mechanism implicated in the loss of paternal 5mC in the early (PN2–PN3) zygotes. In this context we note that both control and DMOG-treated zygotes show normal enrichment of the chromatin-bound XRCC1 DNA repair protein in the paternal pronucleus (Supplementary Fig. 5f), suggesting that the previously reported activation of the base excision DNA repair pathway\(^1,18\) during DNA demethylation in the mouse zygotes does not require Tet3-driven 5mC hydroxylation. In further support of this we observed incorporation of new isotope-labelled deoxycytidine (dC\(^5\)) in pre-replicating zygotes (Supplementary Fig. 5g,h).

Contrary to previous assumptions, we show that zygotes contain \(\text{de novo}\) DNA methyltransferase activities and both maternally inherited Dnmt1 and Dnmt3a are necessary for the accumulation of paternal 5mC. Our findings thus support a model whereby Tet3-driven hydroxylation is predominantly implicated in the protection of the newly acquired hypomethylated state from accumulating new DNA methylation. This is achieved through targeting of newly formed 5mC generated by zygotic Dnmt3a and Dnmt1 enzymes or possibly through preventing Dnmt1-driven methylation maintenance at some regions (Fig. 5b).

Cumulatively, our study explains the previously observed low locus-specific 5mC levels\(^35\), the limited effect of Tet3-KO on zygotic DNA demethylation\(^15,16\) and only limited overlap between 5hmC and parental genomes in mouse zygotes.\(^32,48\) We show that epigenetic reprogramming in the early embryo is a complex process underpinned by a dynamic interplay between active DNA demethylation, \(\text{de novo}\) DNA methylation and Tet3-driven 5mC hydroxylation. Finally, although our study provides information regarding the global dynamics of DNA modifications during zygotic reprogramming, further studies will be necessary to unravel locus-specific modification changes and targeting of the key factors involved in this fascinating process.

**METHODS**

Methods and any associated references are available in the **online version of the paper**.

**Note:** Supplementary Information is available in the **online version of the paper**.

**ACKNOWLEDGEMENTS**

We are grateful to the members of the Hajkova laboratory (especially K. McEwen) and to N. Navaratnam for discussions and revision of the manuscript. We thank T. Carell for providing the isotope-labelled synthetic nucleosides. We would like to acknowledge the MRC CSC Microscopy facility for help with imaging of the embryos, and M. Woodberry, D. Hardy and J. Jegola for mouse husbandry. We would like to thank MRC transgenic facility for their help regarding IVF. The LC-MS analysis was conducted in collaboration with Agilent Technologies, whom we would like to thank for generous support and help. This work was supported by MRC (MC_UU_A652_SPY70) and EpigeneSys network funding to P.H. R.A. was a recipient of the Marie Curie Intra-European Fellowship (FP7). B.N. was a recipient of the Marie Curie Incoming-European Fellowship (FP7).

**AUTHOR CONTRIBUTIONS**

P.H. and R.A. conceived the study and wrote the manuscript with assistance from H.S. and N.R.K. R.A. performed the experiments with the help of Z.D.’s and P.W.S.H.; R.A. and P.H. analysed the data. B.N. carried out micromanipulation of zygotes, and provided technical assistance. S.N. performed SCNT with assistance from B.N. and R.A. R.A. and A.T. performed the LC-MS experiment and analysed the data with the help of V.E. H.S. provided the Dnmt1, Dnmt3a and Dnmt3L KO mice and K.S. performed the IVF experiments. E.N. carried out Tet3 targeting in ESCs and generated Tet3 chimaera. M.N., M.M. and H.K. provided the Tet3 KO mice.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3296

Reprints and permissions information is available online at www.nature.com/reprints

