

LETTERS

A role for the elongator complex in zygotic paternal genome demethylation

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The life cycle of mammals begins when a sperm enters an egg. Immediately after fertilization, both the maternal and paternal genomes undergo dramatic reprogramming to prepare for the transition from germ cell to somatic cell transcription programs¹. One of the molecular events that takes place during this transition is the demethylation of the paternal genome^{2,3}. Despite extensive efforts, the factors responsible for paternal DNA demethylation have not been identified⁴. To search for such factors, we developed a live cell imaging system that allows us to monitor the paternal DNA methylation state in zygotes. Through short-interfering-RNA-mediated knockdown in mouse zygotes, we identified Elp3 (also called KAT9), a component of the elongator complex⁵, to be important for paternal DNA demethylation. We demonstrate that knockdown of Elp3 impairs paternal DNA demethylation as indicated by reporter binding, immunostaining and bisulphite sequencing. Similar results were also obtained when other elongator components, Elp1 and Elp4, were knocked down. Importantly, injection of messenger RNA encoding the Elp3 radical SAM domain mutant, but not the HAT domain mutant, into MII oocytes before fertilization also impaired paternal DNA demethylation, indicating that the SAM radical domain is involved in the demethylation process. Our study not only establishes a critical role for the elongator complex in zygotic paternal genome demethylation, but also indicates that the demethylation process may be mediated through a reaction that requires an intact radical SAM domain.

Global removal of the methyl group from 5-methyl-CpG (5mC) of DNA has been observed in at least two stages of embryogenesis. One occurs in zygotes when the paternal genome is preferentially demethylated^{2,3}; however, imprinted genes are resistant to this wave of DNA demethylation⁶. Instead, this group of genes is actively demethylated in primordial germ cells from embryonic day (E)10.5 to E12.5, which results in the establishment of gender-specific methylation patterns⁷. Given the importance of active DNA demethylation in embryogenesis, reprogramming, cloning and stem cell biology, the identification of the putative demethylase has been one of the major focuses in the field⁴.

The first molecule for which a claim was made for DNA demethylase activity was the methyl-CpG binding protein Mbd2 (ref. 8). However, Mbd2 is not required for paternal genome demethylation, as normal demethylation is still observed in Mbd2-deficient zygotes⁹. Several recent studies in plants^{10,11}, zebrafish¹² and mammalian cells¹³ have suggested that active DNA demethylation can occur through various DNA repair mechanisms. However, it is not known whether any of these proteins affect paternal genome demethylation.

Both Gadd45a and Gadd45b have been implicated in DNA demethylation in somatic cells^{13,14}, but the role of Gadd45a in DNA demethylation has been challenged by some recent studies^{15,16}. To

determine whether Gadd45 proteins are involved in paternal DNA demethylation in zygotes, we performed quantitative polymerase chain reaction with reverse transcription (RT-qPCR) and found that *Gadd45b* is the most highly expressed gene among the Gadd45 family members in zygotes (Supplementary Fig. 1a). Because Gadd45b has been shown to affect DNA demethylation in mature non-proliferating neurons¹⁴, we examined whether loss of Gadd45b function affects zygotic paternal DNA demethylation. Immunostaining with the 5mC antibody indicates that paternal DNA demethylation is not affected by Gadd45b knockout, indicating that Gadd45b is not required for paternal DNA demethylation (Supplementary Fig. 1b).

To facilitate the identification of factors involved in paternal DNA demethylation, we attempted to develop two molecular probes (Supplementary Fig. 2a, b). The methyl-CpG-binding domain (MBD) of Mbd1 and the CxxC domain of Mll1 have high affinity towards methyl-CpG and non-methyl-CpG, respectively^{17,18}. Expectedly, EGFP-MBD exhibited a nuclear dotted pattern, whereas CxxC-EGFP exhibited diffuse nuclear staining in wild-type mouse embryonic fibroblasts (MEFs; Supplementary Fig. 2c, d). In contrast, almost 100% of Dnmt1-null MEFs that lack CpG methylation exhibited punctate nuclear localization of CxxC-EGFP. Unexpectedly, the nuclear dotted pattern of EGFP-MBD was still maintained in ~60% of the double knockout cells (Supplementary Fig. 2c, d). This result indicates that when compared to EGFP-MBD, CxxC-EGFP is the better probe whose subcellular localization pattern can reflect the DNA methylation state. We further confirmed the utility of the CxxC-EGFP reporter by demonstrating that 5-Aza-dC-mediated DNA demethylation resulted in a clear increase in the number and intensity of GFP bright dots in NIH3T3 cells (Supplementary Fig. 2e).

