Naive pluripotency is associated with global DNA hypomethylation

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Naive pluripotent embryonic stem cells (ESCs) and embryonic germ cells (EGCs) are derived from the preimplantation epiblast and primordial germ cells (PGCs), respectively. We investigated whether differences exist between ESCs and EGCs, in view of their distinct developmental origins. PGCs are programmed to undergo global DNA demethylation; however, we find that EGCs and ESCs exhibit equivalent global DNA methylation levels. Inhibition of MEK and Gsk3b by 2i conditions leads to pronounced reduction in DNA methylation in both cell types. This is driven by Prdm14 and is associated with downregulation of Dnmt3a and Dnmt3b. However, genomic imprints are maintained in 2i, and we report derivation of EGCs with intact genomic imprints. Collectively, our findings establish that culture in 2i instills a naive pluripotent state with a distinctive epigenetic configuration that parallels molecular features observed in both the preimplantation epiblast and nascent PGCs.

Pluripotency can be defined as the ability at the single-cell level to engender all somatic cell lineages as well as germ cells. In the preimplantation embryo, pluripotency is established in the epiblast of the late inner cell mass (ICM)1,2. These cells can be captured and maintained in culture as ESCs3–5. Both ICM cells and ESCs can contribute to chimeras and colonize the germline following reintroduction to the embryo, which provides functional proof of their naive pluripotency6–8. Conversely, neither the postimplantation epiblast nor the primed pluripotent stem cells derived from this tissue have the capacity to contribute efficiently to chimeras after blastocyst integration9–11. Pluripotency is lost in the embryo upon somatic differentiation12 and can only be reinstated experimentally by reprogramming strategies13. However, in the developing postimplantation embryo, PGCs can give rise to EGCs14,15, which exhibit all the properties of naive pluripotent stem cells including contribution to chimeras16,17. Thus, preimplantation epiblast and PGCs share the distinctive capacity to give rise to naive pluripotent stem cells under permissive conditions ex vivo.

During their development, PGCs undergo a unique epigenetic reprogramming process that entails global chromatin rearrangement and DNA demethylation18,19. This includes erasure of DNA methylation marks at imprinted loci at embryonic day 11.5 (E11.5)20. Consequently, EGC lines derived from PGCs at or after this stage lack DNA-methylation imprint marks17,21, and this loss of imprinting leads to skeletal abnormalities in high-contribution chimeras21. Previous reports have documented that EGC lines derived from early PGCs before E11.5 also exhibit imprint erasure, although in some cases less extensively than lines derived from later stages22. Consequently, to our knowledge, there has thus far been no report of EGC lines derived with intact genomic imprints, and this feature is widely considered to distinguish them from ESCs17,21.

Besides the loss of genomic imprints, it has been hypothesized that, in connection with their PGC origin, EGCs are also characterized by global DNA hypomethylation23. To investigate what differences, if any, exist between ESCs and EGCs we measured gene expression and global DNA methylation in a large cohort of genetically matched cell lines. We show that ESCs and EGCs are highly similar at the transcriptome level. We further demonstrate that, contrary to previous assumptions, both ESCs and EGCs contain comparable levels of DNA methylation and that EGCs can be derived with imprints intact. We additionally show that global DNA methylation of both ESCs and EGCs is directly responsive to the culture environment and identify Prdm14 as a key factor underlying this epigenetic regulation.

RESULTS

Culture over origin defines the pluripotent cell transcriptome

To assess similarities and differences between ESCs and EGCs that would reflect their distinct embryonic origin, we derived a large cohort of genetically identical EGC and ESC lines. We derived EGC lines from E8.5 mouse embryos by using three different protocols: fetal calf serum (FCS) plus leukemia inhibitory factor (LIF) on mouse embryonic fibroblasts (MEFs); FCS plus LIF on MEFs for 48 h followed by 2i plus LIF; and direct derivation into 2i plus LIF24,25 (Fig. 1a). For direct comparison, ESC lines were derived in either FCS plus LIF on MEFs or in 2i plus LIF conditions, and once established the cell lines were also switched between the two culture environments (hereafter referred to as FCS and 2i, respectively; Fig. 1a).

