Global delay in nascent strand DNA methylation

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Cytosine methylation is widespread among organisms and essential for mammalian development. In line with early postulations of an epigenetic role in gene regulation, symmetric CpG methylation can be mitotically propagated over many generations with extraordinarily high fidelity. Here, we combine BrdU labeling and immunoprecipitation with genome-wide bisulfite sequencing to explore the inheritance of cytosine methylation onto newly replicated DNA in human cells. Globally, we observe a pronounced lag between the copying of genetic and epigenetic information in embryonic stem cells that is reconsolidated within hours to accomplish faithful mitotic transmission. Populations of arrested cells show a global reduction of lag-induced intermediate CpG methylation when compared to proliferating cells, whereas sites of transcription factor engagement appear cell-cycle invariant. Alternatively, the cancer cell line HCT116 preserves global epigenetic heterogeneity independently of cell-cycle arrest. Taken together, our data suggest that heterogeneous methylation largely reflects asynchronous proliferation, but is intrinsic to actively engaged cis-regulatory elements and cancer.

Results

Repli-BS identifies a global delay in methylation of nascent DNA. To investigate the acquisition of CpG methylation on nascent DNA, we combined immunoprecipitation of bromodeoxyuridine (BrdU)-labeled nascent strands followed by sequencing (Repli-seq)21 with bisulfite treatment to measure post-replication cytosine methylation at base pair resolution (Repli-bisulfite seq: Repli-BS, Fig. 1a, Supplementary Fig. 1a and Methods). Human embryonic stem cells (ESCs; male HUES64) were treated for one hour with BrdU and sorted into six S-phase fractions (S1–S6) before BrdU immunoprecipitation followed by bisulfite sequencing (Fig. 1a,b, Supplementary Fig. 1b and Supplementary Dataset 1). We initially pooled data from the six fractions and compared the methylation level of ~24.5 million newly replicated (nascent) CpGs to bulk (non-sorted, no BrdU immunoprecipitation) whole-genome bisulfite sequencing (WGBS) data. Whereas our bulk reference population exhibited a canonical methylation landscape with high CpG methylation (mean of 0.83), the average for DNA synthesized within our 1-h BrdU pulse was globally reduced (mean 0.64; Fig. 1c and Supplementary Fig. 1c). This discrepancy was consistent across early (S1+S2; mean of 0.63), mid (S3+S4; mean of 0.63) and late (S5+S6; mean of 0.66) S-phase stages (Supplementary Fig. 1d). Moreover, we found that different measured genomic features appeared equally affected by this delay, including promoters, enhancers and gene bodies, showing no correlation with gene expression level (Supplementary Fig. 1e,f). CpG density as well as enrichment for the Polycomb repressive complex 2 (PRC2) subunit EZH2 appeared to have some influence on a very small subset of CpGs (Supplementary Fig. 1g–j). We also observed a global delay for non-CpG methylation, which was more apparent for gene bodies,

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Fig. 1 | Repli-BS identifies a global delay in the acquisition of CpG methylation on nascent DNA. a. Schematic of our experimental design. Asynchronously growing human ESCs are labeled with BrdU for 1 h. During the pulse, BrdU (red) is incorporated into newly synthesized nascent DNA (blue) as it is copied from the parental strand (black). Empty circles marked with an ‘x’ (‘to determine’) indicate CpGs investigated by Repli-BS for methylation status. Six gates of equal proportion were used to sort S-phase cells according to DNA content. BrdU immunoprecipitation (BrdU-IP) was performed to capture newly replicated DNA strands, which were treated with bisulfite before library generation and sequencing. b. Genome browser tracks show read density and corresponding replication pattern across each of the six collected S-phase fractions. c. Local regression analysis was performed using methylation values for CpGs within nascent (S1–S6) and bulk genomic DNA. LOESS-smoothed mean methylation levels are displayed as bold lines. Shaded areas show one s.d. d. Single-cell RRBS data for cells sorted by cell cycle stage. Violin plots with overlap display mean methylation per cell for all CpGs with methylation ≥0.8 in bulk cells. Each green dot represents one cell. Despite not isolating nascent DNA, 85% of S-phase cells (>0.8 in bulk) were (Supplementary Fig. 1k,l). Notably, the emergence of nonsymmetric methylation on the nascent strand cannot serve as a template and hence follows an alternative mechanism. Finally, we used single-cell RNA sequencing to determine expression of key epigenetic regulators throughout the cell cycle (Supplementary Fig. 2a and Supplementary Dataset 2) and in parallel applied a new multiplexed single-cell reduced representation bisulfite sequencing (RRBS) approach to measure cytosine methylation across individual cells sorted by DNA content. Cells in S phase showed lower mean methylation values compared to cells in G1 or G2-M, which independently points toward a replication-associated reduction in methylation (Fig. 1d and Supplementary Fig. 2b,c).