We next tested whether the CxxC-EGFP probe can accurately 'report' paternal genome demethylation. Because injected plasmid DNA is transcriptionally inactive in 1-cell zygotes, we adapted an mRNA injection technique that allows for visualization of molecular events in the mammalian zygote as early as 3 h after introduction¹⁹. We generated poly(A) mRNAs for the CxxC-EGFP as well as H2B-mRFP1 (monomeric red fluorescent protein 1) by *in vitro* transcription¹⁹ (Supplementary Fig. 2b). Using the procedure outlined in Supplementary Fig. 3a, the mRNAs were co-injected into the zygotes immediately after *in vitro* fertilization. Time-lapse imaging of the injected zygotes indicates that CxxC-EGFP is visible at the pronuclear stage 2 (PN2) and accumulates throughout the PN3–4 and PN5 stages in the paternal pronucleus (Supplementary Fig. 3b). The dynamics of the paternal pronuclear CxxC-EGFP accumulation mimic paternal DNA demethylation dynamics reported previously^{2,3}. On the basis of this result, we conclude that paternal genome demethylation can be monitored by injection of CxxC-EGFP mRNA in zygotes.

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We next asked whether short interfering RNA (siRNA)-mediated depletion of candidate mRNAs in the oocytes could affect paternal DNA demethylation in zygotes. We first determined the optimal siRNA concentration and the time needed for injected siRNA to become effective using siRNA against lamin A/C. The tests established that effective knockdown required a minimum dose of 2 μ M and incubation time of 8 h before intracytoplasmic sperm injection (ICSI). On the basis of the results from these tests, we established a modified experimental procedure (Fig. 1a). To facilitate early stage pronucleus identification (PN0–2), we also replaced H2B–mRFP1 with H3.3–mRFP1 as H3.3 has been shown to be preferentially deposited in the paternal pronucleus immediately after fertilization²⁰. This modified experimental scheme allows us to monitor H3.3 deposition and DNA demethylation simultaneously with time-lapse imaging. A representative snapshot of the various pronucleus stages with the injection of a scrambled siRNA control is presented in Fig. 1b. This time-lapse imaging system coupled with siRNA knockdown allowed us to test a dozen candidate genes selected based on several criteria that include: (1) their expression in zygotes; (2) the domain/structure motifs they contain; and (3) their potential in catalysing DNA demethylation. Using these criteria, we designed siRNAs targeting a dozen candidate genes. We achieved a knockdown efficiency of at least 80% for six of the candidate genes that include *Cyp11a1*, *Smc6-like*, *Brm* (also called *Smarca2*), *Alkbh5*, *Nfu1* and *Elp3* (Supplementary Fig. 4a). We also attempted to knockdown the recently identified 5mC hydroxylase *Tet1* (ref. 21) but failed to achieve high efficiency. With the exception of *Elp3* knockdown (Fig. 1c), none of the other knockdowns altered the preferential distribution of the reporter to the paternal pronucleus (Supplementary Fig. 4b). To verify this preliminary observation, we performed immunostaining using the anti-5mC antibody. Results shown in Fig. 2a clearly demonstrate that knockdown of *Elp3* prevents paternal DNA demethylation. Furthermore, a second siRNA targeting a different region of *Elp3* also resulted in a similar effect (Fig. 2a).

Although preferential demethylation of the paternal DNA in zygotes is a general phenomenon, the extent of demethylation of individual zygotes is variable (Supplementary Fig. 5). Therefore, we quantified the effect of *Elp3* knockdown on paternal DNA demethylation by analysing a large number of zygotes. After determination of the highest 5mC intensity of the Z-section for both male and female pronucleus

(Supplementary Fig. 6a), a ratio of paternal over maternal 5mC intensity was determined for each zygote (Supplementary Fig. 6b). Analysis of 80 PN4–5 stage zygotes with control injection results in an average ratio of 0.501. However, this ratio is significantly increased (average 0.742, $P = 8.14 \times 10^{-7}$) with injection of siRNAs that target *Elp3* (Fig. 2b). These results indicate that *Elp3* knockdown significantly impairs paternal DNA demethylation as judged by 5mC antibody staining.