1. Wossidlo, M. et al. 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. *Nat. Commun.* 2, 241 (2011).
2. Zhang, P. et al. The involvement of 5-hydroxymethylcytosine in active DNA demethylation in mice. *Biochem. Biophys. Res. Commun.* 416, 104–112 (2012).
3. Gu, T. P. et al. The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. *Nature* 477, 606–610 (2011).
4. Iqbal, K., Jin, S. G., Pfeifer, G. P. & Szabo, P. E. Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. *Proc. Natl Acad. Sci. USA* 108, 3642–3647 (2011).
5. Oswald, J. et al. Active demethylation of the paternal genome in the mouse zygote. *Curr. Biol.* 10, 475–478 (2000).
6. Mayer, W., Nieuwlaat, A., Walter, J., Fundele, R. & Haaf, T. Demethylation of the zygotic paternal genome. *Nature* 403, 501–502 (2000).
7. Santos, F. et al. Active demethylation in mouse zygotes involves cytosine deamination and base excision repair. *Epigenet. Chromatin* 6, 39–50 (2013).
8. Ito, S. et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 333, 1300–1303 (2011).
9. He, Y. F. et al. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science* 333, 1303–1307 (2011).
10. Inoue, A., Shen, L., Dai, Q., He, C. & Zhang, Y. Generation and replication-dependent dilution of 5fc and 5cab during mouse preimplantation development. *Cell Res.* 21, 1670–1676 (2011).
11. Tahiliani, M. et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324, 930–935 (2009).
12. Wossidlo, M. et al. Dynamic link of DNA demethylation, DNA strand breaks and repair in mouse zygotes. *EMBO J.* 29, 1877–1888 (2010).
13. Smith, Z. D. et al. A unique regulatory phase of DNA methylation in the early mammalian embryo. *Nature* 475, 339–344 (2011).
14. Huang, Y. et al. The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. *PLoS ONE* 5, e8888 (2010).
15. Shen, L. et al. Tet3 and DNA replication mediate demethylation of both the maternal and paternal genomes in mouse zygotes. *Cell Stem Cell* 15, 459–470 (2014).
16. Guo, F. et al. Active and passive demethylation of male and female pronuclear DNA in the mammalian zygote. *Cell Stem Cell* 15, 447–458 (2014).
17. Santos, F., Hendrich, B., Reik, W. & Dean, W. Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev. Biol.* 241, 172–182 (2002).
18. Hajkova, P. et al. Genome-wide reprogramming in the mouse germ line entails the base excision repair pathway. *Science* 329, 78–82 (2010).
19. Rougier, N. et al. Chromosome methylation patterns during mammalian preimplantation development. *Genes Dev.* 12, 2108–2113 (1998).
20. Shirane, K. et al. Mouse oocyte methyllomes at base resolution reveal genome-wide accumulation of non-CpG methylation and role of DNA methyltransferases. *PLOS Genet.* 9, e1003349 (2013).
21. Smallwood, S. A. et al. Dynamic CpG island methylation landscape in oocytes and preimplantation embryos. *Nat. Genet.* 43, 811–814 (2011).
22. Kaneda, M. et al. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* 429, 900–903 (2004).
23. Tomizawa, S. et al. Dynamic stage-specific changes in imprinted differentially methylated regions during early mammalian development and prevalence of non-CpG methylation in oocytes. *Development* 138, 811–820 (2011).
24. Bourc’his, D., Xu, G. L., Lin, C. S., Bollman, B. & Bestor, T. H. Dnmt3L and the establishment of maternal genomic imprints. *Science* 294, 2536–2539 (2001).
25. Guerneti, M., Duffie, R., Iranzo, J., Fauque, P. & Bourc’his, D. Plasticity in Dnmt3L-dependent and -independent modes of de novo methylation in the developing mouse embryo. *Development* 140, 562–572 (2013).
26. Hirasawa, R. et al. Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. *Genes Dev.* 22, 1607–1616 (2008).
Genetic evidence for Dnmt3a-dependent imprinting during oocyte growth obtained by conditional knockout with Zp3-Cre and complete exclusion of Dnmt3b by chimera formation. 

Genes Cells 15, 169–179 (2010).

Hata, K., Okano, M., Lei, H. & Li, E. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. 

Development 129, 1983–1993 (2002).

Kurihara, Y. et al. Maintenance of genomic methylation patterns during preimplantation development requires the somatic form of DNA methyltransferase 1. 

Dev. Biol. 313, 335–346 (2008).

Cirio, M. C. et al. Preimplantation expression of the somatic form of Dnmt1 suggests a role in the inheritance of genomic imprints. 

BMC Dev. Biol. 8, 9–22 (2008).

