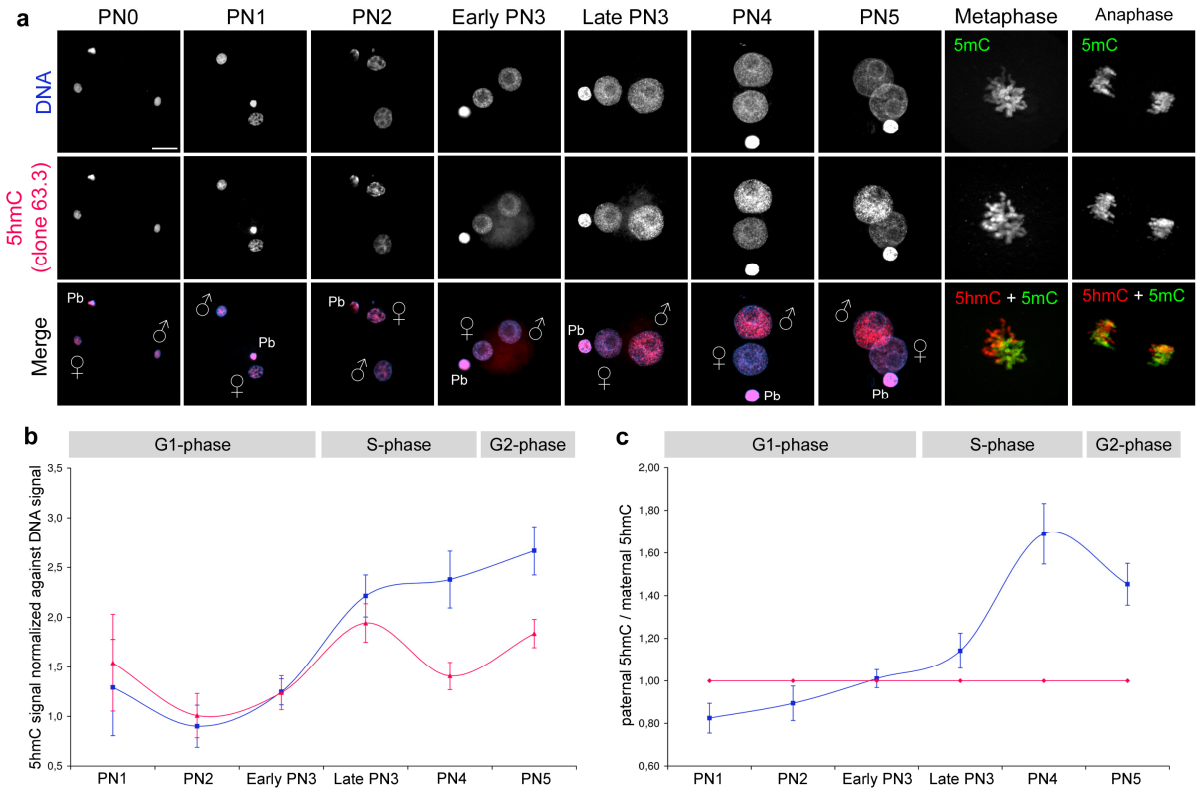
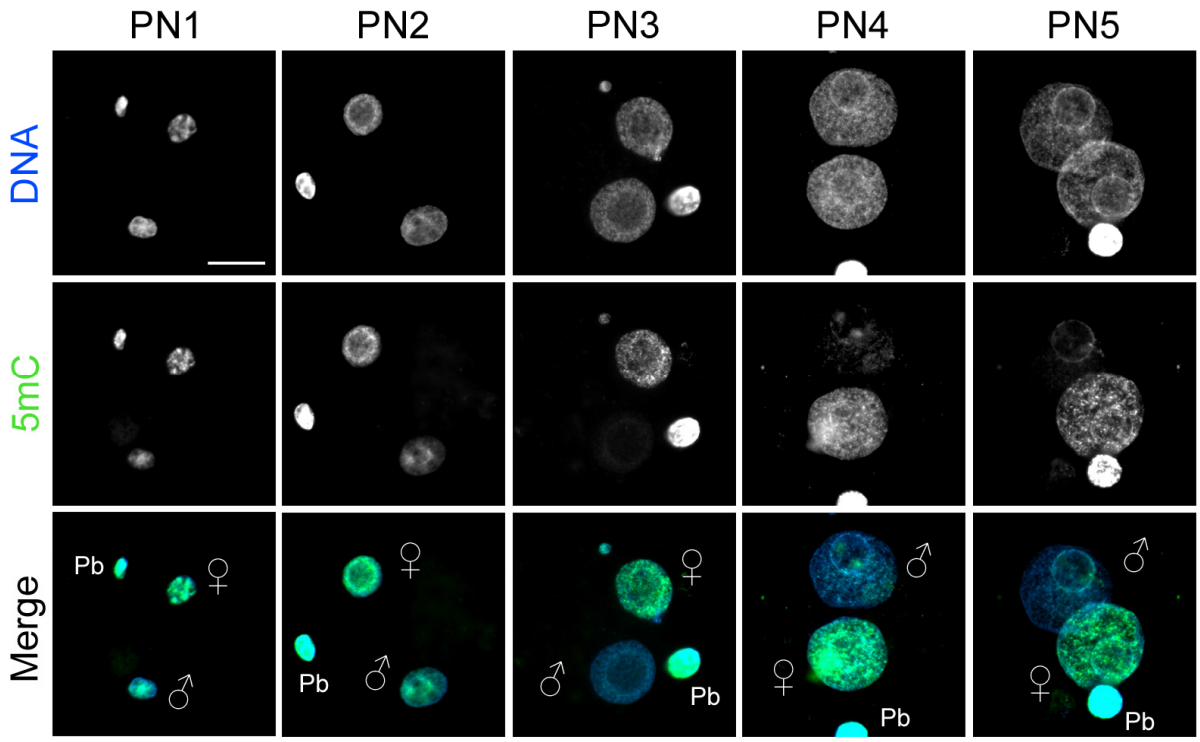