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First, we set out to compare ESCs and EGCs at the transcriptional level. Unsupervised hierarchical clustering of Affymetrix gene-expression data revealed that the major distinction between all cell lines is culture condition and not embryonic origin (Fig. 1b). We observed a dramatic effect of the culture environment on the pluripotent cell transcriptome in both EGCs and ESCs, with 2,016 genes differentially expressed between FCS and 2i (false discovery rate (FDR) < 0.05; fold change > 1.5; Supplementary Table 1 and Supplementary Fig. 1; details in Online Methods). Additionally, EGCs and ESCs clustered on the basis of the maintenance culture condition rather than the derivation procedure (Fig. 1b), which supports the interconvertibility of the two molecular states defined by FCS or 2i (ref. 26). These results thus indicate that the culture environment in which cells are maintained has a substantial and dominant effect over cellular origin with respect to global gene expression.

As separation between ESCs and EGCs may be eclipsed by the prominent difference between FCS and 2i, we performed unsupervised hierarchical clustering of cell lines cultured in FCS only (Supplementary Table 1 and Supplementary Fig. 1a) or in 2i only (Supplementary Table 1 and Supplementary Fig. 1b). Although some separation was observed, the two cell types did not cluster discretely into two groups. Upon statistical analysis, the expression of only 83 genes was significant between ESCs and EGCs. Interestingly, the expression of these 83 genes was not clonally expression (GSEA); normalized enrichment score (NES) = 1.11), although we noted an impact of the culture condition on the expression of this class of genes (Fig. 1c).

Collectively, our data show that ESCs and EGCs are nearly identical at the transcriptional level, thus indicating a lack of appreciable transcriptional memory of the germline origin in EGCs.

**Culture in 2i leads to global DNA hypomethylation**

PGCs are programmed to undergo genome-wide erasure of DNA methylation, including genomic imprints27. Therefore, we asked whether EGCs are also characterized by global DNA hypomethylation in comparison to ESCs. We analyzed global levels of 5-methylcytosine (5mC) by LC-MS and detected no difference between ESCs and EGCs. However, we unexpectedly found that all pluripotent cell lines (ESCs and EGCs) grown in 2i conditions had dramatically decreased global 5mC content (P < 0.0001, unpaired t test; n ≥ 6; Fig. 2a,b). To evaluate this loss of DNA methylation further, we used thin-layer chromatography (TLC), which specifically profiles 5mC present in CCGG sites, a typical feature of CpG islands. A large drop in 5mC was observed under
To elucidate the molecular grounds for the observed global DNA methylation changes occurring upon transfer of pluripotent cells from serum-containing into 2i culture conditions. Dnmt3a and Dnmt3b and their cofactor Dnmt3l in combination with changes in the abundance of factors implicated in the regulation of DNA methyltransferases Dnmt1 remained unchanged. We confirmed these findings by quantitative real-time PCR (qRT-PCR) and found that the Dnmt3a2 variant was specifically altered, whereas Dnmt3a1 was not (Fig. 3a). These changes were also observed at the protein level, with low expression of Dnmt3a2 and Dnmt3b in cell lines cultured in 2i, regardless of their embryonic origin (Fig. 3b,c).

The prominent changes in 5mC content in 2i may be linked to loss of 5mC through Tet-driven hydroxylation. Indeed, our microarray analysis as well as qRT-PCR data showed changes in expression of Tet hydroxylases (Fig. 3a and Supplementary Fig. 3a). However, the hypomethylated cells grown under 2i conditions contained significantly reduced amounts of 5-hydroxymethylcytosine (5hmC; P < 0.01, unpaired t test; Figs. 3d and 2b). Furthermore, the 5hmC/5mC ratio remained similar between cells grown in the different conditions, which suggests that the reduced 5hmC abundance reflects the lower amount of available 5mC substrate (Fig. 3d).