To provide direct evidence that *Elp3* knockdown affects paternal DNA demethylation, we evaluated DNA methylation levels by bisulphite sequencing. Previous studies indicated that the transposable elements *Line1* and *Etn* (early retrotransposons) are actively demethylated in zygotes^{22,23}. We therefore asked whether knockdown of *Elp3* would impair their demethylation. To this end, we injected siRNAs that target *Elp3* before ICSI and isolated paternal pronuclei at the PN3–4 stages when DNA demethylation is at the beginning or is still occurring. We note that this is the latest time that we can still isolate the paternal pronuclei without co-isolating the maternal pronuclei, as the two pronuclei become too close at the PN5 stage. Despite the fact that demethylation is far from completion at the PN3–4 stage, the effect of *Elp3* knockdown on *Line1* and *Etn* demethylation is still obvious (Fig. 2c). However, the methylation state of the imprinted H19 differential methylated region (DMR) or an unmethylated control, *Epha7*, is not affected by *Elp3* knockdown (Supplementary Fig. 7), indicating that the observed effect on *Line1* and *Etn* is specific.

Elp3 is a component of the elongator complex that was initially identified based on its association with the RNA polymerase II holoenzyme involved in transcriptional elongation. Subsequent studies have revealed that the elongator complex has diverse functions that include cytoplasmic kinase signalling, exocytosis and transfer RNA modification⁵. The yeast elongator complex is composed of six subunits, *Elp1*–*Elp6*, which includes the histone acetyltransferase (HAT) *Elp3* (ref. 24). The human elongator purified from HeLa cells is also composed of six subunits although the identities of *Elp5* and *Elp6* are unknown²⁵. To determine whether knockdown of other elongator subunits in oocytes also prevents paternal DNA from demethylation, we performed knockdown on two additional elongator subunits, *Elp1* and *Elp4*. Results shown in Fig. 3 demonstrate that knockdown of these two subunits also significantly affects paternal genome demethylation. Collectively,

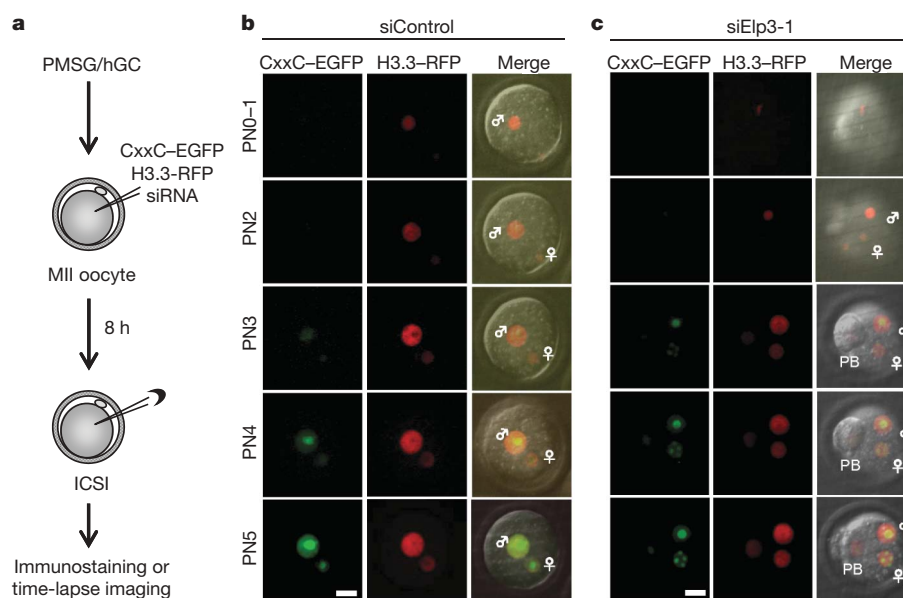


Figure 1 | Knockdown of *Elp3* prevents preferential incorporation of the CxxC–EGFP reporter into the paternal pronucleus. **a**, Scheme of the experimental procedure. **b, c**, Time-lapse imaging of CxxC–EGFP (green; left column) and H3.3–mRFP1 (red; middle column) at various pronucleus

stages of zygotic development in the absence (**b**) or presence (**c**) of siRNA that targets *Elp3*. Male and female symbols indicate male pronucleus and female pronucleus, respectively. PB, polar body. Scale bars, 25 μ m.

