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 gene when co-transfected with Tet2 into HEK293T cells. Interestingly, 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) 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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Li). The inactive mutant Tet2 (HD for H1295Y,D1297A) was described (9). The coding regions of NEIL1 (human), OGG1 (human), MUTYH (human), Nth11 (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 Genecopeia 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 glycosylase, 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. For HPLC analysis, 500 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, Dmnt3b, 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 a rate of 0.6 ml/min, and the detectors were set at 280 nm. Standard C, 5mC, 5hmC, and 5caC 2′-dexoynucleoside 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′-AATTGGTGAACCTTCTTGCAGAGATGGGATTA-3′, X = U, 5hmC, 5fC, or 5caC; 5′-GGTACCATACAGATXGAGTCAGAAGCTT-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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Reactivation of methylated reporters by TDG depends on its DNA glycosylase activity. Moreover, the Tet2 dioxygenase activity might 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 level of 5caC. Unmethylated C from 5fC and 5caC, we found that the oxidation of 5mC was decreased by 48% (from 98.3 to 50.4%) when recovered from cells expressing UNG2 (Fig. 3A). 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 methylstate 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 oxidized 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.SssI-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 catalytically inactive 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 knock-out
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 \textit{H19} and the maternally imprinted gene \textit{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. \textit{Line1} transposon elements and the embryonic stem cell marker gene \textit{Nanog}, which are highly methylated in sperm undergo demethylation in male pronuclei (24, 29), whereas \textit{Dnmt3b} and \textit{Zbtb32} hypermethylated in oocytes lose methylation in female pronuclei (18). Compared with the wild-type zygotes, both \textit{Nanog} and \textit{Line1} retained higher levels of paternal methylation in UNG-deficient zygotes, suggesting that demethylation at these two loci was partially impaired by \textit{Ung} deletion (Fig. 5B). Consistently, \textit{Ung} deletion also partially suppressed demethylation at the two maternally hypermethylated genes, \textit{Dnmt3b} and \textit{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, \textit{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 \textit{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 relative 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 3B).

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. 1C). 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 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.

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.
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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References


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