Transcriptional profiles are reversible between culture conditions; however, DNA methylation is considered to be a relatively stable epigenetic mark. Unexpectedly, we found a complete reversal of global 5mC methylation changes occurring upon transfer of pluripotent cells from serum-containing into 2i culture conditions.

**Figure 3** Characteristics of DNA methylation changes between culture conditions. (a) qRT-PCR of Dnmt and Tet expression in ESCs maintained in 2i or FCS. **P < 0.01; ***P < 0.001 by unpaired t tests; n ≥ 4 independent cell lines for each condition; error bars, s.e.m. (b) Western blot of Dnmt expression in ESCs and EGCs cultured in 2i or FCS for five passages. (c) Immunofluorescence staining for Dnmt3a and Dnmt3b in EGC line EG-2i-1 cultured in FCS or 2i. DNA counterstained with DAPI. Scale bar, 10 μm. (d) Global 5mC levels by LC-MS in undifferentiated (day-0) and day-4 embryoid bodies derived from pluripotent cells grown in FCS or 2i. Oct4 and Sox17 expression shown for progression of differentiation.

**Figure 4** Changes in global DNA methylation upon transfer to 2i culture conditions. (a) Expression, relative to ESC-3 (day-0) normalized to G6pd expression, of Dnmt3a1−2, Dnmt3a1+2, Pou5f1, Sox17 and Lamin B in 2i and FCS. (b) DNA methylation profile of the OCT4 promoter for each condition; error bars, s.e.m. (c) Immunofluorescence staining for Dnmt3a and Dnmt3b expression shown for progression of differentiation.
Global DNA methylation is restored upon differentiation
Previous studies have documented that DNA methylation is dispensable for ESC self-renewal\textsuperscript{15,38} but is essential for the ability of cells to differentiate. As ESCs grown in 2i are not compromised in their ability to differentiate\textsuperscript{24,26}, we investigated the dynamics of Dnmt expression during embryoid-body differentiation. We found that hypomethylated EGCS and ESCs grown in 2i efficiently upregulated both Dnmt3a2 and Dnmt3b upon differentiation to levels similar to those seen after differentiation of cells grown in FCS (Fig. 3f). In agreement with this, global 5mC levels were equivalent in embryoid bodies derived from cells cultured in 2i or FCS (Fig. 3g).

Pдрм14 is a critical regulator of DNA hypomethylation
Dnmt3b has been described as a direct target of Prdm14 in mouse ESCs\textsuperscript{39}. We found that Pдрм14 was highly upregulated in 2i as a response to both inhibitors (Figs. 4a and 1c), which suggests a plausible mechanism leading to Dnmt3b suppression and subsequent reduced DNA methylation in 2i. To test this hypothesis, we derived Prdm14\textsuperscript{−/−} ESCs from heterozygous Prdm14\textsuperscript{+/−} intercrosses, using 2i (N.G., J. Tischler, H.G.L. and M.A.S., unpublished data). We established by qRT-PCR that Dnmt3b was highly upregulated in Prdm14\textsuperscript{−/−} ESCs compared to wild-type ESCs grown in 2i conditions (P < 0.05, unpaired t test; Fig. 4b). Additionally, we observed a slight upregulation of Dnmt3a1 (P < 0.01, unpaired t test) but not of Dnmt1 or Dnmt3a2 (Fig. 4b). LC-MS analysis revealed that despite being maintained in 2i conditions, Prdm14\textsuperscript{−/−} ESCs had global DNA methylation comparable to that of wild-type ESCs cultured in FCS (Fig. 4c). These findings thus demonstrate that Prdm14 is a key driver of the DNA hypomethylation observed in 2i cultures.