Pfeiffer, M. J. et al. Proteomic analysis of mouse oocytes reveals 28 candidate factors of the 'reprogrammome'. 

J. Proteome Res. 10, 2140–2153 (2011).

Jackson-Grusby, L. et al. Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. 

Nat. Genet. 27, 31–39 (2001).
METHODS

Mice. Outbred MF1 mice and B6CBAF1 used for the in vitro fertilization procedure were purchased from Charles River or Harlan. B6CBAF1 mice were superovulated by intraperitoneal injection of 5 U pregnant mare’s serum (PMS) and 5 U of human chorionic gonadotropin (HCG) 48 h later. Dmnt-deficient mice used in this study have been described previously: (Dmnt1<lox<lox>, Zp3-Cre) (ref. [22]), (Dmnt3a<lox<lox>, Zp3-Cre) (ref. 22) and (Dmnt3l<lox<lox>, Zp3-Cre) (ref. 28).

Tet3 conditional knockout (KO) mice were generated using a two-step strategy described in Fig. 3a. The targeting construct, which generates a null allele, is composed of exon 8 (ENSMMUSE00000238680), exon 9 (ENSMUSE00000238677) and a FRT-flanked neomycin cassette, flanked by two loxP sites. Following insertion the KO allele was rescued in heterozygous Tet3<+/-> by crossing with a transgenic mouse expressing Flp recombinase. Tet3-maternally deleted oocytes (Tet3<+/->) were obtained by crossing Gdf9-Cre Tet3<+/-> males with Tet3<+/-> females and by superovulation of 3-week-old Gdf9-Cre Tet3<+/-> females.

All animal experiments were carried out under a UK Home Office Licence in a Home Office-designated facility.

**In vitro fertilization of mouse oocytes.** The procedure was carried out as previously described40. The sperm was isolated from dissected epididymis and capacitated for 1.5 h in HTF fertilization medium (Millipore) supplemented with 4 mg ml⁻¹ methionine (Fluka) or isotope-labelled (13C) methionine (Sigma) and kept in this medium until use. After a 15 min recovery period, sperm were washed three times in HTF and used for fertilization of mouse oocytes. For restriction inhibition, 3 μg ml⁻¹ a-aminocolin (Sigma-Aldrich, dissolved in dimethylsulphoxide) was added to the fertilization medium. As a control, zygotes were incubated with the matching concentration of dimethylsulphoxide (0.1–0.3%).

For the quantification of maintenance and de novo DNA methylation, oocytes were incubated for at least 40 min before fertilization with 7.5 mg l⁻¹ unlabelled methionine (Fluka) or isotope-labelled (13C,15N) methionine (Sigma) and kept in this supplemented medium during IVF. De novo DNA methylation was monitored by adding 2 μg ml⁻¹ a-aminocolin to the HTF medium.

**Immunofluorescence staining of zygotes.** Zygotes collected from natural matings of outbred MF1 mice were cleaned from cumulus cells by 5 min incubation in Medium (Millipore) containing 4 mg ml⁻¹ BSA supplemented with 300 μg ml⁻¹ hyaluronidase, and fixed for 20 min by 4% paraformaldehyde (PFA) in PBS, followed by three 10 min washes in PBS, 1% BSA. Permeabilization was performed for 30 min at room temperature in PBS, 1% BSA, 0.5% Triton X-100. Zygotes were then incubated overnight in PBS, 1% BSA, 0.1% Triton X-100 containing the first antibody, washed three times for 10 min with PBS, 1% BSA, 0.1% Triton X-100 and incubated for 1 h in the dark with Alexa Fluor 405-, 488- or 568-conjugated IgG secondary antibody (dilution 1:300, Molecular Probes) in the same buffer. Zygotes were mounted with ProLong Gold mounting medium containing DAPI (Life Technologies) as imaging channel for 5mC and 5hmC (using single-antibody staining of zygotes). In all cases, the results confirmed our findings presented in Fig. 1c.