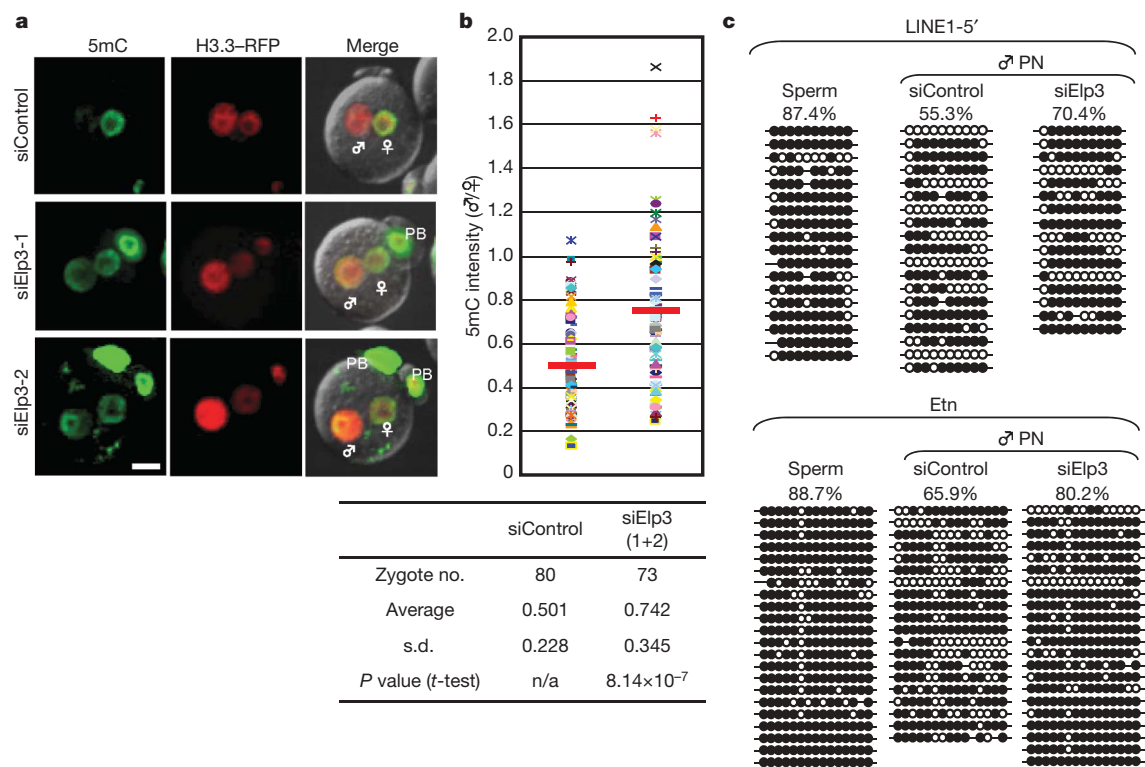


Figure 2 | Knockdown of *Elp3* impairs DNA demethylation in the paternal pronucleus. **a**, siRNA-mediated knockdown of *Elp3* resulted in increased 5mC staining (green) in the PN5 paternal pronucleus. H3.3–mRFP1 (red) serves as a nuclear marker. The H3.3–mRFP1 signal is more intense in male pronuclei than in female pronuclei owing to preferential incorporation of H3.3 to the paternal genome. Male and female symbols indicate male pronucleus and female pronucleus, respectively. PB, polar body. Scale bar, 25 μ m. **b**, Quantification of the ratio (male/female) of 5mC intensity in *Elp3*

knockdown and control groups. Each data point represents a zygote. Red bars represent the average ratio of each group. The statistics of the injections are presented in the table. **c**, Bisulphite sequencing of Line1-5' and Etn indicates that knockdown of *Elp3* impairs paternal DNA demethylation. Open circles and filled circles represent unmethylated and methylated CpG, respectively. Each line represents an individual clone. A total of 10 CpGs and 15 CpGs were analysed for Line1-5' and ETn, respectively.