ESCs and EGCS can maintain DMR methylation in 2i
Genomic imprinting is regulated by DNA methylation at imprinting control regions\textsuperscript{40}. Using bisulfite sequencing, we found that ESCs grown in 2i maintain methylation at imprinted differentially methylated regions (DMRs) despite global DNA hypomethylation (Fig. 5a and Supplementary Fig. 4a). This is consistent with unchanged Dnmt1 levels between culture conditions and the described involvement of Dnmt1 in maintenance of DNA methylation at imprinted DMRs\textsuperscript{40}. Whereas Dnmt3a and Dnmt3b double-knockout ESCs do show abnormalities in imprinted DNA methylation at high passage number\textsuperscript{36}, low but detectable levels of Dnmt3a and Dnmt3b enzymes, combined with unaltered Dnmt1 levels, appear sufficient to prevent loss of methylation at these loci in 2i conditions.

Figure 4 Prdm14 regulates DNA methylation in pluripotent stem cells. (a) Pдрм14 expression as assessed by qRT-PCR in cells grown in FCS or 2i or in the presence of a single Erk (PD) or Gsk3b (CH) inhibitor (LIF was added in all conditions). (b) qRT-PCR analysis of Dnmt and Tet expression in Prdm14\textsuperscript{−/−} ESCs (two independent cell lines) in comparison to representative ESC lines grown in FCS or 2i (three independent cell lines for each condition). *P < 0.05; **P < 0.01. Results of unpaired t tests are shown for comparison to Prdm14\textsuperscript{−/−} ESCs only; error bars, s.e.m. (c) Analysis of global 5mC in Prdm14\textsuperscript{−/−} ESCs in comparison to representative ESC lines grown in FCS or 2i. Each data point represents the average of two technical replicates for an independent cell line. *P < 0.05 by unpaired t tests.

Figure 5 Imprinted DMR methylation status of ESC and EGC lines. (a) Summary of bisulfite sequencing of imprinted DMRs in ESC line ES-2i-4 cultured in 2i or FCS. (b) Summary of bisulfite sequencing of imprinted DMRs in EGCs derived in 2i or FCS, showing that EGCs derived in 2i exhibit two distinct methylation patterns. (c) Bisulfite sequencing of imprinted DMRs in an EGC line derived in 2i following an interspecific cross (C57BL/6 × Mus castaneous), allowing the parental origin of each allele to be determined. Blue line represents the paternal allele and red the maternal allele. Filled and open circles represent methylated and unmethylated CpGs, respectively. (d) Image of chimeras generated by injection of EG-2i-1 (agouti) into C57BL/6 blastocysts (black).
Previous reports have indicated that EGCs derived at E8.5 show variability in the extent of erasure of DNA methylation marks at imprinted DMRs\(^\text{17,22}\). However, most lines exhibit a high degree of imprint erasure, and Kcnq1ot1 DMR methylation is invariably erased in all EGC lines tested thus far\(^\text{22}\). Consistent with this, our EGCs derived by using the traditional protocol exhibited erasure of methylation at the Kcnq1ot1 and Peg3 DMRs but retained methylation at the intergenic DMR (IG-DMR) (Fig. 5b and Supplementary Fig. 4a). Our data hence confirm that the derivation of EGC lines by using traditional conditions leads to the loss of genomic imprints. Unexpectedly, EGC lines derived by using 2i exhibited two distinct methylation patterns: two lines displayed almost complete imprint erasure, and two lines retained a completely intact imprint methylation pattern (Fig. 5b and Supplementary Fig. 4a). Moreover, using EGCs derived in 2i from an interspecific cross, we confirmed the presence of allele-specific DMR methylation (Fig. 5c). Thus in total, we have derived three EGC lines with intact imprint marks by using 2i, identifying a fundamental difference from historically derived EGC lines. The observed methylation profiles were retained through differentiation in embryoid bodies and were also generally stable upon switching the culture condition (data not shown).