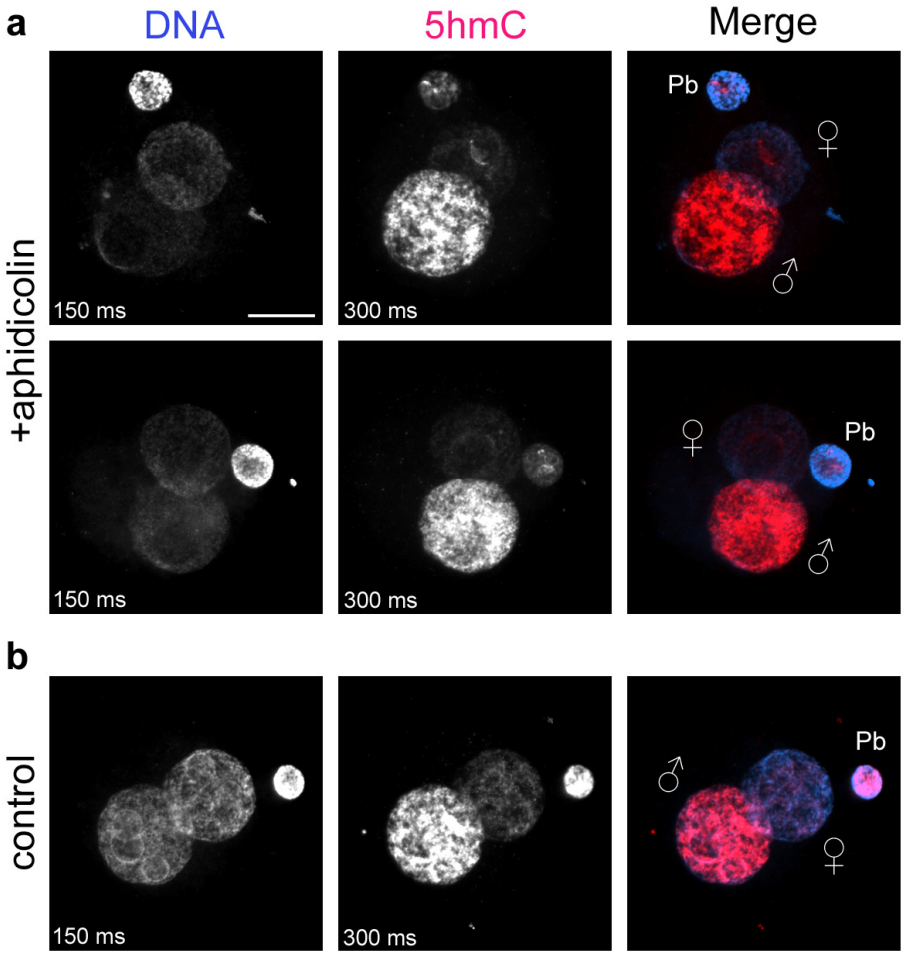
Supplementary Figure S1

5hmC (clone 63.3) immunostaining on mouse zygotes. a, Representative images of indirect immunostainings using antibodies against 5hmC (clone 63.3, red) and DNA (blue)/5mC (green) on IVF derived zygotes at distinct pronuclear stages (PN0 to PN5, Metaphase and Anaphase). **b**, Quantification of 5hmC signal (clone 63.3) normalized against DNA antibody signal. 3 to 9 precisely staged embryos per pronuclear stage from 2 to 3 IVF experiments were analyzed. **c**, Comparison of paternal and maternal 5hmC signal (maternal signal set to 1) normalized against DNA antibody signal. Error bars represent standard deviations. Blue line = paternal, red line = maternal signal, Scale bar = 20 µm, ♂=male and ♀=female pronucleus, Pb = polar body.



