Uracil-DNA Glycosylase UNG Promotes Tet-mediated DNA Demethylation*

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In mammals, active DNA demethylation involves oxidation of 5-methylcytosine (5mC) into 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) by Tet dioxygenases and excision of these two oxidized bases by thymine DNA glycosylase (TDG). Although TDG is essential for active demethylation in embryonic stem cells and induced pluripotent stem cells, it is hardly expressed in mouse zygotes and dispensable in pronuclear DNA demethylation. To search for other factors that might contribute to demethylation in mammalian cells, we performed a functional genomics screen based on a methylated luciferase reporter assay. UNG2, one of the glycosylases known to excise uracil residues from DNA, was found to reduce DNA methylation, thus activating transcription of a methylation-silenced reporter. UNG2, one of the glycosylases known to excise uracil residues from DNA, was found to reduce DNA methylation, thus activating transcription of a methylation-silenced reporter. UNG2 could decrease 5caC from the genomic DNA and a reporter plasmid in transfected cells, like TDG. Furthermore, deficiency in Ung partially impaired DNA demethylation in mouse zygotes. Our results suggest that UNG might be involved in Tet-mediated DNA demethylation.

Cytosine methylation in DNA, one of the major epigenetic modifications, contributes to multiple processes such as transposon control, genomic imprinting, and X chromosome inactivation in mammals (1–3). Dysregulation of cytosine methylation has been implicated in a number of diseases, including developmental defects and cancer. DNA methylation correlates with specific chromatin structure and transcriptional activity (4, 5). Locus-specific DNA methylation patterns in the mammalian genome are established during development and cell differentiation and are stably maintained during cell proliferation, assuming its role in epigenetic inheritance.

Although methylation at promoters and enhancers in general represses gene transcription, demethylation appears to be essential for achieving reactivation of previously silenced genes. Mechanisms of DNA demethylation have been proposed but no demethylase has been identified convincingly (6, 7). Tet family proteins came into the limelight for their ability to catalyze the hydroxylation of methylated cytosine (5mC)3 into 5-hydroxymethylcytosine (5hmC) (8), 5-formylcytosine (5fC), and 5-carboxycytosine (5caC) (9–11). 5fC and 5caC are recognized and excised by the DNA glycosylase TDG and replaced with unmethylated cytosine via a base excision repair (BER) pathway (9, 12). Tet and TDG-mediated demethylation has been confirmed to be operative in mouse embryonic stem cells and neurons (13–16). Although TDG function is required for the restoration to unmodified cytosine from 5mC in embryonic stem cells and induced pluripotent stem cells (9, 14, 17), recent work by Guo et al. (18) showed that the zygotic demethylation process is unaffected by TDG deletion from the zygotes. This observation has suggested the existence of as yet unknown factors responsible for the demethylation process downstream of the Tet-mediated 5mC oxidation.

We sought out to search for proteins capable of antagonizing the transcriptional repression by DNA methylation in cooperation with Tet enzyme. We previously showed that in vitro methylation of a luciferase reporter plasmid confers transcriptional repression by more than 100-fold when transfected into cells but overexpression of ectopic Tet dioxygenase together with TDG alleviates the repression (17, 19). In this work, we report the identification of uracil DNA glycosylase UNG2 by taking advantage of the methylated reporter assay and present evidence that UNG2 is able to counteract DNA methylation in cooperation with Tet.

Experimental Procedures

Animals—All animal experiments were approved by the Animal Care and Use Committee of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences.