The connection between loss of genomic imprinting in EGCs and compromised viability of high-contribution chimeras has been well established\(^\text{21}\). Consistently, blastocyst injection of our EGCs derived and maintained in FCS produced very few live-born pups (3 from 79 total blastocysts) and no chimeras (Supplementary Fig. 4b). Although heavily pregnant females were observed, the pregnancies were either spontaneously aborted or the pups immediately cannibalized. EG-2i-4, an EGC line derived in 2i, with its lack of imprints, similarly failed to produce larger chimeras (Supplementary Fig. 4b). To the contrary, and similar to our control ESC line (derived and maintained in 2i), we obtained robust contribution to chimeras upon injection of EG-2i-1, which showed normal methylation patterns (Fig. 5d and Supplementary Fig. 4a,b). The chimeras generated from EG-2i-1 were also test mated and produced germline transmission (Supplementary Fig. 4c). Our data thus clearly show that 2i culture conditions allow for derivation of EGC lines with intact genomic imprints, and hence neither global DNA methylation nor lack of genomic imprinting can be considered a distinguishing feature of pluripotent EGCs.

**DISCUSSION**

Our results reveal that both EGCs and ESCs reach a transcriptionally similar identity despite their distinct embryonic origins. We furthermore show that, despite previous assumptions, EGCs and ESCs show comparable global levels of DNA methylation, and EGC lines can be derived with intact genomic imprints. We thus establish that neither global DNA methylation nor presence of genomic imprints is a distinguishing feature of pluripotent cells derived from PGCs or the ICM. Our findings document that different culture conditions are associated with profound changes in global DNA methylation. We demonstrate that pluripotent cells (both ESCs and EGCs) grown under 2i conditions are characterized by DNA hypomethylation and downregulated expression of the de novo DNA methyltransferases Dnmt3a and Dnmt3b as well as Dnmt3l. As the naive pluripotent state in 2i is characterized by global DNA hypomethylation, and subsequent differentiation is accompanied by the accumulation of DNA methylation, the higher methylation observed in FCS cultures might be indicative of a more advanced differentiation status\(^\text{8}\). Additionally, the observed pronounced effect of Dnmt3a and Dnmt3b on global DNA methylation (Figs. 2 and 3) supports previous suggestions regarding the importance of a combination of both de novo and maintenance DNA methyltransferases to sustain genome-wide DNA methylation\(^\text{41}\) and might be indicative of an as-yet-unappreciated dynamic nature of DNA methylation.

Notably, downregulation of both Dnmt3a and Dnmt3b is observed in cells of the early preimplantation epiblast\(^\text{42,43}\), and the blastocyst represents the nadir in global DNA methylation during early development\(^\text{44,45}\) (Fig. 6a). Aspects of this molecular state, such as downregulation of Dnmt3a and Dnmt3b and upregulation of pluripotency genes, are also recapitulated upon PGC specification.\(^\text{46}\) We therefore propose that a characteristic feature of cells with the inherent capacity to generate pluripotency is protection from de novo methylation. This naive state is sustained or recapitulated in vitro under 2i conditions (Fig. 6b).

Our data demonstrate that the PGC determinant Prdm14 is upregulated in 2i conditions and has a critical role in maintaining the observed DNA hypomethylation in vitro. Prdm14 is expressed in both the ICM of the preimplantation embryo and in nascent PGCs\(^\text{47}\), and it is thus conceivable that it influences global DNA methylation also in these contexts. We also note that Prdm14 might act through multiple downstream effectors; almost half (106/231) of the Prdm14 target genes that are responsive to Prdm14 knockdown in ESCs\(^\text{39}\) are significantly affected by culture condition (FDR < 0.05; fold change > 1.5; Supplementary Fig. 3c). Prdm14 targets include numerous epigenetic modifiers. Consistent with these findings, altered expression of several of these factors in 2i conditions was observed alongside changes in the abundance of relevant histone modifications, some of which have been directly implicated in the regulation of de novo DNA methylation (Supplementary Fig. 3). Similar changes in epigenetic regulators and histone modifications are observed in both ICM and nascent PGCs expressing Prdm14 in vivo, which suggests a common epigenetic signature in cells with the capacity to generate pluripotency.

