TET2 promotes histone O-GlcNAcylation during gene transcription

Qiang Chen1, Yibin Chen1, Chunjing Bian1, Ryoji Fujiki2,3 & Xiaochun Yu1

Ten eleven translocation (TET) enzymes, including TET1, TET2 and TET3, convert 5-methylcytosine to 5-hydroxymethylcytosine and regulate gene transcription1–4. However, the molecular mechanism by which TET family enzymes regulate gene transcription remains elusive5–6. Using protein affinity purification, here we search for functional partners of TET proteins, and find that TET2 and TET3 associate with O-linked β-N-acetylglucosamine (O-GlcNAc) transferase (OGT), an enzyme that by itself catalyses the addition of O-GlcNAc onto serine and threonine residues (O-GlcNAcylation) in vitro5,6. TET2 directly interacts with OGT, which is important for the chromatin association of OGT in vivo. Although this specific interaction does not regulate the enzymatic activity of TET2, it facilitates OGT-dependent histone O-GlcNAcylation. Moreover, OGT associates with TET2 at transcription start sites. Downregulation of TET2 reduces the amount of histone 2B Ser112 GlcNAc marks in vivo, which are associated with gene transcription regulation. Taken together, these results reveal a TET2-dependent O-GlcNAcylation of chromatin. The double epigenetic modifications on both DNA and histones by TET2 and OGT cooperate to regulate gene transcription.

Using protein affinity purification, we sought functional partners to the TET family enzymes. Mass spectrometry analysis showed that both TET2 and TET3 associate with OGT, an enzyme that catalyses O-GlcNAcylation7,8 (Fig. 1a, Supplementary Table 1 and Supplementary Fig. 1a, b). To confirm this interaction, we performed a coimmunoprecipitation assay with overexpressed TET1, TET2, TET3 and OGT. TET2 and TET3, but not TET1, associated with OGT (Fig. 1b and Supplementary Fig. 2a). To study the endogenous interaction, we used mouse embryonic stem (ES) cells in which TET2, but not TET3, was expressed1,6,9. Again, TET2 associated with OGT endogenously in ES cells (Fig. 1c and Supplementary Fig. 2d). To map the regions of interaction between TET2 and OGT, we generated a series of TET2-deletion mutants, and found that the carboxy-terminal catalytic double-strand β-helix (DSBH) domain of TET2 interacts with OGT (Fig. 1d and Supplementary Fig. 2b). The tertiary structure of OGT has been examined10–12. There are two major functional domains in OGT. One is the amino-terminal regulatory domain that is composed of 13.5 tetratricopeptide repeats (TPR); the other is the C-terminal catalytic domain that transfers GlcNAc from UDP-GlcNAc to Ser/Thr residues of the substrate. Using the internal-deletion mutants, we found that TPR5 and TPR6 of OGT are required for the interaction with TET2 (Fig. 1e and Supplementary Fig. 3c, e, f). We also generated baculoviruses encoding either TET catalytic domain that (TET2CD) or OGT. After co-infecting S9 cells with these two types of baculovirus, the recombinant proteins of streptavidin-binding peptide (SBP)-tagged TET2CD and glutathione S-transferase (GST)-tagged OGT could be co-purified at 1:1 stoichiometry from cell lysates, suggesting TET2 tightly binds OGT (Fig. 1f). Moreover, the internal-deletion mutants D4 (which lacks TPR9 and TPR10) and D5 (which lacks TPR11 and TPR12) of OGT slightly reduced the interaction with TET2, indicating that other TPR repeats may also regulate the interaction between OGT and TET2 (Fig. 1e and Supplementary Fig. 2f). In addition, recombinant TET3 catalytic domain also directly binds OGT (Supplementary Fig. 2g). Taken together, these results demonstrate that TET2 and TET3 form a complex with OGT both in vitro and in vivo.