**Antibodies.** XRCC1 (Serotec) 1:200, 5mC (clone 3D3, Diagenode) 1:5,000, H3K9me2 (47-044, Upstate) 1:400, H3K27me3 (gift from T. Jenuwein, Max Planck Institute of Immunobiology and Epigenetics, Germany) 1:500, H3K4me2 (07-030, Upstate) 1:500, H3K36me3 (gift from P. Wade, Tokyo Institute of Technology, Japan) 1:50, TET3 (C-term, Abcam) 1:200, Dnmt3a (igenex, IMG-268A) 1:200, and Dnmt3l (Abnova, PAB2230) 1:100 were used.

**Bisulphite sequencing.** Polyrne zygotes of oocytes obtained by IVF were removed by micromanipulation and zygotes (10–20 per experiment) were snap frozen in liquid nitrogen. Alternatively, paternal pronuclei were isolated by micromanipulation (10–15 per experiment). Bisulphite sequencing was subsequently carried out using the agarose bead embedding method as described in ref. 40 or by using the Imprint DNA modification kit (Sigma-Aldrich). The following primers were used for the amplification of Line1 elements: F1: 5’-GGTAGAGAATTTGTATGTTTGGAG TTAGG-3’; R1: 5’-CCTAAACAAAACGTTTCTCAAACATATAT-3; F2: 5’-TGGGAATAATGTTGATATAGTGGAAGGT-3; R2: 5’-TCTAACATATATACTTAAACATCCA-3. The semi-nested approach was used: first PCR (F1,R1 primers), second PCR (F1,R2 primers). PCR conditions: 95 °C 5 min, 95 °C 1 min, 56 °C 1 min, 72 °C 1 min) x 35, 72 °C 5 min. The primers amplify L1Md_Tf and L1Md_Gf Line1 subtypes. The P values were calculated using the Mann–Whitney U-test.

**Dot blot and competition assay.** Plasmid DNA was amplified using a mixture of dATP, dGTP, TTP (Roche) together with either dCTP (Roche), 5-methyl-dCTP (Fermentas) or 5-hydroxymethyl-dCTP (BioSciences). PCR products were denaturated using 0.1% NaOH at 95 °C for 5 min before cooling on ice. Fourfold serial dilutions were spotted on a nylon membrane (Hybond-N°, GE Healthcare); the membrane was ultraviolet-crosslinked (254 nm, 1,200 mJ m⁻²). blocked in TBS, 0.1% Tween-20, 5% milk and incubated with primary antibodies overnight at 4 °C in a萌。After washing, membranes were incubated with HRP-conjugated IgG secondary antibodies for 1 h at room temperature in the same buffer. Alternatively, peroxidase-conjugated secondary antibodies were used for blotting. The 5mC/5hmC kinetics was also independently assessed using the same imaging channel for 5mC and 5hmC (using single-antibody staining of zygotes).

**Recombinant TET1 protein**. Recombinant TET1 protein (0.5 μg; Actif Motif, No. 31363) was incubated with 100 ng of 5mC-enriched PCR product according to the manufacturer's instructions for 3 h at 37 °C in the presence of DMOG (10 or 25 mM) or DFX (10 or 20 mM). The reaction was then spotted on a nylon membrane and processed for dot blotting.

**Somatic cell nuclear transfer.** The parental ESC line E14Tg2a.4 (referred to as WT-ESC) was obtained from BayGenomics (MMRRC no. 015890-UCD-ULTRA). DNA methyltransferase triple KO ES cells (Dmnt1<−/−>, Dmnt3a<−/−>, Dmnt3l<−/−>) were used as recipients.
Euparytom a piezo-driven system (Prime Tech). After removal of metaphase chromosomes, oocytes were transferred into 10 ml of the same medium covered with mineral oil (Sigma-Aldrich) on the lid of a culture dish. All micromanipulations were performed under an inverted microscope equipped with Hoffman optics using a piezo-driven system (Prime Tech). After removal of metaphase chromosomes, a wild-type or DNMT-TK0 ESC arrested at metaphase was introduced into the perivitelline space of the enucleated oocyte.6 The fusion of the donor and recipient was induced by a d.c. pulse of 2,500 V cm⁻¹ for 10 ms using an ECM 830 (BTX) in 300 ml mammintol, 0.1 mM MgSO₄, 0.1 mg ml⁻¹ polyvinyl alcohol and 3 mg ml⁻¹ bovine serum albumin. The fusion rates were determined 1 h after the pulse by microscopic examination. Fused pairs were treated with 10 mM SrCl₂ in calcium-free CZB medium for 1 h. The reconstructed embryos were cultured in M16 medium (Millipore) in 5 % CO₂ in air at 37 °C.