Finally, we demonstrate that, similar to the ICM in vivo, imprints can be maintained in pluripotent cells in 2i even in the context of global DNA hypomethylation. In fact, the derivation of EGCs with normal imprints is actually facilitated by 2i, and, to our knowledge, this is the first report of EGCs with intact genomic imprint marks. The difference in imprint status between EGC lines that were obtained from the same derivation experiment reflects either molecular heterogeneity of...
the starting PGC population at E8.5 or emerges during the derivation process. In either case, our results demonstrate that loss of imprints is not a prerequisite for EGC derivation. As imprint instability has been demonstrated in some ESC lines, we suggest that imprint status is not a reliable method to discriminate between ESCs and EGCs.

Collectively our results provide new insights into the unique epigenetic status of naïve pluriptotency, with distinct mechanistic parallels to the epigenetic changes occurring during developmental acquisition of pluripotent capacity in the mouse embryo (Fig. 6b).

METHODS

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

Accession codes. The microarray data are available in the Gene Expression Omnibus (GEO) database, under the accession number GSE43398.

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

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AUTHOR CONTRIBUTIONS

The study was conceived of and designed by H.G.L., K.R.M. and P.H. Experiments and Agilent T echnologies for their support regarding the LC-MS analysis. W .M., N.G. and J.G.K. provided reagents. A.S. and M.A.S. provided critical feedback. H.G.L., K.R.M. and P.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animal studies. Animal studies were authorized by the Cambridge University ethics review committee and a UK Home Office Project License and carried out in a Home Office–designated facility.

Cell culture. FCS medium consists of DMEM-F12 (Gibco) supplemented with 15% FCS, 0.1 mM MEM nonessential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol and mouse LIF 10 µg/ml (prepared in house). Cells cultured in serum were maintained on a MEF feeder layer. For downstream analysis, MEFs were removed by serial panning and the purity of EGCs or EGCs confirmed by alkaline phosphatase staining. The 2i medium consists of the MEF inhibitor PD0325901 (PD) at 1 μM, the GSK3 inhibitor CHIR99021 (CH) at 3 μM and mouse LIF at 10 µg/ml (as above) in N2B27 medium. Cells cultured in 2i were maintained on laminin (10 µg/ml, Sigma). Cells were passaged by dissociation with trypsin and replating every 2–3 d. EGCs were derived in serum conditions or in 2i as described previously. EGCs were derived as described previously.

All cell lines were derived from embryos produced by crossing mixed background Oct4APE-GFP transgenic males with strain-129SvEv female mice.

Passage numbers. The passage number for all the cell lines shown in Fig. 1a ranges between 7 and 15. The EGCs which were split into different conditions were cultured side by side for five passages in 2i and FCS. In Fig. 3e, the cells were either maintained in their usual culture condition (for instance, 2i) or switched to the alternative condition (FCS, and vice versa) for five passages. Therefore, the passage numbers for this experiment range between 17 and 22.

Chimeras and embryos. Mouse chimeras were produced by microinjection of EGCs or ESCs (agouti) into E3.5 C57BL/6 blastocyst. Chimerism was assessed by agouti coat color. ICMs were isolated from E4.0 blastocysts by immunosurgery as described previously.

Affymetrix microarrays and data analysis. RNA (100 ng), isolated by using the Qiagen RNeasy Mini kit, was processed with the Ambion WT Expression kit and scanned with the GeneChip scanner. At least four biological replicates were included for each condition (Fig. 1a). Quality control was undertaken by using Affymetrix Expression Console software. Preprocessing by robust multiaarray averaging (RMA) was performed in R using the Bioconductor package linear models for microarray data (limma), and batches were corrected by using Combat. Statistical testing was performed by using limma log2-transformed data; differentially expressed genes were identified by using thresholds of Benjamini-Hochberg FDR < 0.05 and fold change > 1.5. GSEA was used for statistical testing of differential expression of gene sets. Hierarchical clustering and heat maps were generated by using Partek software, version 6.6.