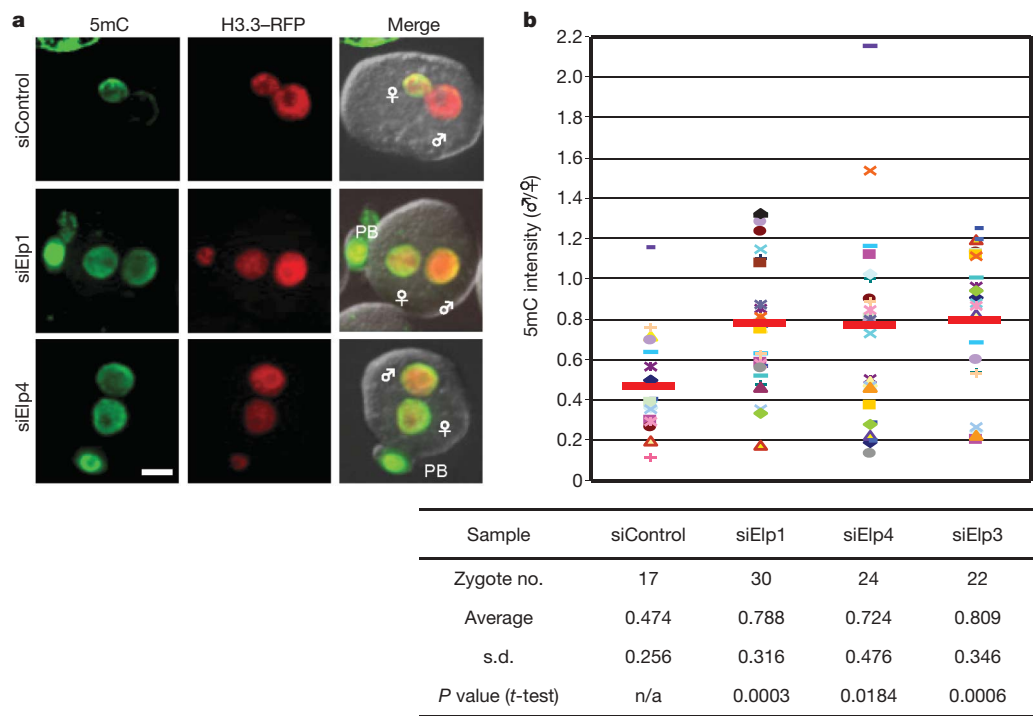


Figure 3 | Knockdown of the elongator components *Elp1* and *Elp4* also impairs paternal DNA demethylation in zygotes. **a**, siRNA-mediated knockdown of *Elp1* and *Elp4* resulted in increased 5mC staining (green) in the PN5 paternal pronucleus. H3.3–mRFP1 (red) serves as a nuclear marker. Male and female symbols indicate male pronucleus and female pronucleus, respectively. PB, polar body. Scale bar, 25 μ m. **b**, Quantification of the ratio (male/female) of 5mC intensity in *Elp1*, *Elp4* and *Elp3* knockdown and control groups. Each data point represents a zygote. Red bars represent the average ratio of each group. The statistics of the injections are presented in the table.

respectively. PB, polar body. Scale bar, 25 μ m. **b**, Quantification of the ratio (male/female) of 5mC intensity in *Elp1*, *Elp4* and *Elp3* knockdown and control groups. Each data point represents a zygote. Red bars represent the average ratio of each group. The statistics of the injections are presented in the table.