Biphasic remethylation of nascent DNA. To better understand the kinetics with which nascent DNA restores methylation over time, we chased BrdU-pulsed cells for 1–16 h before performing Repli-BS (Fig. 1e and Supplementary Dataset 1). The most notable methylation increase occurred within the first hour following replication, which could be associated with active recruitment of DNMT1 to the replication fork, followed by a slower, incremental period that stabilized to bulk reference levels after ~4 h (Fig. 1e and Supplementary Fig. 2d). The delayed kinetics of the second phase could reflect a replication-uncoupled search for unevenly dispersed hemimethylated targets. These dynamics appear to affect the genome equally (Supplementary Fig. 2e); however, while the mean methylation and rate of increase were remarkably similar between replicates (Fig. 1e), methylation typically emerged on distinct CpGs (Supplementary Fig. 2f). This finding suggests that the temporal order of adding this modification is not genetically encoded and may instead be determined by the molecular limits of DNMT1 activity near the replication fork, where additional processes may act as obstructions. The gain of methylation over time can also be measured at single-molecule resolution by determining the read-level progression from partial to fully methylated states (Supplementary Fig. 2g,h), which confirmed a high correlation between neighboring CpGs that would be consistent with in vitro measurements reporting a processive activity for DNMT1 (refs 6,21) (Supplementary Fig. 2i).
DNMT3A and DNMT3B have a limited effect on post-replication methylation rate. As previously observed, genetic deletion of DNMT1 in mouse and human ESCs results in rapid, global loss of CpG methylation, thus confirming the central role of DNMT1 in methylation maintenance. Long-term passaging of DNMT3A and DNMT3B double knockout (DKO) ESCs results in a gradual loss of methylation, showing that the de novo methyltransferases are required to compensate for the incomplete maintenance fidelity of DNMT1. After approximately 20 passages, our human DKO cells showed a global decrease in CpG methylation of 10% (Supplementary Fig. 3a). To assess the contribution of the de novo DNMTs to post-replication nascent strand methylation, we repeated the 1-h BrdU pulse-chase Repli-BS experiment in the DKO cells and observed that DNMT1 is able to methylate nascent DNA to DKO bulk levels in the absence of any de novo activity (Supplementary Fig. 3b–d). In fact, the nascent strand methylation rate appeared slightly faster than that in wild-type cells, which may be caused by the reduced number of targets (hemimethylated CpGs) in the comparatively hypomethylated DKO line.