Expression and Reporter Plasmids—The catalytic domain of mouse Tet2 (amino acids 1042–1912, GenBank™ NM_001040400) was cloned into pCAG vector (a kind gift from En

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¶ The abbreviations used are: 5mC, methylated cytosine; 5hmC, 5-hydroxymethylcytosine; 5fC, 5-formylcytosine; 5caC, 5-carboxycytosine; TDG, thymine DNA glycosylase; BER, base excision repair; PN, pronuclei; BS-seq, bisulfite sequencing; MAB-seq, M.SssI-assisted bisulfite sequencing; DMR, differentially methylated region.
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Li). The inactive mutant Tet2 (HD for H1295Y,D1297A) was described (9). The coding regions of NEIL1 (human), OGG1 (human), MUTYH (human), NhH1 (mouse), Mbd4 (mouse), Tdg (mouse), and Ung2 (mouse) were cloned into a FLAG tag vector. The coding region of human SMUG1 was cloned into a Myc tag vector. The plasmids encoding human NEIL2 and MPG were purchased from Genecopoeia Co. Ltd. The human NEIL3 plasmid was described previously (20). The CMV promoter from pcDNA3.1 (Invitrogen) was subcloned into Cpg-free pCPGL-Basic plasmid (21) to generate the pCPGL-CMV-firefly luciferase reporter plasmid. The firefly luciferase coding sequence was replaced by the Renilla luciferase coding region to generate the control reporter pCPGL-CMV-Renilla luciferase plasmid. The pCPGL-CMV-firefly luciferase plasmid was methylated in vitro using M.SssI (New England Biolabs) and then purified by QIAquick Nucleotide Removal Kit (Qiagen). Complete methylation of the pCPGL-CMV-firefly luciferase plasmid was verified by restriction assay using methylation sensitive enzyme Tail (Fermentas). Oxi-5mC reporter plasmid was prepared by in vitro oxidation of the methylated firefly luciferase plasmid using human TET2 recombinant protein (22) (a kind gift from Dr. Yanhui Xu) and purified by using a QIAquick Nucleotide Removal Kit (Qiagen). The oxidation efficiency was quantified by MAB-seq as described later.

Cell Transfection and Luciferase Reporter Assay—In the dual-luciferase reporter assay, 5 ng of methylated pCPGL-CMV-firefly luciferase plasmid was co-transfected with 500 ng of Tet2 plasmid and different amounts of glycosylase plasmid using FuGENE HD (Promega) into HEK293T cells on a 12-well plate with 0.5 ng of pCPGL-CMV-Renilla plasmid as an internal control. To ensure a relative comparable protein expression level of different glycosylases, the amount of plasmid DNA used was adjusted: 100 ng for UNG2, 200 ng for TDG, OGG1, MPG, Mbd4, NTHL1, and SMUG1, 300 ng for NEIL1 and NEIL3, and 500 ng for NEIL2 and MUTYH. All luciferase readings for cell lysates were acquired following addition of LARII and Stop & Glo® reagent (Promega) according to the vendor’s instruction manual on the Envision instrument (PerkinElmer Life Science). Each experiment was repeated at least three times. Relative luciferase activity was calculated by dividing the firefly luciferase activity by the Renilla luciferase activity, which was normalized to the value from the mock transfection using the two luciferase plasmids and an empty expression vector. For HPLC analysis, 500 ng of Tet2CD plasmid and 200 ng of TDG or 100 ng of UNG2 plasmid were transfected in a 6-well plate. For BS-Seq and MAB-Seq, 1 ng of 5mC-firefly plasmid or 1 ng of 5caC-firefly plasmid was co-transfected with 1 μg of Tet2CD plasmid and 500 ng of TDG or 250 ng of UNG2 plasmid also in a 12-well plate. After transfection, cells were cultured at 37 °C for 44 h and harvested for further analysis.

Antibodies—The monoclonal FLAG antibody (Sigma, catalogue number F3165, 1:5000 dilution), polyclonal GADPH antibody (Cell Signaling, catalogue number 2118S, 1:5000 dilution), and monoclonal Myc antibody (Santa Cruz, catalogue number sc-40, 1:10000 dilution) were used for Western blot analysis. For immunofluorescence staining, the polyclonal anti-5caC antibody (1:500 dilution) was generated as described (18). The monoclonal anti-5mC (catalogue number 39649, 1:500) and polyclonal anti-5hmC (catalogue number 39791, 1:500) antibodies were purchased from Active Motif. The monoclonal anti-single-strand DNA antibody (catalogue number MAB3868, 1:50) was purchased from Millipore.