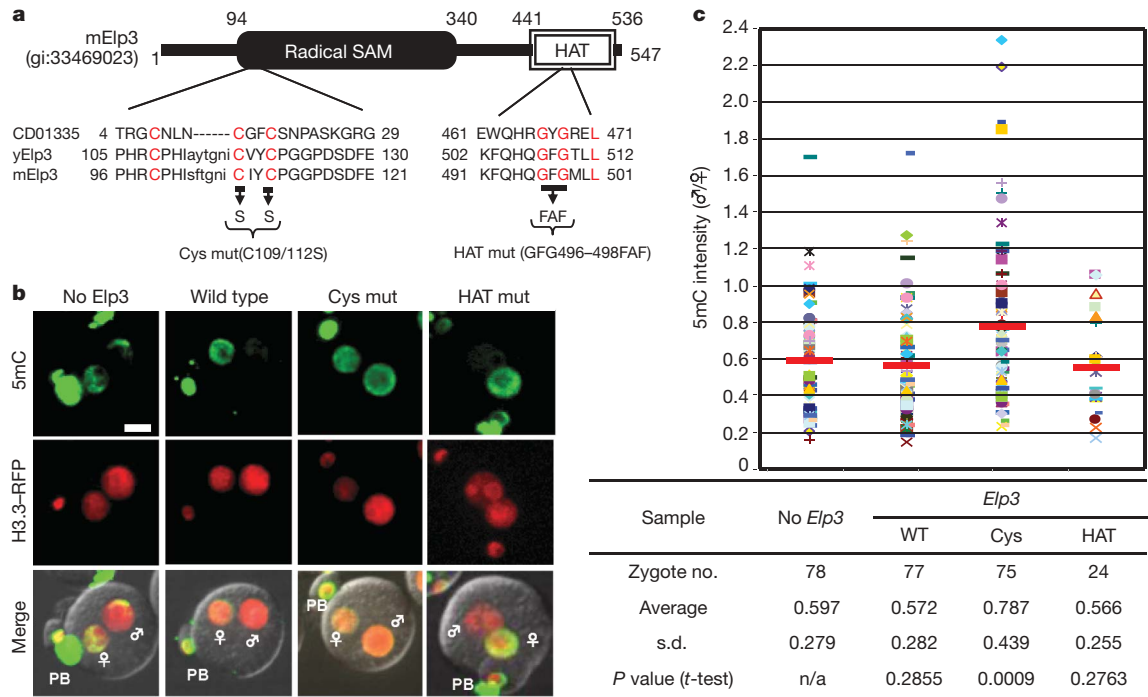


Figure 4 | Mutation of the cysteine-rich radical SAM domain of Elp3 impairs paternal DNA demethylation. **a**, Schematic representation of wild-type and mutant mElp3. The conserved domain (CD) of Elp3 protein sequences from NCBI are aligned with Elp3 sequences from budding yeast and mouse. Conserved amino acid residues are colour coded in red. Lowercase letters indicate amino acids that are not present in the *C. elegans* Elp3. **b**, Overexpression of the Cys mutant, but not the HAT mutant or wild type,

these results indicate that paternal DNA demethylation in zygotes requires the elongator complex.

In addition to a HAT domain, Elp3 also contains a domain that shares significant sequence homology with the radical SAM domain present in the radical SAM superfamily (Fig. 4a). Members of this superfamily contain an iron-sulphur (Fe-S) cluster and use S-adenosylmethionine (SAM) to catalyse a variety of radical reactions²⁶. Interestingly, a recent study confirmed the presence of this Fe₄S₄ cluster in the Elp3 protein of the bacterium *Methanocaldococcus jannaschii*²⁷. To determine whether any of the two conserved domains of the mouse Elp3 is required for paternal DNA demethylation, we used a dominant-negative approach and generated mRNAs that harbour mutations in the cysteine-rich motif and the HAT domain, respectively (Fig. 4a). Injection of the cysteine mutant mRNA, but not the wild-type or HAT mutant mRNA, significantly impaired paternal DNA demethylation (Fig. 4b, c), indicating that the cysteine-rich motif, but not the HAT domain, is important for paternal DNA demethylation.

Using a live cell imaging reporter system coupled with siRNA knock-down, we uncovered a critical function of the elongator complex in paternal DNA demethylation. Several lines of evidence support our conclusion. First, three independent assays (reporter localization, 5mC staining, bisulphite sequencing) indicate that knockdown of *Elp3* impairs paternal DNA demethylation (Figs 1 and 2). Second, knockdown of additional components of the elongator complex, *Elp1* and *Elp4*, also impaired paternal DNA demethylation (Fig. 3). Third, a dominant-negative approach identified the radical SAM domain, but not the HAT domain, of Elp3 to be critical for the demethylation to occur (Fig. 4). Consistent with the involvement of the elongator complex in zygote demethylation, mRNA levels of Elp1–4 are upregulated 3–9-fold in the PN1–2 stages before the start of paternal DNA demethylation at PN3 (Supplementary Fig. 7). Considering the fact that it takes ~3 h for injected mRNA to be expressed, the upregulation of the Elp mRNA levels at PN1–2 stages is consistent with

blocked paternal DNA demethylation. Representative images from PN5 stage are shown. Male and female symbols indicate male pronucleus and female pronucleus, respectively. PB, polar body. Scale bar, 25 μ m. **c**, Quantification of the ratio (male/female) of 5mC intensity in control and *Elp3* (wild type, Cys mutant, or HAT mutant) mRNA injected groups. Each dot represents a zygote. Red bars represent the average ratio of each group. The statistics of the injections are presented in the table.

demethylation of the paternal DNA at PN3–5 stages. Although the exact molecular mechanism by which the elongator complex participates in the demethylation process has yet to be determined, the demonstration that a specific protein complex is involved in paternal genome demethylation in zygotes has set the stage for further studies. The fact that the radical SAM domain is required for demethylation to occur points to a potential mechanism that involves the generation of a powerful oxidizing agent, 5'-deoxyadenosyl radical, from SAM. 5'-Deoxyadenosyl radical could then extract a hydrogen atom from the methyl group of 5mC to generate 5mC radical for subsequent reactions. Although this potential mechanism is attractive, currently we do not have evidence indicating that Elp3 directly acts upon DNA as a DNA demethylase. We note that reconstitution of the enzymatic activity may not be trivial, as the maternal genome in the same zygote is not subject to demethylation, indicating that certain features of the paternal genome might be required for the demethylation reaction to occur. Regardless of how the elongator complex participates in the demethylation process, our studies not only uncover a novel function for the elongator complex, but also set the stage for understanding the functional significance of paternal genome demethylation.