Reverse transcription and quantitative PCR analysis. Total RNA was purified using TriZol (Life Technologies) following the manufacturer's instructions. Random-primed reverse transcription was performed using PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time, Takara Bio). cDNA corresponding to 1.5–3 zygotes in 3 μl was added to 10 μl of quantitative PCR mix containing SensiMix SYBR No-ROX (Bioline). Real-time quantitative PCR reactions were performed on a CFX96 real-time PCR detection system (Bio-Rad). The standard curve method using ESC cDNA was used for quantification. Primers: H3F₃ₙ₉₋₅’-CCATGCCGACCTGACCA-3’, H3F₃₉₋₅’-TACCCTTGACGACGAGAAGA-3’, Tet₁ exom 3’_5’-CCGTGGACCGATTTCTCGA-3’, Tet₂ exom 11’_5’-TGGATGCTGAACCCAGGTTAC-3’, Tet₃ exom 11’_5’-TGAAGGATCCAGGTTC-3’. Two-tailed unpaired t-test was performed by using GraphPad Prism software.

Parthenogenetic activation of oocytes. MII oocytes were incubated with KSO medium where Ca²⁺ had been replaced with 10 mM strontium (Sigma) supplemented with 5 μg ml⁻¹ cytosolamin B (Sigma) for 1.5 h. Parthenogenetically activated oocytes were then transferred into HTF medium supplemented with 2 μg ml⁻¹ aphidicolin and 5 μg ml⁻¹ cytosolamin B. Completion of S phase was monitored by incubating the parthenotes for 45 min in HTF medium supplemented with 400 μM Edu before staining as described below.

Edu staining in zygotes using Click-IT chemistry. To verify replication inhibition by aphidicolin and the completion of S phase in parthenogenetically activated oocytes, embryos were incubated with 400 μM of 5-ethyldeoxyuridine (Edu, Life Technologies) during development before removal of the zona pellucida and fixation for 20 min in PFA 4% at room temperature. When indicated, a pulse of Edu was performed by incubating the zygotes for 30 min before zona pellucida removal and fixation.

Edu staining followed the manufacturer’s instruction (Click-IT Nascent RNA capture kit, Life Technologies). Briefly, zygotes were permeabilized in PBS, 1% BSA, 0.5% Triton X-100 for 30 min at room temperature, washed twice in PBS, 1% BSA and incubated for 1 h in dark with a Click-IT reaction mix containing 2.5 μl of Alexa Fluor Azide 488. Zygotes were subsequently washed several times in PBS, 1% BSA and mounted in ProLong DAPI mounting medium.

DNA isolation from sperm, oocytes and early-stage embryos. Sperm DNA from B6CBAF1 was isolated using a protocol modified from ref. 44. Fresh sperm from dissected epididymis was left to settle in HTF medium. Supernatant containing active sperm was centrifuged for 2 min at 1,100g and resuspended in 200 μl of solution A (75 mM NaCl and 25 mM EDTA) and 200 μl of solution B (10 mM Tris-HCl pH 8, 10 mM EDTA, 2 mM SDS) and sonicated at 80 W for 1 min. DNA was further purified by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation; DNA pellet was resuspended in LC-MS-quality-grade water (Fisher Scientific).

Genomic DNA from oocytes and two-cell embryos was isolated using a DNA Micro Kit (Qiagen) according to the manufacturer’s instructions, without RNA carrier. DNA was eluted in LC-MS-quality-grade water (Fisher Scientific). For samples of zygotes (when indicated), both polar bodies were first carefully removed by micromanipulation (Narishige) before DNA extraction.