Isolation of Genomic DNA—Transfected HEK293T cells were harvested and incubated with buffer C1 (Blood & Tissue Midi Kit, Qiagen) on ice for 10 min and centrifuged at 1300 × g for 15 min to obtain cell nuclei. The nuclei were then lysed and genomic DNA was purified using Wizard genomic DNA purification kit (Promega) according to the vendor’s instruction.

Bisulfite Sequencing (BS-seq) and M.SssI-assisted Bisulfite Sequencing (MAB-seq)—For BS-seq, 50 ng of genomic DNA or 40–60 pronuclei were treated with the EZ DNA Methylation Direct Kit (Zymol Research) according to the vendor’s instruction manual. For MAB-Seq, 50 ng of genomic DNA was first methylated by M.SssI (New England Biolabs) and then used for bisulfite conversion as described above. The bisulfite-treated DNA sample was then subjected to PCR amplification. PCR primers for Nanog, Line1, Dnmt3b, and Zbtb32 promoters were described previously (18). The primers for the CMV promoter were described previously (19). PCR products were purified for restriction analysis or cloned into pMD19-T (TaKaRa) for Sanger sequencing.

HPLC Analysis of Genomic DNA—10 μg of genomic DNA was treated with RNase A and RNase T1 overnight at 37 °C to completely remove RNA and then hydrolyzed with nucleosome P1 at 37 °C for 4 h. Calf intestinal alkaline phosphatase (TaKaRa) was then added for incubation at 37 °C for at least 2 h. The samples were subjected to HPLC analysis using an Agilent 1260 HPLC instrument (Agilent Technologies) with an AQ-C18 column (5-μm particle size, 25 cm × 4.6 mm). The mobile phase was 10 mM KH2PO4 (pH 3.75) running at the rate of 0.6 ml/min, and the detectors were set at 280 nm. Standard C, 5mC, 5hmC, and 5caC 2’-deoxygenucleoside were used as references (9).

Base Excision Assay—The glycosylase activity of UNG2 toward different dsDNA substrates was analyzed with the “nicking” procedure described previously (9). The substrate DNA containing a single G/U mismatch or a chemically modified cytosine at the MspI site (CCGG) or G/5hmU (5caU) mismatch at the TaqI site (TCGA) was prepared by annealing a modified oligonucleotide (5′-AATTGGTGAACCTCTTCCX-GGAGATGGGATTA-3′, X = U, 5hmC, 5fC, or 5caC; 5′-GGTACCATCACAGTATXGAATCTCAGAAGCTT-3′, X = 5hmU or 5caU) end-labeled by [γ-32P]ATP with an equal molar amount of a complementary oligonucleotide.

Isolation of Pronuclei—Female mice 4 to 8 weeks old superovulated with intraperitoneal injections of pregnant mare serum gonadotropin and human chorionic gonadotropin were mated with wild-type males. Zygotes were harvested and the PN stages of individual zygotes were classified on the basis of microscopic observation of the size of two pronuclei and the distance between them. Male pronuclei were first harvested from zygotes of PN3–PN4 stages by breaking the zona using a Piezo drive (Prime Tech) and aspirating using a micromanipulator. Female pronuclei were extracted afterward. For BS-seq, 40–60 pronuclei, or sperm and oocytes were used.
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**Results**