Real-time quantitative PCR. Random-prime reverse transcription was performed by using the Invitrogen Superscript III kit on 1 µg RNA for cell-line samples or 250 ng RNA for embryoid-body samples. Volumes of 3 µl of 1:40 dilutions (1:10 for embryoid body samples) were added to 10-µl reaction mixes (Bioline Sensimix SYBR No-Rox), and qRT-PCR was performed by using the standard-curve method. Cycling conditions were 95 °C 10 min; 40 cycles of 95 °C 15 s; 60 °C 15 s; and 72 °C 15 s, followed by a melting curve from 55 °C to 95 °C (1 °C increments). Four genes were tested for their use as a normalization gene (glucose-6-phosphate dehydrogenase X-linked (G6pd); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); β-actin; and hypoxanthine guanine phosphoribosyltransferase). G6pd was selected, owing to its consistency of expression between the sample sets shown in Fig. 1a. GAPDH was selected for Prednim14 knockout sample comparisons. Major satellites were assessed by semiquantitative PCR, owing to the presence of multiple PCR products. All primer pairs (sequences available upon request) were annealed at 60 °C. Two-tailed unequal t tests were performed by using GraphPad Prism software.

Immunofluorescence staining. For 5mC and 5hmC staining, cells grown on Labtech slides were briefly washed in PBS and fixed in 4% paraformaldehyde (PFA, prepared in PBS) for 15 min at room temperature. The cells were subsequently permeabilized by using PBS, 1% BSA, 0.5% Triton X-100 (30 min), washed in PBS and incubated with RNase A (20 mg/ml in PBS) for 1 h at 37 °C. Following subsequent PBS washes, the DNA was denatured in 4 M HCl for 10 min at 37 °C, and the slides were neutralized by using extensive PBS washes, blocked in PBS, 1% BSA, 0.1% Triton X-100 (30 min) and incubated with the indicated antibody (5mC; Active Motif 39791 (polyclonal), 1:400; 5hmC; DsRed G4081100 (monoclonal), 1:4000) in the same buffer at 4 °C overnight. The slides were subsequently washed three times in PBS, 1% BSA, 0.1% Triton X-100 and incubated with Alexa fluorophore–conjugated secondary antibodies (Invitrogen) for 1 h at room temperature in the dark and washed in PBS, 1% BSA, 0.1% Triton X-100 and twice in PBS. Finally, the slides were mounted in Vectashield containing DAPI (Vector Laboratories) and imaged by using a Leica SP5 confocal microscope.

Immunofluorescence detection of Dnmts and Oct4 expression was carried out as above without the RNase A and HCl-denaturation steps (Dnmt3a, Imgenex IMG-268A (monoclonal), 1:10; Dnmt3b, Imgenex IMG-268A (monoclonal), 1:400; Oct4, Abcam Ab19857 (polyclonal), 1:100). DNA was visualized by DAPI.

Western hybridization. For DNA methyltransferase western hybridizations, cells were lysed in buffer containing 150 mM NaCl 50 mM Tris-HCl pH 7.5, 0.5% Triton X-100 (v/v) and protease-inhibitor cocktail (Roche). Homogenized protein (10 μg) was loaded onto an 8% acrylamide/bis gel and transferred to a PVDF membrane after electrophoresis. Following blocking with 5% nonfat milk for 1 h, membranes were incubated at 4 °C overnight with primary antibodies at the following dilutions: Dnmt1, Imgenex IMG-261 (monoclonal) 1:500; Dnmt3a, Imgenex IMG-268A (monoclonal), 1:500; Dnmt3b, Imgenex IMG-184A (monoclonal), 1:20,000; lamin B, (C-20) Santa Cruz sc-6216 (polyclonal), 1:10,000. HRP-conjugated secondary antibodies were incubated for 1 h at room temperature. Amersham ECL western blotting analysis systems were used for detection on a GE ImageQuant LAS 4000 mini.