TET enzymes were originally identified as homologues of JBP1 and JBP2 in Trypanosoma brucei; these two thymidine hydroxylases are involved in the synthesis of the modified DNA base β-N-glucosylhydroxymethyluracil (also known as base J)13. Synthesis of base J consists of two steps in which it is directly converted from thymidine bases incorporated in DNA strands. In the first step, JBP1 and JBP2 catalyse the oxidation of thymidine to hydroxymethyluracil. During the second step, a yet unknown enzyme transfers a glucose residue onto the hydroxyl group of hydroxymethyluracil to form base J13. We wondered whether OGT could catalyse the glycosylation on hydroxyl groups of 5-hydroxymethylcytosine (5hmC). To test this hypothesis, 5-hydroxymethylcytosine (5mC) was incubated with recombinant TET2 and OGT in vitro. However, OGT failed to convert 5hmC into any new base in vitro (Supplementary Fig. 3a, b). Co-expression of OGT with TET2 in cells did not alter the TET2-dependent 5hmC synthesis (Supplementary Figs 3c and 4). Furthermore, we could not detect glycosylated 5hmC in vivo using mass spectrometry (Supplementary Figs 3d, e and 5). These results suggested that OGT does not affect TET2-dependent 5hmC synthesis.

Next, we asked whether TET2 regulates the function of OGT. We fractionated ES cell lysates using different salt concentrations and pH levels. A subset of TET2 and OGT could only be eluted from the chromatin using 300 mM NaCl or 0.2 M HCl, indicating that these TET2 and OGT species are tightly associated with the chromatin (Supplementary Fig. 6a). Interestingly, knockdown of TET2 by short hairpin RNA (shRNA) in ES cells abolished the chromatin-associated OGT, suggesting that TET2 may target OGT to chromatin (Supplementary Fig. 6a). To verify this phenomenon, we used 293T cells stably expressing TET2. Because the exogenous TET2 level was much higher than the endogenous level (Supplementary Fig. 7), the chromatin-bound OGT was markedly increased in 293T cells stably expressing TET2 (Supplementary Fig. 6b). Moreover, because the D2 mutant of OGT abolished the interaction with TET2, only wild-type OGT but not the D2 mutant existed in the chromatin fraction, suggesting that the interaction with TET2 is important for the chromatin localization of OGT (Supplementary Fig. 6c). In addition, knockdown of OGT by shRNA did not significantly affect the chromatin retention of TET2 (Supplementary Fig. 6d). Collectively, these results suggest that TET2 recruits OGT to chromatin.

Recently, it has been shown that histones can be modified by OGT at different sites14–17. In particular, OGT regulates O-GlcNAcylation of histone 2B (H2B) at Ser 112 in vivo15. Because TET2 targets OGT to chromatin, we wondered whether TET2 regulates OGT-dependent H2B O-GlcNAcylation. With a specific antibody for H2B Ser 112 GlcNAc, we found that H2B Ser 112 GlcNAc was significantly reduced in TET2-depleted ES cells (Fig. 2a). Moreover, TET2 also regulates
TET2 forms a complex with OGT. A. Purification of TET2- and TET3-associated proteins. TET2- and TET3-associated proteins were analysed by mass spectrometry and are shown in the Supplementary Fig. 1a. B, OGT interacts with TET2 and TET3 but not with TET1. SBP-tagged TET1–3 proteins were expressed and examined with indicated antibodies. Whole cell lysate (WCL) was used as the input. C, OGT interacts with TET2 endogenously in ES cells. Irrelevant IgG was used as the immunoprecipitation control (ctrl); PALB2 was used as the negative control. D, The DSBH domain of TET2 interacts with OGT. Deletion mutants of TET2 mutants were expressed. The F3 mutant containing the core-DSBH domain (catalytic domain) interacts with OGT, TPR5 and TPR6 of OGT interact with TET2. The D2 mutant of OGT, which lacks TPR5 and TPR6, abolished the interaction with TET2. F, OGT directly binds TET2. S9 cells were infected with baculoviruses encoding SBP–OGT and/or GST–TET2CD. The protein complex was purified by streptavidin beads or GST beads and examined by Coomassie blue staining.