**UNG2 and Tet2 Together Increases Expression of a Methylated Reporter Gene in a Glycosylase Activity-dependent Manner**—To identify factors that are able to counteract DNA methylation and thus might be candidates for active demethylation, we carried out a luciferase screen on an expression library using a cell-based assay. A firefly luciferase reporter plasmid that includes a CpG-containing CMV promoter in CpG-free pCpGL-basic (21) was used. The reporter plasmid contains only a bacterial replication origin, which is unable to replicate in mammalian cells. Therefore, the reactivation of the in vitro methylated reporter may depend on active demethylation. We assayed the firefly luciferase activity in HEK293T cells transfected with Tet2 and an expression construct from an arrayed library of at least 3000 mammalian genes primarily for nuclear proteins, along with the methylated reporter and a control Renilla luciferase plasmid. As a positive control, co-expression of TDG and Tet2 registered an increase of the firefly luciferase activity by about 36-fold over the control with empty expression vectors as expected (Fig. 1A). Among the screened genes that showed increased reporter activity by more than 5-fold, we found another DNA glycosylase UNG2 having a strong effect (58-fold) when co-transfected with Tet2 (Fig. 1, A and B). Assay of the 11 mammalian glycosylases indicated that TDG and UNG2 displayed strong reactivation activity. NEIL1 seemed to have a weaker reactivation activity than TDG, as reported in a previous study (25). Protein expression of NEIL2 and MUTYH was hard to detect. Therefore, we focused on characterizing the role of UNG2 in DNA demethylation. UNG2 is the nuclear form encoded by Ung and differs from the mitochondrial form Ung1 in the 41 amino acids of the N-terminal region due to alternative promoter usage and splicing (26). Dual luciferase assay showed time-dependent reactivation of the methylation-silenced reporter in cells transfected with UNG2 or TDG together with Tet2 (Fig. 1C). UNG2 overexpression alone did not show any reactivation of the reporter gene.

Reactivation of methylated reporters by TDG depends on its enzymatic activity in removing Tet-generated 5fC or 5caC from DNA and initiating BER to accomplish DNA demethylation (17, 19). To examine whether UNG2-mediated reactivation of the methylated reporter relies on its DNA glycosylase activity, a catalytically inactive mutant of mouse UNG2 was tested. An aspartate residue in the conserved water-activating loop motif GQDPPY of UNG2 is known to be critical for its catalytic activity (27). We thus introduced a point mutation in the loop motif, which substitutes asparagine for aspartate at position 147 (D147N). As a result, the mutation rendered UNG2 incapable of reactivating the methylated reporter (Fig. 1, D and E), indicating that the reactivation function of UNG2 depends on its glycosylase activity. Moreover, the Tet2 dioxygenase activity was also required for UNG2 to exhibit its effect as co-expression with an inactive Tet2 mutant did not result in any reactivation (Fig. 1D, bottom panel). By contrast, UNG2 in combination with Tet2 had no effect on expression of the unmethylated reporter gene (Fig. 1D, upper panel). These data suggest that UNG2 might be involved in oxidative demethylation initiated by Tet enzyme.

**UNG2 Glycosylase Promotes Removal of Tet2-generated 5caC in the Genomic DNA of HEK293T Cells**—Ectopic Tet enzymes could generate readily detectable amounts of 5fC and 5caC, in addition to 5hmC in HEK293T cells (9, 10). We next examined whether UNG2 can possibly contribute to DNA demethylation by promoting the removal of 5mC oxidation intermediates in the active DNA demethylation process. The genomic DNA from cells co-transfected with UNG2 and Tet2 was analyzed for the levels of 5caC, 5mC, and 5hmC by HPLC. Similar to TDG, ectopic expression of wild-type UNG2 significantly reduced the amount of 5caC (Fig. 2A). The protein level of transfected Tet2 was not altered by UNG2 overexpression (Fig. 2B). Drastic reduction in 5fC and 5caC levels was confirmed by triple quadrupole mass spectrometry quantification, by 34.1 and 71.2%, respectively (Fig. 2C). These data indicate that UNG2 is able to facilitate 5fC or 5caC removal from the genomic DNA.

**UNG2 Promotes Tet-based DNA Demethylation on Methylated Reporter Plasmid**—Because the methylated reporter gene showed increased transcription in cells expressing UNG2 and Tet2, we next analyzed the methylation state of the CMV promoter driving the luciferase reporter. BS-seq analyses revealed that overexpression of UNG2 and Tet2 reduced the level of bisulfite-insensitive cytosines (5mC or 5hmC) within the analyzed region by at least 11% (from 99.52 to 88.46%), at a greater degree than the difference made by co-expression of TDG with Tet2 (6.3%) (Fig. 3A).