For histone-modification western hybridizations, histones were extracted by using Triton extraction buffer (PBS containing 0.5% Triton X-100 (v/v), 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (w/v) NaN3). After 10 min on ice, samples were centrifuged, washed in Triton extraction buffer, then incubated in 0.2 N HCl overnight at 4 °C. Supernatants were measured by Bradford assay following centrifugation. Histone protein (0.5 μg) was loaded on 20% acrylamide/bis gels and after electrophoresis was transferred to PVDF membranes. Blocking was performed with 5% BSA for 1 h at room temperature. Membranes were then incubated for 1 h with primary antibodies at the following dilutions: H3K9me2, Upstate 07-441 (polyclonal), 1:5,000; H3K9me3, Abcam ab8898 (polyclonal), 1:5,000; H4K20me3, Upstate 07-463 (polyclonal), 1:10,000; H3K27me3, Cell Signaling 97565 (polyclonal), 1:10,000; H3K4me2, Upstate 04-030 (polyclonal), 1:10,000; H3K4me3, Abcam ab8580 (polyclonal), 1:10,000; H3K36me3, Upstate 05-801 (monoclonal), 1:10,000. Secondary antibodies were incubated for 1 h at room temperature. Blots were developed by using Amersham ECL and signal was detected by using standard chemiluminescent Kodak BioMax light film.

Thin-layer chromatography. Genomic DNA (500 ng) was digested with the restriction enzyme MspI overnight at 37 °C. Oligonucleotides containing only mC or dC were used as controls and subjected to the same treatment as genomic DNA. Following incubation for 1 h at 37 °C with 2.5 U of antarctic phosphatase, samples were purified by using the Qiagen QIAquick PCR purification kit. Eluate (80 ng) was end labeled for 1 h at 37 °C with [32P]ATP by using 10 U T4 polynucleotide kinase. Unincorporated isotopes were removed by QIagen column purification. DNA quantity equivalent of 50,000 counts was aliquoted and denatured by heating at 99 °C. The reaction was incubated with 2 U of nuclease P1 and 10 mM ammonium acetate (pH 5.3) for 2 h at 45 °C in a 5-µl reaction volume. Digested DNA (1.5 µl) was spotted onto a cellulose TLC plate and developed for approximately 8 h in isobutric acid/water/30% ammonium hydroxide (66:18:3) in a sealed TLC tank. The TLC plate was dried and incubated in an autoradiography cassette overnight. Radioactivity levels were recorded by using a Fujifilm FLA-5100 phosphorimager.

Mass spectrometry. Up to 500 ng genomic DNA was denatured by heating at 100 °C for 3 min. Samples were incubated with 1/10 volume of 0.1 M ammonium acetate, pH 5.3, and 2 U of nuclease P1 for 2 h at 45 °C. A 1/10 volume of 1 M ammonium bicarbonate and 0.002 units of phosphodiesterase I were added, followed by incubation for 2 h at 37 °C. Finally, samples were incubated for 1 h at 37 °C with 0.5 U alkaline phosphatase. Samples were subsequently diluted in

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2 mM ammonium formate, pH 5.5. The nucleosides were separated on an Agilent RRHD Eclipse Plus C18 2.1 × 100 mm 1.8u column by using the HPLC system 1200 (Agilent) and were analyzed by using an Agilent 6490 triple quadrupole mass spectrometer. To calculate the concentrations of individual nucleosides within the samples analyzed, standard curves with known amounts of synthetic nucleosides were generated and used to convert the peak-area values to corresponding concentrations.

**Additional methods.** Additional methods are provided in the Supplementary Note.

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Corrigendum: Naive pluripotency is associated with global DNA hypomethylation

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In the version of this article initially published online, a bar connecting the two central data sets in Figure 3e was inadvertently included. The error has been corrected for the print, PDF and HTML versions of this article.