Liquid chromatography-mass spectrometry. Genomic DNA of 100 to 600 cells was used for quantification of DNA modifications. DNA was digested to nucleosides using 1 U of benzonase (Novagen), 0.5 mM phosphodiesterase I (Sigma) and 200 nM of alkaline phosphatase in 20 mM Tris-HCl pH 7.9, 4 mM MgCl₂ for a minimum of 6 h at 37 °C, or using a digestion enzymatic mix (NEB). Samples were pre-cooked by acetone precipitation. All samples and standard curve points were spiked with isotope-labelled synthetic nucleosides (100 fmol of dC and dG, 5 fmol of 5mcG, 500 amol of 5mC) obtained from T. Carell (Center for Integrated Protein Science at the Department of Chemistry, Ludwig-Maximilians-Universität München, München, Germany). The nucleosides were separated on an Agilent RRHD Eclipse Plus C18 2.1 × 100 mm 1.8 μm column by using the UHPLC 1290 system (Agilent) and analysed using an Agilent 6490 triple quadrupole mass spectrometer. To calculate the concentrations of individual nucleosides, standard curves representing the ratio of the peak response known of amounts of synthetic nucleosides spiked with the same matrix as the samples and the peak response of the isotope-labelled nucleosides were generated and used to convert the peak-area values to corresponding concentrations. Thresholds for quantification are: signal-to-noise (calculated with a peak-to-peak method) above 10 and the limit of quantification as detailed for each nucleoside in the Supplementary Fig. 4a. For non-quantifiable peaks (n.d.), an overestimation of the 5mCdG or 5mCrdG ratio is calculated based on the limit of detection for both nucleosides.

Reproducibility of experiments. This journal’s policy precludes the use of statistical analysis for data where n < 3 biological replicates. However, in our LC-MS experiments, we observed a very low variation between biological replicates with a coefficient of variation below 10 for 5mC (Figs 2b,c and 3e,f, and Supplementary Fig. 4e,h). Additionally, LC-MS results are technically supported by immunofluorescence data (Figs 2a and 3d and Supplementary Figs 2b and 4j) and bisulfite sequencing analysis (Supplementary Figs 3d-f and 4j), and conceptually by alternative approaches (conditional KO mouse model and small-molecule inhibitors of dioxygenases). It should also be noted that each LC-MS measurement reflects DNA modification quantification of a pool of about 100 embryos, analysed in technical duplicates. LC-MS data (Fig. 2b, DNA modifications in zygotes without polar bodies) represent three biological replicates (except for the DMOG-treated data point); a replicate of this experiment (zygotes with polar bodies) is shown in Supplementary Fig. 4e. LC-MS results presented in Figs 2c and 4a and Supplementary Figs 4c,e,h and 5b are based on two biological replicates (each carried out in technical duplicates).

Immunofluorescence data presented in Fig. 2a and Supplementary Fig. 2b were reproduced four times independently, Figs 3d and 4c,d and Supplementary Figs 2f, 3c and 5b twice independently. It should be noted that for the quantification of DNA modifications in zygotes by immunofluorescence, each embryo represents a biological replicate.