Based on its effect on genomic 5caC in the transfected cells, UNG2 might also stimulate the removal of 5fC and 5caC on the reporter plasmid. To address this possibility, we prepared oxi-5mC (5fC and 5caC containing) reporter plasmids by performing in vitro Tet oxidation following CpG methylation with M.SssI. We determined whether oxi-5mC in the CpG sites of the CMV promoter would decrease in cells overexpressing UNG2. By using the recently developed M.Sssl-assisted bisulfite sequencing (MAB-seq) assay, which could distinguish unmodified C from 5fC and 5caC, we found that the oxi-5mC level (5fC and 5caC combined) was reduced by 48% (from 98.4 to 50.4%) when recovered from cells expressing UNG2 (Fig. 3B). As a positive control, reduction in TDG-overexpressing cells was 53.2%. Compared with the wild-type UNG2, the catalytic mutant UNG2 brought about a 19.3% reduction of 5fC/5caC due to its remaining activity. Taken together, UNG2 might contribute to DNA demethylation by promoting removal of the higher oxidation products of 5mC.

**UNG2 Possesses Weak 5caU Excision Activity from DNA in Vitro**—Although UNG2 might contribute to promote Tet-based DNA demethylation, it has not been shown to excise 5caC in vitro (9). However, deamination of C, 5hmC, and 5caC could occur spontaneously or be catalyzed by unknown deaminase in mammalian cells. The resulting potentially mutagenic mispairs of uracil (U), 5hmU, or 5caU with guanine (G) are substrates for repair by various DNA glycosylases. To detect
whether UNG2 may act as a glycosylase to excise deamination intermediates of oxidized 5mC species, such as 5hmU or 5caU, we purified the FLAG-UNG2 protein and its inactive mutant from transfected HEK293T cells and performed the glycosylase activity assay of UNG2 toward different DNA substrates. Incubation of a 32-nucleotide oligomer (32-mer) G/U mismatch substrate with purified protein resulted in a 17-mer cleavage product, resulting from the removal of the U base and thus an abasic site that was broken by hot alkaline treatment (Fig. 4).

Besides a strong activity on G/U mismatch DNA, UNG2 also showed a weak glycosylase activity on the G/5caU mismatch pair. By contrast, the excision activity could be hardly detected for the UNG2 D147N mutant protein. These results indicate that UNG2 may function as a glycosylase to excise 5caU, which is the deamination intermediate of 5caC, thus contributing to DNA demethylation.

UNG Deficiency Partially Impairs DNA Demethylation in Mouse Zygotes—Because TDG is dispensable for zygotic DNA demethylation (18), alternative factors operating downstream of Tet3 need to be identified. Because UNG is highly expressed in the mouse early embryo (28), we examined whether UNG might be involved in zygotic demethylation. Ung

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**FIGURE 1.** UNG2 glycosylase reactivates methylation-silenced reporter in transfected cells. A, luciferase activity assay to test mammalian DNA glycosylases in the reactivation of an in vitro methylated reporter. The methylated firefly luciferase reporter and unmethylated Renilla luciferase reporter were co-transfected into HEK293T cells alone (mock) or with the expression constructs for Tet2 and the indicated genes. The activity of co-transfected Renilla luciferase was used as internal control for normalization. The luciferase activity is normalized relative to that of the mock sample, which is set to 1. Data represents the mean ± S.E. of three independent experiments. B, Western blot showing the expression of Tet2CD (upper panel) and the 11 epitope-tagged glycosylases (middle panel) in transfected cells. The GAPDH level served as a loading control (bottom panel). C, time-dependent reactivation of the in vitro methylated reporter in cells co-transfected with UNG2 and Tet2. Luciferase activity was measured 24, 36, and 48 h after transfection, respectively. Mock denotes transfection of the luciferase reporter plasmid only. Data represents the mean ± S.E. of three independent experiments. D, requirement of the glycosylase activity for UNG2 to reactivate the methylated reporter expression. An unmethylated (upper panel) or methylated (bottom panel) form of the pCpGL-CMV-firefly luciferase plasmid was transfected into HEK293T cells together with Tet2, TDG, and UNG2 or their catalytic inactive mutant as indicated. Data represents the mean ± S.E. of three independent experiments. E, Western blot confirming the expression of wild-type or mutant Tet2CD, TDG as well as wild-type or mutant UNG2 in transfected cells.
mice (23) were used to isolate male and female pronuclear DNA from developing zygotes at PN3–4 stage. Methylation analysis of the paternally imprinted gene H19 and the maternally imprinted gene Peg1 validated the isolation and methylation analysis of male and female pronuclear DNA (Fig. 5A). We and others have previously shown Tet3-dependent active removal of methylation at specific sequences. Line1 transposon elements and the embryonic stem cell marker gene Nanog, which are highly methylated in sperm undergo demethylation in male pronuclei (24, 29), whereas Dnmt3b and Zbtb32 hypermethylated in oocytes lose methylation in female pronuclei (18).