METHODS SUMMARY

Oocyte/zygote preparation, 5mC staining and time-lapse imaging. MII oocytes, collected from female BDF1 mice treated with pregnant mare serum gonadotrophin (PMSG; Harbour-UCLA) and human chorionic gonadotrophin (hCG; Sigma Aldrich), were cultured in M16 medium (EmbryoMax, Millipore) or Chatot–Ziomek–Bavister (CZB) medium at 37 °C with 5% CO₂ before being used in experiments. For 5mC staining, zygotes were fixed with 4% para-formaldehyde and then permeabilized with 0.4% Triton X-100 for 30 min at room temperature. Cells were then washed with PBS containing 0.05% Tween20 (PBST), treated with 4 N HCl for 30 min, and then neutralized with 0.1 M Tris-HCl (pH 8.5) for 10 min before being washed with PBST containing 0.5 M NaCl. After blocking with 1% BSA in PBST, cells were incubated with anti-5mC antibody (Eurogentec) for 0.5–1 h at 37 °C, and the signal is detected by fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (Jackson

Immunoresearch). Fluorescent images and time-lapse imaging were taken using a confocal microscope with a spinning disk (CSU-10, Yokogawa). Images were acquired as multiple 2 μm Z-axis intervals, and stacked images were reconstituted using Axiovision (Zeiss) or MetaMorph (Universal Imaging Co).

mRNA and siRNA injection, RT-qPCR and bisulphite sequencing. About 3–5 μl of siRNAs (2 μM) purchased from Ambion (Supplementary Table 1) were co-injected with H3.3-mRFP1 (25 $\mu\text{g ml}^{-1}$) and Cx3C-EGFP mRNAs (25 $\mu\text{g ml}^{-1}$). After 8 h of cultivation, cells were subjected to ICSI. To determine knockdown efficiency, RNA isolated from 10–20 zygotes at PN4–5 stage was used for RT-qPCR using the SuperScript III cDNA synthesis kit and SYBR GreenER (Invitrogen). For bisulphite sequencing, *Elp3* or control siRNA was co-injected with H3.3-mRFP1 mRNA to MII oocytes followed by ICSI. Male pronuclei were harvested at PN3–4 stages. Forty-three and forty-seven male pronuclei from control or siElp3-injected zygotes were used for bisulphite conversion using the EZ DNA Methylation-Direct Kit (Zymo Research), respectively.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions Y.Z. conceived the project. Y.Z. and Y.O. designed the experiments and prepared the manuscript. Y.O. performed the majority of the experiments. K.Y. and K.H. helped with some of the experiments. T.W. provided the instruments and reagent for Y.O.'s technical training.

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METHODS

DNA constructs. cDNA that encodes the CxxC domain (amino acids 1144–1250) of mouse Mll-1 (NCBI accession NP_005924) was cloned by RT-PCR. cDNA for H3.3 was provided by Y. Nakatani²⁸. These cDNAs were subcloned into a pcDNA3.1-poly(A)₈₃ vector with a C-terminal EGFP or mRFP1. pcDNA3.1-EGFP-MBD-poly(A)₈₃ and pcDNA3.1-H2B-mRFP1-poly(A)₈₃ were previously described¹⁹. These plasmids were used for *in vitro* transcription using the RiboMAX Large Scale RNA production System T7 (Promega). Synthesized mRNAs were purified with Illustra MicroSpin G-25 columns (GE Healthcare) before being used for injection. The mouse *Elp3* cDNA was amplified by RT-PCR and was subcloned into a pcDNA3.1-poly(A)₈₃ vector with a Flag tag at the N terminus. Both the cysteine and the HAT mutants of *Elp3* were generated by PCR-based mutagenesis and confirmed by sequencing. The primers used for generation of these mutants were as follows: Cys-F, 5'-ACAGGGAATATATCTATATACTCCCCGGAGGACCTG-3'; Cys-R, 5'-CAGGTCCTCCGGGGAGTATATAGATATATTCCTGT-3'; HAT-F, 5'-AATTTGAGCATCAGTTCCGCTTCATGCTGCTGATGG-3'; HAT-R, 5'-CCATCAGCAGCATGAAGCGGAACCTGATGCTGAAATT-3'. The underlined nucleotides are substituted in the mutants.