Distinct origins of global intermediate methylation. In both cell lines, our data show that the delay in methylation of nascent DNA reduces global methylation levels. Given that ~35% cells within an unsynchronized ESC population are undergoing replication at any given time, we hypothesized that the intrinsically lower global methylation of S-phase cells may contribute to the intermediate methylation (values other than 0% or 100%) observed in bulk measurements, which are often attributed to intercellular heterogeneity. To investigate this possibility, we arrested ESCs in prometaphase and performed WGBS to determine whether absence of cells in S phase reduces the fraction of intermediately methylated CpGs (Fig. 2a, Supplementary Fig. 4a,b and Supplementary Dataset 1), which comprise the majority (58%) of CpGs in proliferating ESCs (Fig. 2b). In line with our hypothesis, the number of unmethylated CpGs (with 0% methylation) remained roughly constant between proliferating and arrested cells (5.6 and 6.9%). In contrast, intermediate methylated CpG sites were reduced from 58% in proliferating cells to 25% in arrested cells, with a concomitant increase in the number of fully methylated CpGs from 36% to 69% (Fig. 2b). In line with our hypothesis, the number of unmethylated CpGs (with 0% methylation) remained roughly constant between proliferating and arrested cells (5.6 and 6.9%). In contrast, intermediate methylated CpG sites were reduced from 58% in proliferating cells to 25% in arrested cells, with a concomitant increase in the number of fully methylated CpGs from 36% to 69% (Fig. 2b). In line with our hypothesis, the number of unmethylated CpGs (with 0% methylation) remained roughly constant between proliferating and arrested cells (5.6 and 6.9%). In contrast, intermediate methylated CpG sites were reduced from 58% in proliferating cells to 25% in arrested cells, with a concomitant increase in the number of fully methylated CpGs from 36% to 69% (Fig. 2b). In line with our hypothesis, the number of unmethylated CpGs (with 0% methylation) remained roughly constant between proliferating and arrested cells (5.6 and 6.9%). In contrast, intermediate methylated CpG sites were reduced from 58% in proliferating cells to 25% in arrested cells, with a concomitant increase in the number of fully methylated CpGs from 36% to 69% (Fig. 2b). In line with our hypothesis, the number of unmethylated CpGs (with 0% methylation) remained roughly constant between proliferating and arrested cells (5.6 and 6.9%). In contrast, intermediate methylated CpG sites were reduced from 58% in proliferating cells to 25% in arrested cells, with a concomitant increase in the number of fully methylated CpGs from 36% to 69% (Fig. 2b). In line with our hypothesis, the number of unmethylated CpGs (with 0% methylation) remained roughly constant between proliferating and arrested cells (5.6 and 6.9%). In contrast, intermediate methylated CpG sites were reduced from 58% in proliferating cells to 25% in arrested cells, with a concomitant increase in the number of fully methylated CpGs from 36% to 69% (Fig. 2b). In line with our hypothesis, the number of unmethylated CpGs (with 0% methylation) remained roughly constant between proliferating and arrested cells (5.6 and 6.9%). In contrast, intermediate methylated CpG sites were reduced from 58% in proliferating cells to 25% in arrested cells, with a concomitant increase in the number of fully methylated CpGs from 36% to 69% (Fig. 2b). In line with our hypothesis, the number of unmethylated CpGs (with 0% methylation) remained roughly constant between proliferating and arrested cells (5.6 and 6.9%). In contrast, intermediate methylated CpG sites were reduced from 58% in proliferating cells to 25% in arrested cells, with a concomitant increase in the number of fully methylated CpGs from 36% to 69% (Fig. 2b). In line with our hypothesis, the number of unmethylated CpGs (with 0% methylation) remained roughly constant between proliferating and arrested cells (5.6 and 6.9%). In contrast, intermediate methylated CpG sites were reduced from 58% in proliferating cells to 25% in arrested cells, with a concomitant increase in the number of fully methylated CpGs from 36% to 69% (Fig. 2b). In line with our hypothesis, the number of unmethylated CpGs (with 0% methylation) remained roughly constant between proliferating and arrested cells (5.6 and 6.9%). In contrast, intermediate methylated CpG sites were reduced from 58% in proliferating cells to 25% in arrested cells, with a concomitant increase in the number of fully methylated CpGs from 36% to 69% (Fig. 2b).
Fig. 3 | Cancer cells and active TF binding sites display inherent cell-to-cell methylation heterogeneity. **a**, Simplified schematic analogous to Fig. 2d, showing an alternative hypothetical scenario that represents CpG methylation heterogeneity arising from cell-to-cell differences. Filled circle, methylated CpG; empty circle, unmethylated CpG. Numbers below indicate the expected mean methylation for the CpGs at that position based on the illustrated pattern. **b**, Left, histograms displaying methylation levels for bulk and arrested CpGs with intermediate methylation in HCT116 cells. Right, pie charts summarizing the distribution of unmethylated, intermediate and fully methylated CpGs in proliferating and arrested HCT116 cells for CpGs covered at ≥15×. **c**, Top, ChIP-seq tracks (chr. 5: 163,394,518–163,756,719) for FOXA2 in ESCs differentiated for 5 d into endoderm (dEN), PAX6 in ESCs differentiated for 5 d into ectoderm (dEC) and NANOG in undifferentiated pluripotent ESCs. Diff., differentiated; pluri., pluripotent. Peaks indicate total number of peaks called by MACS2. Ratio is the average number of CpGs per peak. Middle, WGBS tracks for proliferating and arrested ESCs. For each TF, methylation levels in proliferating and arrested ESCs that show a shift to high methylation levels (as in the ‘genome’ example) for nonbound sites but no shift in the engaged NANOG binding site are shown. Genome, chr. 5: 163,477,070–163,491,961; PAX6, chr. 5: 163,434,313–163,439,131; FOXA2, chr. 5: 163,527,525–163,531,337; NANOG, chr. 5: 163,721,080–163,726,485. **d**, Global analysis of the phenomena described locally in **c**. When considering all CpGs or the ones found at not actively bound (but experimentally confirmed) TF binding sites, we observed a similar decrease in the percentage of CpGs with intermediate methylation levels (decrease of 33% for all CpGs, 22% for FOXA2 and 20% for PAX6; ‘+ shift observed’), whereas sites that are bound by NANOG in ESCs do not show this behavior and remain largely unchanged despite the arrest (decrease of only 4%; ‘– shift observed’).
in association with reduced global methylation levels\textsuperscript{27,28} (Fig. 3a). Considering our results, it seemed possible that the elevated fraction of intermediate methylation levels in cancer cells could be associated with the highly proliferative nature of these transformed cells. To explore this, we arrested the colon cancer cell line HCT116 and found an expected increase in mean methylation levels (from eliminating contribution of nascent strands with reduced methylation but not a shift toward full methylation, as observed in ESCs (Fig. 3b and Supplementary Fig. 4e,f). Although we only investigate HCT116 cells here, our data suggest that the epigenetic heterogeneity in cancer cells is a combination of both replication-associated and elevated cell-to-cell variation.