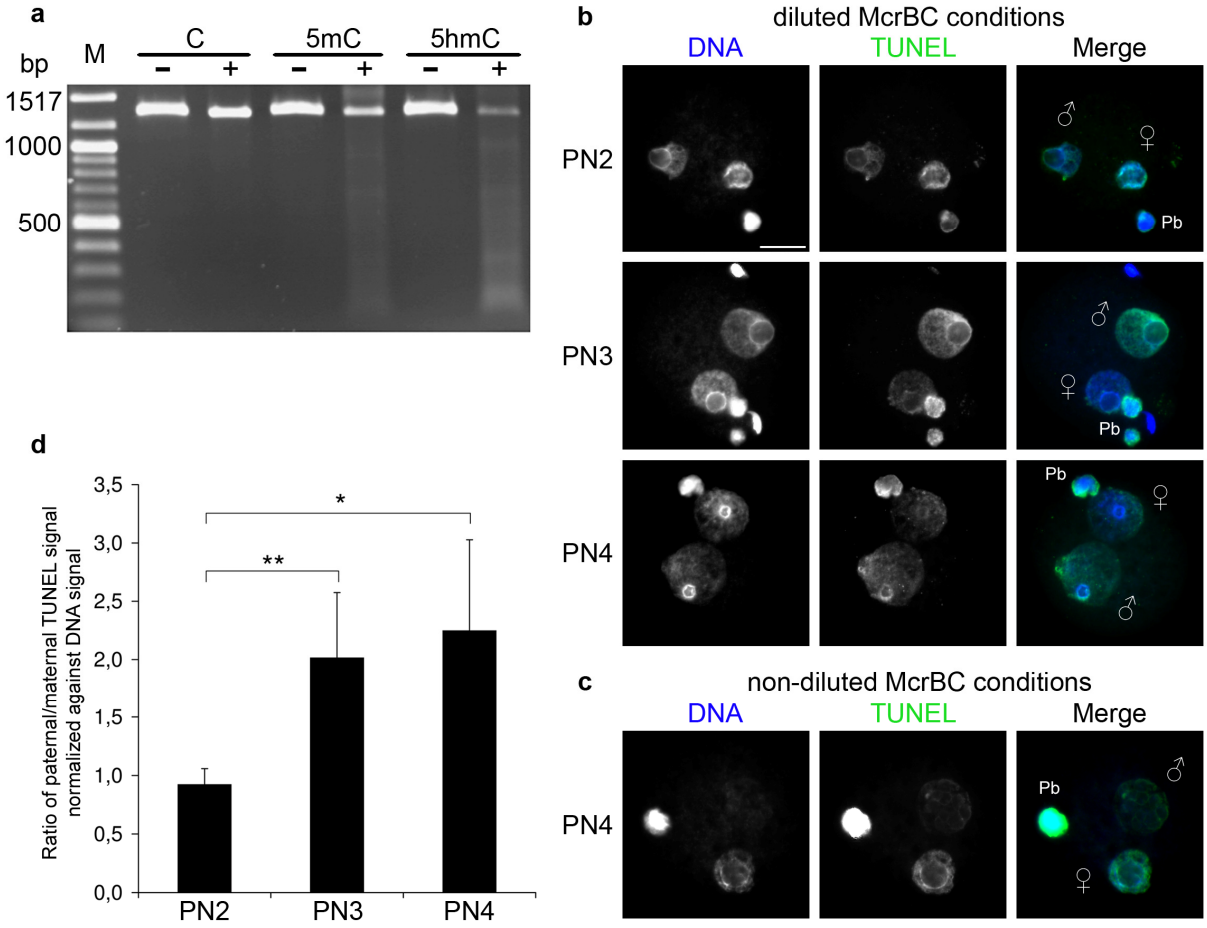
Supplementary Figure S2

Dynamic loss of 5mC signal in the paternal pronucleus during zygotic development. Representative images of 5mC (green)/DNA (blue) immunostainings at distinct pronuclear stages. Scale bar = 20 µm, ♂=male and ♀=female pronucleus, Pb = polar body.



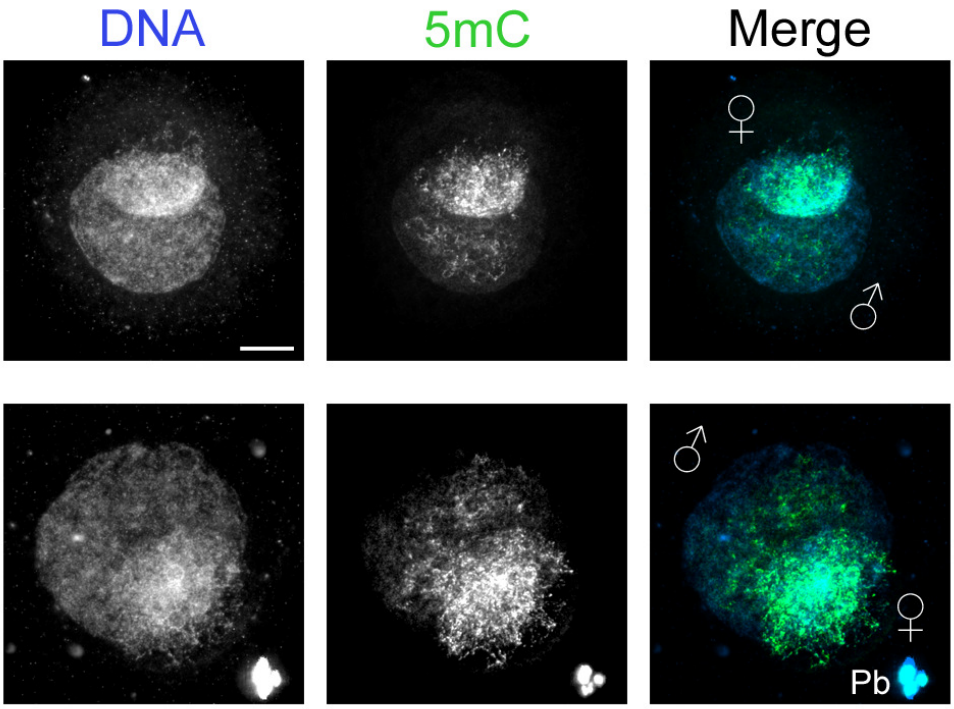
Supplementary Figure S3

Appearance of 5hmC is independent of DNA replication. a, IVF derived zygotes were treated with aphidicolin from late G1-phase onwards (over S-phase into G2-phase), fixed at PN5 stage and analysed for 5hmC (red) by immunostaining. Paternal and maternal pronuclei still show increased levels of 5hmC signal similar to the untreated control (**b**). Note the decreased DNA signal in the aphidicolin treated group compared to the control group due to the inhibited S-phase by blocked DNA polymerases. N>20, Scale bar = 20 µm, ♂=male and ♀=female pronucleus, Pb = polar body.