Mice and oocyte/zygote preparation. All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee (IACUC protocol 07-006) and the Animal Experiment Handbook at the Kobe Center for Developmental Biology (RIKEN). Four-to-twelve-week-old BDF1 mice (C57BL6 × DBA2, Charles River or Japan SLC) were used for all the experiments. MII oocytes, collected from female mice treated with PMSG (Harbour-UCLA) and hCG (Sigma Aldrich), were cultured in M16 medium (EmbryoMax, Millipore) or CZB medium at 37 °C with 5% CO₂ before being used in experiments. Gadd45b-deficient zygotes were obtained by mating of Gadd45b knockout mice pairs¹⁴.

5mC staining and time-lapse imaging. Zygotes were fixed with 4% paraformaldehyde for at least 1.5 h at 4 °C. After washing with PBS, the zygotes were permeabilized with 0.4% Triton X-100 for 30 min at room temperature. Cells were then washed with PBS containing 0.05% Tween20 (PBST) and treated with 4 N HCl for 30 min at room temperature before being neutralized with 0.1 M Tris-HCl (pH 8.5) for 10 min, and then washed with PBST containing 0.5 M NaCl. In the following procedure, all solutions and buffers contain 0.5 M NaCl. After blocking with 1% BSA in PBST, cells were incubated with anti-5mC antibody (1:100 dilution, Eurogentec) for 0.5–1 h at 37 °C, and the positive signal was detected by FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch). Fluorescent images were taken using a confocal microscope with a spinning disk (CSU-10, Yokogawa). The same confocal microscope system, combined with an on-stage incubation chamber, was used for time-lapse imaging. For both live and fixed zygotes, images were acquired as multiple 2 μm Z-axis intervals, and stacked images were reconstituted using Axiovision (Zeiss)

or MetaMorph (Universal Imaging Co). The intensity of 5mC in each pronucleus was calculated by MetaMorph as shown in Supplementary Fig. 6.

mRNA and siRNA injection, RT-qPCR and bisulphite sequencing. About 3–5 pl of siRNAs (2 μM) purchased from Ambion (Supplementary Table 1) were co-injected with H3.3-mRFP1 (25 μg ml⁻¹) and CxxC-EGFP mRNAs (25 μg ml⁻¹) simultaneously. After 8 h of cultivation, cells were subjected to ICSI (Fig. 2a). For determination of knockdown efficiency, RNA isolated from 10–20 zygotes at PN4–5 stage was used for reverse transcription using SuperScript III Cell Direct cDNA synthesis kit (Invitrogen) followed by quantitative PCR (qPCR) using SYBR GreenER (Invitrogen). To determine the expression dynamics of *Elp1–4* during pronuclear stages (Supplementary Fig. 6), MII oocytes were subjected to *in vitro* fertilization. Groups of zygotes (100–120) were collected at 4 h after insemination (PN1–2), and then every 2 h (PN3, 4, 5, respectively) followed by acidic Tyrode's treatment to remove cumulus cells. The extracted RNAs were subject to reverse transcription. Results were normalized with 18S rRNA as a standard. Primer sequences for qPCR are listed in Supplementary Table 2.

For bisulphite sequencing, either *Elp3* siRNA or control siRNA was co-injected with H3.3-mRFP1 mRNA to MII oocytes followed by ICSI after 6–8 h of siRNA/mRNA injection. Male pronuclei, which were distinguished from female pronuclei based on their size, distance from polar bodies, and more intense H3.3-mRFP1 fluorescence, were harvested from zygotes of PN3–4 stages by breaking the zona and cytoplasm using Piezo drive (Prime Tech) and aspirating using a micromanipulator. Forty-three male pronuclei from control siRNA-injected zygotes and 47 male pronuclei from si*Elp3*-injected zygotes were collected and subjected to bisulphite conversion using the EZ DNA Methylation-Direct Kit (Zymo Research). Nested PCR was performed using Platinum Taq DNA polymerase (Invitrogen) or ExTaq HS (TaKaRa). The sequences of the PCR primers and PCR conditions are listed in Supplementary Table 3 (refs 29, 30).

Cell culture and transfection. Immortalized *p53* knockout and *p53/Dnmt1* double knockout MEFs were previously described³¹. The knockout MEFs, double knockout MEFs, and NIH3T3 cells were maintained in DMEM supplemented with 10% FBS. pcDNA3-EGFP-pA83 plasmids containing the MBD domain and CxxC motif were transfected using Eugene6 (Roche). NIH3T3 cells that stably express CxxC-EGFP were selected under 1 mg ml⁻¹ G418. 5-Aza-2'-deoxycytidine (Sigma Aldrich) was applied at the concentration of 5 μM for 72 h.

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