**Intermediate methylation at bound transcription factor targets points to intercellular heterogeneity.** Prior studies have also suggested that intermediate levels of methylation are frequently found around transcription factor (TF) binding sites\textsuperscript{3,12,26,29}. To compare these local events with the global patterns described above, we re-examined our bulk and arrested ESC datasets and used previously determined TF binding sites determined by chromatin immunoprecipitation followed by sequencing (ChIP-seq) experiments in ESCs as well as their differentiated derivatives. We first identified TF targets that are engaged and thus actively regulated in pluripotent cells (OCT4 or NANOG) as well as differentiation-specific target sites (PAX6 in ectoderm, FOXA2 in endoderm, GATA6 in mesoderm and EOMES in mesendoderm) that are not bound in undifferentiated ESCs and should therefore behave like the genomic average (Fig. 3c and Supplementary Fig. 4g,h). We then compared methylation levels for bulk and arrested ESCs and found that differentiation-specific regions behaved like the greater genome, whereas actively engaged sites preserved their intermediate methylation status. This finding suggests that TF binding may play a direct role in establishing local intercellular heterogeneity (Fig. 3c,d and Supplementary Fig. 4g,h).

**Discussion**

We applied a genome-wide approach to measure cytosine methylation levels of nascent DNA (Repli-BS) and found a persistent lag in the genome-wide lag between the copying of genetic and epigenetic information adds further insight to our understanding of how epigenetic information is mitotically inherited.

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41594-018-0046-4.

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**Author contributions**

J.C., T.L.D. and A.M. designed the study with input from Z.D.S. T.L.D., J.C., R.P. and V.A. performed the experiments. H.G. and A.G. developed the multiplexed single-cell RRBS protocol, generated the sequencing libraries and helped with experimental design and analysis. K.C., S.K., B.T. and M.J.Z. assisted in data processing. J.C. performed bioinformatics analyses. A.M.T. performed the single-cell RNA-seq cell cycle analysis. D.P.S. and E.K. performed the MN differentiation, characterization and sample collection. J.C., T.L.D., Z.D.S., A.G. and A.M. interpreted the data. J.C., T.L.D., Z.D.S. and A.M. wrote the manuscript with assistance from the other authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Methods

Cell culture. Human embryonic stem cells (male HUES64; obtained from Harvard University) and the modified variant DNMT3A–/–DNMT3B–/– (HUES64 DKO) cells were grown in feeder-free conditions using Geltrex (Thermo Fisher Scientific) and mESR (STEMCELL Technologies). HCT116 cells (obtained from S. B. Baylin at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins) were grown in McCoy's 5A (modified) medium supplemented with 10% FBS and 1% penicillin and streptomycin. All cell lines tested negative for mycoplasma.