Supplementary Figure S4

McrBC preferentially cuts the paternal genome at late zygotic stages. a, The restriction endonuclease McrBC preferentially cuts DNA containing 5hmC. M = DNA molecular weight marker 100 bp DNA ladder, C = control (1305 bp PCR product containing unmodified nucleotides), 5mC and 5hmC = PCR products with cytosines substituted by either 5mC or 5hmC respectively. (-) no McrBC, (+) PCR products treated with McrBC. **b**, Representative images of the modified HELMET assay using McrBC on chromatin decondensed zygotes at distinct PN stages and detection of DNA strand breaks by TUNEL signal (green). McrBC under diluted conditions preferentially digests the paternal genome at PN3 and PN4 stages resulting in higher TUNEL signal in paternal pronuclei. **c**, McrBC in higher concentrations nearly completely digests DNA preferentially in paternal pronuclei at PN4 stages as it is shown by the decrease of DNA signal (blue). **d**, Quantification of HELMET assay images acquired after treatment with diluted McrBC. TUNEL integral signal densities were normalized against DNA signals. Shown are the ratios of paternal versus maternal pronuclei TUNEL signals of PN2, PN3 and PN4 stage zygotes (4 to 5 zygotes per pronuclear stage). Error bars represent standard deviations. Scale bar = 20 µm, ♂=male and ♀=female pronucleus, Pb = polar body.



Supplementary Figure S5

Decrease of 5mC signal in paternal pronuclei of rabbit zygotes. Representative Images of 5mC (green)/DNA (blue) immunostaining on rabbit G2-phase zygotes. N=15, Scale bar = 20 µm, ♂=male and ♀=female pronucleus, Pb = polar body.

Supplementary Table

Supplementary Table S1

siRNA sequences

Gene name	Sense sequence (5´-3´)
Tet1	AAGAAGCAGTGTACACATAAT
Tet1	GCAGATGGCCGTGACACAAAT according to[14]
Tet2	AAGGCCTGTGATGCTGATAAT
Tet2	GGATGTAAGTTTGCCAGAAGC according to[14]
Tet3	AAGCGCAACCTATTCTTGGAA
Scrambled (negative control)	GCCTGAGCGATTATAACCTTT

Supplementary Methods

Activity test of McrBC restriction enzyme on substrate DNA containing either unmodified cytosines, 5mC or 5hmC (Supplementary Figure S4a). 250 ng of 1305 bp PCR product (50% GC-content), encompassing arabinose inducible promoter amplified from pBAD plasmid vector (Invitrogen), were treated with 5 units of McrBC (New England Biolabs) for 2 h at recommended conditions. The deoxynucleotide-triphosphate mixtures used contained dCTP to amplify control DNA substrate; 5mdCTP (Fermentas) or 5hmdCTP (Bioline) were used to amplify DNA substrates containing 5mC or 5hmC, respectively.

Modified HELMET assay (Supplementary Figure S4b-d). The HELMET assay protocol according to Koji *et al.*[34] was modified as follows: IVF derived zygotes were partially permeabilised in PBS containing 0.2% Triton X100 for 30 sec, then incubated in M2 medium supplemented with 0.5 mM spermin, 4 mM spermidine and 1.5mg/ml heparin for 20 min at 37°C in CO2 incubator. After removing the *zona pellucida* zygotes were fixed in 3.7% PFA for 25 min at RT, washed in permeabilisation solution and equilibrated in McrBC buffer for 5 min. The McrBC treatment was carried out in 10 µl of mineral oil covered reaction mix containing either 5 (non-diluted) or 0.25 (1:20 diluted) units of McrBC (New England Biolabs) for 2 h at 37°C. After McrBC treatment the zygotes were washed in TUNEL wash buffer (permeabilisation buffer containing 5 mM EDTA and 0.5% BSA) and equilibrated in TUNEL Label Mix (Roche) for 5 min at RT. TUNEL labelling was carried out in 10 µl of mineral oil covered TUNEL Label / TUNEL Enzyme reaction mix (Roche) for 1 h at 37°C. After two washes in TUNEL wash buffer the zygotes were mounted in DAPI containing mounting solution and analysed under fluorescent microscope.

Supplementary Reference

34. Koji, T., Kondo, S., Hishikawa, Y., An, S. & Sato, Y. In situ detection of methylated DNA by histo endonuclease-linked detection of methylated DNA sites: a new principle of analysis of DNA methylation. *Histochem Cell Biol* **130**, 917-925 (2008).