Compared with the wild-type zygotes, both Nanog and Line1 retained higher levels of paternal methylation in UNG-deficient zygotes, suggesting that demethylation at these two loci was partially impaired by Ung deletion (Fig. 5B). Consistently, Ung deletion also partially suppressed demethylation at the two maternally hypermethylated genes, Dnmt3b and Zbtb32, as they retained higher methylation levels in UNG-null zygotes. However, the global 5mC, 5hmC, and 5caC signal intensity in the pronuclei detected by immunostaining did not appear to be significantly different in UNG-deficient zygotes at PN4–5 (Fig. 5C). These observations indicate that UNG is required for zygotic DNA demethylation at specific genomic loci.

**Discussion**

In this study, we present several lines of evidence for a potential function of UNG glycosylase in Tet-mediated DNA demethylation. First, UNG2 synergizes with Tet to activate the methylated reporter gene in transfected HEK293T cells (Fig. 1). Second, UNG2 reduced DNA methylation of the reporter plasmid when co-transfected with Tet2 into HEK293T cells (Fig. 3A). Third, UNG2 could decrease 5fC/5caC from the genomic DNA and a reporter plasmid in transfected HEK293T cells (Figs. 2 and 3B). Last, Ung-deficient mouse zygotes show impaired demethylation at specific genomic loci.

UNG is commonly known as a member of uracil-DNA glycosylase (UDG) family to remove uracil from DNA (30). The function of UNG2 in removing misincorporated uracils at replication and those generated by cytosine deamination in antibody diversification has been well established (23, 31, 32). However, UNG in the zygotes has also been implicated in activation-induced deaminase-initiated DNA demethylation, which supposedly involves 5mC deamination but is independent of Tet-mediated 5mC oxidation (33). In addition, knockdown of ung in zebrafish showed impaired post-fertilization DNA demethylation, repressed transcriptional activity, and embryonic lethality (34). These previous reports are consistent with our observation that UNG2 is capable of de-repressing a methylated reporter in an expression library screen. However, the evidence in zebrafish for the role of UNG in DNA demethylation is relatively weak due to limitation of the anti-5mC immunofluorescence method, which is not quantitative and is only indicative of the global DNA methylation. Using our reporter system, we showed the increased luciferase activity upon UNG2 overexpression is correlated with the decreased methylation level of the CMV promoter, which drives the luciferase reporter. Moreover, Wu et al. (34) did not present any evidence on the mechanism of UNG in DNA demethylation. Although Santos et al. (33) proposed a role of UNG2 in excising G/U mismatch and initiating long-patch BER after activation-induced deaminase-mediated cytosine deamination, our work...
has suggested that UNG2 may contribute to DNA demethylation by promoting the removal of 5mC oxidation products 5fC and 5caC (Figs. 2 and 3).