Bulk versus arrested population measurements. Cells were either grown with no treatment (bulk) or arrested using 2 µg/ml nocodazole for 16 h, then collected and washed twice with PBS. Arrested cells were stained using the live-dead Fixable Blue Dead Cell Stain kit (Thermo Fisher Scientific) according to manufacturer's recommendations, then fixed by adding 100% ethanol dropwise to the cell suspension until a final ethanol concentration of 75% was achieved. Fixed arrested cells were then counterstained with propidium iodide (PI) before FACS sorting to collect cells in G2-M phase.

BrDU treatment. To label nascent strands of DNA, BrdU (5-bromo-2'-deoxyuridine; BD Biosciences) was added to cell culture medium at a final concentration of 50 µM. Cells were incubated for 40 h at 37°C to allow incorporation of BrdU/ nucleotides into newly synthesized DNA. Cells were washed three times with basal media (DMEM-F12), then either collected immediately or incubated at 37°C for the duration of the chase before collection. To collect, cells were detached from tissue culture plates and disaggregated into single cells using StemPro Accutase Cell Dissociation Reagent (Thermo Fisher Scientific) for ESCs and 0.05% trypsin for HCT116. Cells were then pelleted, resuspended in PBS and either fixed in ethanol as described above or snap frozen.

Enrichment for BrdU-labeled DNA (original). Genomic DNA was isolated by incubating cells in SDS–PK buffer (0.5% wt/vol) sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, 0.01 M EDTA, 1 M NaCl and 0.2 mg/ml proteinase K) at 56°C for 2 h. Phenol–chloroform extraction was performed, and genomic DNA was precipitated in one volume of isopropanol at –20°C. DNA was pelleted, washed with ethanol and resuspended in TE buffer. Genomic DNA was fragmented to an average size of 200 bp using a Branson Digital Sonifier (Model 250) and a Branson 101-148-063 microtip. DNA was sonicated using 40% amplitude for a total of 4 min. A double-antibody immunoprecipitation was performed to isolate nascent (BrdU labeled) strands of DNA. For each immunoprecipitation reaction, at least 120 ng of sonicated DNA was used. DNA was first heat denatured at 95°C for 5 min and rapidly cooled on ice. 12.5 µg primary antibody (mouse anti-BrdU, BD Biosciences Pharmingen) was added to the ssDNA suspension with constant rocking for 20 min and then 20 µg of secondary antibody (rabbit anti-mouse IgG, Sigma, cat. no. M-7023) was added and stained for 20 min. Antibody–DNA complexes were then pelleted, resuspended and incubated in digestion buffer (50 µM Tris-HCl, 0.01 M EDTA, 0.5% SDS and 0.25 mg/ml proteinase K) at 37°C overnight. Nascent ssDNA was purified by phenol–chloroform extraction and ethanol precipitation.

Enrichment for BrdU-labeled DNA (optimized). The above protocol was adapted to decrease the length of sample processing specifically for non-fixed, non-sorted cells. DNA was isolated using the Quick-DNA Universal kit (Zymo Research). Sonication was performed for 40% amplitude in 200 µl, 4 min for samples resuspended in 500 µl or in a 96-well plate using a Covaris 96-well sonicator (EZ20) for 75 s per sample. BrdU immunoprecipitation was performed for 60 min and 30 min for primary and secondary antibodies listed above, respectively, and then nascent ssDNA was purified using the Agencourt AMPure XP system (Beckman Coulter).

FACS and cell cycle analysis. Fixed cells, (in some cases BrdU labeled and/or with live–dead stain) were pelleted and washed twice in FACS buffer (1% FBS in PBS) and then stained with PI for 20–30 min. Cells were also coincubated with RNase A (0.250 mg/ml). Prior to sorting, cells were passed through 40 µm filter FACS tubes. FACS was performed using a BD FACS Aria Cell Sorter with a 100 µm nozzle under low pressure. To identify G1, S and G2-M, -M phase G1, S and G2-M cell populations, three sequential gating strategies were applied to the bulk sample population. Cell debris were removed using a forward versus side scatter comparison, dead cells were removed based on live-dead Fixable Blue stain intensity, and, finally, single cells (singlets) were enriched using a PI signal width versus height comparison strategy.