Statistics derived from bisulfite sequencing analysis (Supplementary Figs 3d-f and 4j) rely on the number of clones amplified; experiments presented in Supplementary Fig. 3d were reproduced twice independently.
**Supplementary Figure 1** (a) Tet3 staining in zygotes shows an accumulation of Tet3 in the paternal pronucleus from PN2 and a gradual enrichment from PN3 to PN5. Tet3 protein is not detectable following the 2-cell stage. Representative images are shown (replicates indicated on the figure). (b) Expression of Tet1/2/3 in mouse preimplantation embryos (based on 39). (c) 5fC and 5caC stainings in PN3 and PN4 zygotes. Note that in the early zygote 5fC (and 5caC) is observed in the maternal pronucleus only (see also Supplementary Fig. 2c,d). DNA stained with PI. Representative images are shown (replicates indicated on the figure). (d) Dot blot analysis of C-, 5mC- and 5hmC-containing PCR products (amplified with dCTP, 5mdCTP and 5hmdCTP, respectively) to assess the sensitivity and cross-reactivity of anti-5mC and anti-5hmC antibodies. 5hmC antibody shows about 10,000 fold greater sensitivity than the 5mC antibody. (e) Competition assay to assess specificity of 5hmC antibody using genomic DNA from 3T3 cells overexpressing Tet1 or Tet3 protein. Competitor nucleosides (200-fold excess) are 5-methyldeoxycytidine (5mC) and 5-hydroxymethyldeoxycytidine (5hmC). © 2016 Macmillan Publishers Limited. All rights reserved.
**Supplementary Figure 2** (a) TET1 *in vitro* activity assay in the presence of DMOG. Conversion of 5mC to 5hmC is assessed by dot blot. (b) Dynamics of 5mC and 5hmC in mouse zygotes treated with DMOG. Note the initial disappearance of paternal 5mC despite the absence of 5hmC in DMOG-treated zygotes. The accumulation of 5mC in DMOG-treated zygotes is detectable only in the late PN stages and correlates with the appearance of 5hmC in control zygotes (n=192 zygotes). 5fC (n=8 PN3 and n=9 PN4 control zygotes; n=8 PN3 and n=6 PN4 DMOG-treated zygotes) (c) and 5caC (n=6 PN3 and n=8 PN4 control zygotes; n=8 PN3 and n=7 PN4 DMOG-treated zygotes) (d) stainings in DMOG-treated PN3 and PN4 zygotes. Quantification is represented as a ratio between the pronuclear signals (pat/mat). Note that in the early zygote 5fC (and 5caC) is observed in the maternal pronucleus only (see also Supplementary Figure 1c). (Scale bars, 5um.) (e) 5hmC staining in pre-implantation embryos derived from *in vitro* fertilisation in the presence of DMOG. 5hmC is not detectable at 2-cell stage following DMOG treatment which is in agreement with the absence of detectable Tet3 protein at this stage (Supplementary Fig. 1a). 5hmC is gradually detectable from 4-cell stage onwards. (Scale bars, 10um.) (f) Lack of zygotic 5hmC (DMOG treatment during IVF) does not affect the developmental potential of mouse embryos (n>20). ♀, female pronucleus; ♂, male pronucleus; 5fC, 5formylcytosine; 5caC, 5carboxylcytosine; DMOG, dimethyloxallyl glycine; PI, propidium iodide. Statistical analysis was carried out using Student’s t-test (two-sided). Error bars indicate s.d. **, p<0.01; ***, p<0.001.
Supplementary Figure 3 (a) Histone modification asymmetry is maintained in DMOG-treated zygotes as assessed by staining with specific antibodies for H3K4me2 (n=5 zygotes), H3K9me2 (n=5 zygotes), and H3K36me3 (n=5 zygotes) and H3K27me3 (n=8 zygotes). Representative images are shown. (b) TET1 in vitro activity assay in the presence of Fe²⁺ chelator deferoxamine (DFX). Conversion of 5mC to 5hmC is assessed by dot blot. (c) Inhibition of TET activity using DFX leads to decrease in paternal 5hmC without affecting the loss of paternal 5mC. Values are represented as a ratio between the signals in parental pronuclei (pat/mat). For 5mC staining, n=8 control and n=8 DFX-treated zygotes; for 5hmC staining, n=8 control and n=8 DFX-treated zygotes. Statistical analysis was carried out using Student’s t-test (two-sided). Error bars indicate s.d. (d) Bisulphite sequencing analysis of the 5’UTR of Line1 repetitive elements (L1Md_Tf and L1Md_Gf subtypes) indicating the percentage of methylated and hydroxymethylated CpG dinucleotides in zygotes without polar bodies (n=22 and n=20 amplified clones derived from control and DMOG-treated zygotes, respectively). Note that bisulphite sequencing does not distinguish between 5mC and 5hmC. (e) Bisulphite sequencing analysis of the 5’UTR of Line1 repetitive elements (L1Md_Tf and L1Md_Gf subtypes) on the paternal pronuclei of control and DMOG-treated zygotes (late PN3-PN4, 7-10hpf) extracted by micromanipulation (n=44 and n=42 paternal pronuclei extracted from control and DMOG-treated zygotes, respectively; 2 independent biological replicates are shown). (f) Bisulphite sequencing analysis of Line1 repetitive elements (L1Md_Tf and L1Md_Gf subtypes) in wild-type (Tet3WT) and Tet3 maternally depleted (Tet3mat-/pat+) zygotes recovered at PN4, 9hpf (n=31 and n=31 amplified clones, respectively). For bisulphite experiments, p values were calculated using Mann-Whitney U-test. **, p<0.01. ♀, female pronucleus; ♂, male pronucleus; DMOG, dimethyloxallyl glycine; DFX, deferoxamine; PI, propidium iodide. (Scale bars, 5um.)