Currently, the mechanism of UNG in reducing 5fC and 5caC is not understood. DNA repair through BER has been proposed as a core component of genome-wide DNA demethylation (35, 36). The BER pathway is initiated by one of at least 11 distinct DNA glycosylases, depending on the type of lesion (30). Like TDG, UNG2 is found to be able to strongly reactivate methylated reporter with Tet2. Because UNG2, unlike TDG, shows no glycosylase activity on 5caC in vitro (9), a few plausible explanations for the ability of UNG2 in reducing 5fC and 5caC can be considered. First, UNG2 may require some unknown partners or cofactors to exert its glycosylase activity in vivo. Second, UNG2 may have stimulated the endogenous TDG glycosylase activity in HEK293T cells. However, this is less likely as the catalytic activity is indispensable for UNG2 to reactivate a methylated reporter (Fig. 1). Alternatively, UNG2 may act as a glycosylase to excise deamination products of oxidized 5mC species, such as 5caU (Fig. 4), thus contributing to demethylation. But this pathway based on sequential oxidation and deamination of 5mC lacks support as no deaminase has been identified to generate these intermediates from the corresponding 5mC oxidative derivatives (37). In addition, overexpression of UNG2 in HEK293T cells may indirectly regulate some factors to induce 5fC/5caC removal. Finally, the possibility for UNG2 to contribute to 5caC decarboxylation cannot be ruled out fully. Notably, the UNG2 protein expressed in

![FIGURE 3. UNG2 promotes DNA demethylation through DNA oxidation on the co-transfected reporter plasmid. A, cytosine modification analysis of the initially in vitro methylated reporter by Combined Bisulfite Restriction Analysis (COBRA) and BS-seq. The data are showing a CMV promoter region of the reporter plasmid, which was in vitro methylated and transfected into HEK293T cells with genes indicated at the left. In COBRA, the relative intensity of the undigested band reflects the amount of converted cytosines (C, 5fC, or 5caC) analyzed at the Tail restriction site. In the BS-seq profiles, percentages of the bisulfite-resistant sites (5mC or 5hmC) are given. Downward arrows indicate the CpG sites that overlap the Tail restriction sites used in COBRA. B, cytosine modification analysis of the initially in vitro methylated and oxidized reporter by COBRA of M.SssI-incubated oxi-plasmids and MAB-seq. The oxi-reporter plasmid was prepared by in vitro M.SssI methylation and Tet oxidation to form 5fC or 5caC at the CpG sites and then transfected into HEK293T cells alone (mock) or with the indicated genes. Recovered reporter DNA was assayed for the same region as described in panel A. CpG sites retaining 5fC and 5caC in the recovered DNA are resistant to M.SssI methylation in vitro but sensitive to subsequent bisulfite treatment are thus indigestible in COBRA and read as C (orange fill) in MAB-seq, whereas all other forms (5mC, 5hmC, and C) are resistant to bisulfite treatment upon M.SssI methylation and are thus cleavable in COBRA and read as 5mC (blue fill) in MAB-seq. The percentages of 5fC and 5caC combined (orange sites) are shown. Arrows indicate CpGs overlapping the Tail restriction sites used in COBRA.](image)

![FIGURE 4. UNG2 possesses weak 5caU-DNA excision activity in vitro. FLAG-UNG2 and its inactive mutant proteins were purified from transfected HEK293T cells and their glycosylase activity was assayed on different DNA substrates as indicated. 10 nm DNA substrate was incubated at 30 °C with 4 nM UNG2 protein in a total volume of 10 μl of reaction for 30 min.](image)
HEK293T cells appears to contain post-translational modifications such as phosphorylation (38), which could modulate its functions. In addition, impairment in zygotic demethylation caused by Ung deletion is less severe compared with Tet3 deletion (Fig. 5B) (18, 24), suggesting the existence of redundant factors. Future work is needed to determine the exact role of UNG in DNA demethylation and its relative contribution in the methylation dynamics in mouse development.

**Author Contributions**—J. H. X., G. L. X., and Y. R. D. designed the experiment, J. H. X., G. F. X., T. P. G., G. D. C., B. B. H., and Y. R. D. performed and analyzed the experiments. M. B. provided the NEIL3 expression plasmids. H. E. K. provided the Ung-deficient mice. Z. M. X. contributed to mouse breeding. G. L. X. and Y. R. D. wrote the paper. M. B. and H. E. K. revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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