Motor neuron differentiation. Human ESC colonies were dissociated using Accutase and plated at a density of 74,000 cells/cm² with 10 µM ROCK inhibitor (Y-27632, DSNK International) in mESR for 48 h. Media was replaced on day 0 with N2B27 medium (50% DMEM:F12, 50% Neurobasal, supplemented with NEAA, B27, Insulin, G418 (0.2 µm), N2 and B27; Gibco, Life Technologies) for 8 days. The N2B27 medium supplemented with 1 µM RA, 1 µM SAG, 5 µM DAPT (DSNK International) and 4 µM SU5402 (DSNK International). Cells were fed daily until day 14 of differentiation. At day 6 and 14, cells were collected for WGBS.

Multiplexed single-cell RRRS. ESCs were collected, fixed in 75% ethanol and stained with PI. Cells were then sorted by cell cycle phase into G1, early S, mid S, late S and G2-M phase to obtain one cell per well in a 96-well plate containing 5 µl 0.1X Cutsmart buffer (New England Biolabs) per well (480 cells total). Sorted cells were lysed at 50°C for 2 h in a reaction containing 0.2 µM proteinase K (New England Biolabs), 0.2% Triton X-100 (EMD Millipore) and 1X Cutsmart buffer. After heat-inactivation of proteinase K at 75°C for 30 min, we added 2 µl of digestion buffer consisting of 1X Cutsmart buffer and 0.5 µl Mspl (20 µU, New England Biolabs) directly to each cell lysis reaction. The digested DNA was end-repaired and adenylated by adding 2 µl of a mixture containing 2.5 U Klenow fragment (3'-5' exo-, NEB), 0.4 µl of dNTP mixture (100 mM dATP, 1 mM dCTP and 1 mM dGTP) and 1X Cutsmart buffer to each sample and incubating at 30°C for 25 min, then at 37°C for another 30 min. After heat inactivation at 70°C for 10 min, adenylated DNA fragments were ligated with 5 µM substituted indexed adapters overnight at 16°C through distributing to each well 3 µl of reaction containing 800 U of T4 ligase (NEB), 0.1 µl of 100 mM ATP (Roche), 1.5 µl of 0.1 µM methylated indexed adapter and 1X Cutsmart buffer. 24 indexed ligation reactions were pooled, purified on Ampure XP beads and subjected to sodium bisulfite treatment using an EpiTect Fast Bisulfite kit (QIAGEN) following the manufacturer’s recommendations with extended conversion time (20 min each cycle). The bisulfite converted multiplex RRRS library was amplified for a total of 15 cycles using KAPA HiFi Uracil DNA Pol HiFi Polymerase. The PCR products were size selected, purified using a 0.1% agarose gel, and the final library DNA was eluted in 12 µl of 1X ddH2O.

Single-cell RNA-seq. Two 96-well plates of human ES and mesoderm single cells were used to make libraries for single-cell RNA-seq. Library preparation and analysis was done as described in ref. 2. Briefly, RNA-seq reads were first trimmed using Trimmomatic. Trimmed reads were aligned to the RefSeq hg38 genome and transcriptome (GRCh38.2) using Bowtie2 (ref. 3) and TopHat4, respectively. The resulting transcriptome alignments were processed by RSEM to estimate the abundance of RefSeq transcripts, in transcripts per kilobase million (TPM). All cells with less than 4,000 detectable annotated transcripts were removed from further analysis. Detectable transcripts were defined as transcripts with TPM > 1. After removing apoptotic cells and background differentiation, we ordered cells according to progress in the cell cycle, as done previously. Expression values displayed in Supplementary Fig. 2a were averaged using a moving window of 20 cells.