Supplementary Figure 4 (a) Table summarising limit of detection (LOD), limit of quantification (LOQ) and signal-to-noise (S/N) ratio (in brackets) of LOQ peak for analysed nucleosides in our LC/MS method. (b) Parthenogenetically activated oocytes were incubated for a short pulse with EdU post-activation to control for the completion of S-phase. (n=4 parthenotes.) Representative images are shown. (Scale bars, 5um.) (c) LC/MS quantification of 5mC/dG ratio in MII oocytes and parthenogenetically activated oocytes blocked for replication, showing relative stability of genome-wide DNA methylation on the maternal genome (2 biological replicates and 2 technical replicates, 100 embryos (or oocytes) used per measurement). (d) Calculation of paternal 5mC levels based on the assumption that 5mC signal on the maternal pronucleus remains stable in the zygote (according to measurements in Fig. 2b). Error bars indicate s.d. (e) LC/MS measurement of 5mC/dG and 5hmC/dG ratio in sperm, control and DMOG-treated zygotes (with polar bodies) confirming results shown in Fig. 2b. Each value represents the average of 2 independent experiments with 2 technical replicates; 100 zygotes used per measurement. (f) Inhibition of replication in aphidicolin-treated zygotes assessed by EdU incorporation. (g) 5mC and 5mC staining in control and aphidicolin-treated zygotes. Loss of 5mC and increase in 5hmC signals occur independently of DNA replication. Each data point represents an independent zygote (n=9 control and n=12 aphidicolin-treated zygotes). Statistical analysis was carried out using Student’s t-test (two-sided). Error bars indicate s.d. (h) LC/MS measurement of 5mC/dG ratio in aphidicolin-treated zygotes (with polar bodies). Each value represents the average of 2 independent experiments with 2 technical replicates. 100 zygotes used per measurement. (i) 5mC kinetics in WT and Tet3 maternally depleted zygotes (Tet3mat-/pat+). (j) Bisulphite sequencing of the 5'UTR of Line1 repetitive elements (L1Md_Tf and L1Md_Gf subtypes) indicating the percentage of modified CpG dinucleotides in control (DMSO) and azadC-treated zygotes after removal of both polar bodies (PN4, 9hpf). p value was calculated using Mann-Whitney U-test. © 2016 Macmillan Publishers Limited. All rights reserved.
Supplementary Figure 5 (a) Dnmt3a and Dnmt3L are detectable in both zygotic pronuclei from early PN3. DNA counter-stained with DAPI. Representative images are shown (n=9 zygotes) (Scale bars, 5um.) (b) Analysis of DNA modifications in zygotes derived from in vitro fertilisation of [Dnmt3L−/−] oocytes by wild-type sperm. Quantification is represented as signal intensity in parental pronuclei. Each data point represents an independent zygote. (n=11 WT and n=11 KO zygotes). Statistical analysis was carried out using Student’s t-test (two-sided). Error bars indicate s.d. ***, p<0.001. (Scale bars, 5um.) (c) Immunostaining of wild-type (WT) or triple Dnmt knockout [Dnmt1−/− Dnmt3a−/− Dnmt3b−/−] (TKO) ES cells with 5mC and 5hmC antibodies. (Scale bars, 15um.) (d) Total (-pre-extraction) and chromatin-bound (+Triton pre-extraction) XRCC1 in control and DMOG-treated zygotes reflecting the activation of BER pathway independently of 5hmC accumulation. DNA counter-stained with DAPI. Representative images are shown. (g) LC/MS quantification of isotope-labelled dC (dC*) ratio over dG in early pre-replicative PN3 zygotes and PN3 zygotes treated with aphidicolin (5.5hpf). Experiment carried out in technical and biological duplicates. (h) Replication kinetics visualised by EdU pulse in pre-replicative (5.5hpf) and post-replicative (6.5hpf) zygotes. Representative images are shown (n=8). (Scale bars, 5um.) ♂, male pronucleus; ♀, female pronucleus; §, male pronucleus; DMOG, dimethyloxallyl glycine; hpf, hours post-fertilisation, n.d., non-detected.