Quantifying gene expression cycling in G1, S and S phase. We ordered all G1-S and S-phase cells according to progress along the cell cycle, found the 20-cell window with highest and lowest expression for each gene and calculated a t test P value. We then permuted the order of all G1-S and S-phase cells at random and calculated the highest and lowest expression 20-cell windows for each gene and corrected for false discovery. For each gene, we used a gene with a treatment P value was measured by counting the number of genes with a more significant P value in the control experiment with permuted cell order normalized to the total number of control gene experiments. Genes were ranked in their cycling of expression using the FDR measure, ordering from smallest to largest.

Bisulfite sequencing. Either total genomic DNA (for WGBS) or DNA fragments recovered from the BrdU antibody pulldown (for Repli-BS) was concentrated by ethanol precipitation (final volume 20 µl). For the motor neuron samples, genomic DNA was fragmented using a Covaris S2 for 6 min according to the manufacturer’s protocol: duty cycle 5%; intensity 10; cycle per burst 200. The sheared genomic DNA was fragmented using a Covaris S2 for 6 min according to the manufacturer’s recommendations with slight modifications. Specifically, we optimized the PCR cycle number required for library DNA enrichments by performing a range of PCR cycles (10, 12, 14, and 16) using 10% of the post-ligation DNA. The minimal cycle number required for library DNA enrichments by performing a range of PCR cycles was determined to be 14 cycles (10, 12, 14, and 16) using 10% of the post-ligation DNA. The minimal cycle number was then used for the sample PCR to generate the sequencing libraries. PCR products were cleaned using the Agencourt AMPure XP system (Beckman Coulter), and the final library DNA was eluted in 12 µl elution buffer. Using the Agilent Bioanalyzer we confirmed the absence of adaptor–dimers. If residual traces were detected, we performed an agarose gel size-selection to further clean up the final library. Up to 12 indexed sequencing libraries were pooled together for high-throughput sequencing.
sequence using a 100-cycle paired-end (PE) run on an Illumina HiSeq 2500 or HiSeq 4000. For six nascent wild-type ESC time course samples (replicate 1, Fig. 1c), 75 base pair-paired-end sequencing was performed.

Data processing and analysis. Raw sequencing reads were aligned against human genome version hg19/GRCh37 using BSMAP. Custom Perl scripts were used to remove read pairs that aligned to different chromosomes. Paired-end read alignments rates varied between 71–94%, mean = 86% (Supplementary Dataset 1). Methylation calls were made by comparing the sequenced reads to the reference genome using custom Perl scripts. Unless otherwise indicated, only CpGs with a minimum coverage of ≥5x were used for the downstream analysis.

For comparison between nascent and bulk methylation levels, methylation ratios were generated by dividing the mean level of methylation for all nascent CpGs by the mean level of methylation for matched bulk CpGs. For non-CpGs, cytosine methylation levels were generated using BSMAP. To visualize read enrichment, IGV was used, and maximum coverage values were set to 20 for all tracks (scaled by 1,000,000 per total number of mapped reads). CpG density was calculated as the percentage of CpG dinucleotides per 100 bp.

To identify replication-timing domains specific to each S-phase fraction (S1–S6), we used a 10-kb sliding window to identify regions in which the average read density was 1.5x greater than the genomic average within each fraction. For the methylation analysis in Supplementary Figs. 1d and 2e, regions from S1 and S2, S3 and S4, and S5 and S6 fractions were merged and termed early, mid, and late fractions, respectively.

For genome methylation plots, LOESS smoothing and s.d. calculations were derived using the mean package in R (v1.0.1) with span set to 0.04 for the 120-kb region on chromosome 17 and 0.1 for the POU5F1 (OCT4) locus. All genomic regions displayed are euchromatic. Violin plots were created using the R package `vioplot` using standard parameters. Boxplots and heat maps in supplementary data were created using R. For boxplots, the median is shown in bold, the box displays interquartile range and whiskers extend to the most extreme data point that is no more than 1.5x the interquartile range.

For analysis of single-cell RRBS data, only CpGs that had methylation ≥0.8 in the ESC bulk sample were used to study methylation levels in single cells. This was done because RRBS captures predominantly regions with high CpG density, which are typically lowly methylated CpGs. When comparing replicates, we confirmed the absence of coverage-dependent effects by comparing methylation of matched CpGs with ≥10x coverage in both samples. For the time course heat maps, only CpGs with ≥5x coverage in the combined replicate dataset and with ≥5x coverage in the bulk dataset were included. For the genomic feature comparisons, CpGs from nascent or bulk DNA were intersected using BedTools v2.25.0 with: CpG islands, as previously defined in ref. 42, H1-specific typical or superenhancers, exons, introns, high CpG density or low CpG density promoters (ref. 41), long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) downloaded from the UCSC Genome Browser. H3K27me3, H3K4me3, H3K27ac and EZH2 annotations were downloaded from UCSC. CpG density was calculated for 100-bp tiles as the total number of CpG dinucleotides per 100 bp.

To compare methylation levels between gene bodies of expressed versus non-expressed genes, bulk RNA-seq data were used from Gifford et al.6. Genes were binned into not expressed (FPKM < 1), expressed (FPKM > 10) and highly expressed (FPKM > 100). For all three categories, CpGs within gene bodies (using the UCSC RefSeq gene annotation) of the respective genes were taken, and nascent and bulk DNA methylation levels were compared.

For individual read analyses, we first extracted individual methylation read patterns using custom scripts written in Perl and applied custom R scripts to determine the CpG methylation status of consecutive sites for each read. Only reads with a minimum of three CpGs were considered for this analysis.

To account for differential coverage among samples (for example, arrested and bulk methylation levels; Supplementary Dataset 1), we downsampled the higher coverage sample to match the number of aligned reads in the comparison set. Specifically, for the arrested to bulk comparison, 28.5% of reads within the ESC bulk DNA dataset were selected at random, and methylation ratios were called as described above.

To investigate CpG methylation within transcription factor binding sites we used our previously described TF ChIP peak sets in human ESCs and their derived germ cell layers (GSE61475): NANOG (ESC; GSM1505698), OCT4 (ESC; GSM1505724), FOXA2 (endoderm; GSM1505693), EOMES (mesendoderm; GSM1505630), GATA6 (mesoderm; GSM4150661) and PA6 (ectoderm; GSM1505715). Bulk and arrested datasets were intersected with the peak sets to obtain only CpGs located within TF binding sites. The proportion of CpGs that were equal to 0, 1 or intermediate was then calculated in bulk and arrested conditions. We repeated this analysis for only CpGs covered with at least ten reads and confirmed no coverage-dependent effects.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. Data have been deposited in the Gene Expression Omnibus (GEO) under accession GSE82045. Other published datasets used in this study include: HUES64 (GSM1112841), HUES64 DNMT3A and DNMT3B DKO (GSM1545007), TF binding data (GSE61475) and motor neuron WGBS data (GSM3039935, WGBS_hESC_WT_D14_R4 (MN day 1), and GSM3039931, WGBS_hESC_WT_D14_R4 (MN day 14)). Other data that support the findings of this study are available from the corresponding author upon reasonable request.

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# Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

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## Experimental design

1. **Sample size**
   - Describe how sample size was determined.
   - Our study did not require sample size determination and is based on sequencing measurements of cell populations.

2. **Data exclusions**
   - Describe any data exclusions.
   - No data was excluded.

3. **Replication**
   - Describe the measures taken to verify the reproducibility of the experimental findings.
   - We replicated our BrdU pulse-chase experiment in WT human ESCs three times with reproducible outcome.

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - There was no randomization required in this study.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - Blinding was not required.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. **Statistical parameters**
   - For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).
   - n/a  | Confirmed
   - The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided
   - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - Test values indicating whether an effect is present
   - Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.
   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

IGV was used to visualize data. Tools for data analysis are described in the methods section with the appropriate reference.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

There are no restrictions, material available upon request.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Two antibodies were used for the BrdU immunoprecipitation:
1. mouse anti-BrdU, BD Biosciences Pharmingen, cat. no. 555627
2. rabbit anti-mouse IgG, Sigma, cat. no. M-7023

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

HUES64 (Harvard University)
HCT116 (Stephen Baylin)

b. Describe the method of cell line authentication used.

Trusted sources of the cell lines were used, all data matched the expectations for the respective cell lines. No additional authentication was performed.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines tested negative for mycoplasma.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

None are listed.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

No animals were used.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not involve human research participants.