O-GlcNAcylation of other histones (Supplementary Fig. 8). Concurrent with TET2 overexpression inducing OGT association with chromatin, H2B Ser 112 O-GlcNAcylation was increased (Fig. 2b). Interestingly, the TET2 enzymatic-dead mutant (His1382Tyr/Asp1384Ala) that still interacted with OGT also increased H2B Ser 112 O-GlcNAcylation, suggesting that the enzymatic activity of TET2 is not required for the regulation of H2B O-GlcNAcylation (Fig. 2b and Supplementary Fig. 9). Moreover, only wild-type OGT but not the enzymatic-dead mutant of OGT (Gly482Ser) nor the D2 mutant lacking the interaction with TET2 could induce H2B S112 GlcNAc and anti-mouse TET2 antibodies. We validated our results using ChIP-quantitative PCR (qPCR) to examine 45 different loci that represent a broad range of ChIP-seq fragment counts (Supplementary Fig. 11). One possibility is that glycosylated H2A and H2B suppresses H3 and H4 glycosylation by OGT. Alternatively, the glycosylation sites on H3 and H4 either in the histone octamer or as mono-nucleosomes are not well exposed to the enzyme. In contrast to many other enzymes, OGT only efficiently glycosylates the substrates that it associates with. Thus, it is likely that TET2 recognizes the chromatin and recruits OGT to the chromatin, and the chromatin-associated OGT glycosylates nucleosomal histones at its vicinity. Consistently, only TET2 but not OGT recognizes double-stranded DNA or mono-nucleosome with 5mC (Supplementary Fig. 12).

To examine the distribution of OGT and TET2 on the chromatin of ES cells, we performed genome-wide chromatin immunoprecipitation (ChIP) sequencing analysis (ChIP-seq) using anti-OGT, anti-H2B Ser 112 GlcNAc and anti-mouse TET2 antibodies. We validated our results using ChIP-seq results using ChIP-quantitative PCR (qPCR) to examine 45 different loci that represent a broad range of ChIP-seq fragment counts ( Supplementary Fig. 11). Next, we compared the TET2 target genes associated with high- and intermediate-density CpG promoters (Supplementary Fig. 14b, c), which are also positive for the trimethylated form of histone H3 at lysine 4 (H3K4me3) (Supplementary Fig. 14d). Gene Ontology analysis showed that OGT, H2B Ser 112 GlcNAc and TET2 are involved in a
Figure 2 | TET2 enhances histone glycosylation. a. Downregulation of TET2 impairs H2B O-GlcNAcylation in ES cells. H2B O-GlcNAcylation was examined by immunoprecipitation with an anti-H2B antibody and western blot with an anti-GlcNAc antibody (RL2) or anti-H2B Ser 112 GlcNAc antibody. Histogram shows the relative level of H2B Ser 112 GlcNAc in TET2-downregulated cells (shTET2) compared to that in control-shRNA-treated cells (shCtrl). b. Upregulation of wild-type TET2 or TET2 enzymatic-dead mutant (His1382Tyr/Asp1384Ala) induced H2B Ser 112 O-GlcNAcylation in 293 cells.

The His1382Tyr/Asp1384Ala mutant could not restore 5hmC at TET2 target genes (Fig. 3f and Supplementary Fig. 18). In addition, the downregulation of a number of OGT, H2B Ser 112 GlcNAc and TET2 (Supplementary Fig. 16). Moreover, we examined alterations to the gene transcription profile in TET2 knockdown ES cells. The percentage of downregulated genes that were occupied by OGT, H2B Ser 112 GlcNAc and TET2 was significantly higher than that of genes not occupied by OGT, H2B Ser 112 GlcNAc and TET2 (Fig. 3a), and similar binding profiles to TET2 at transcriptional start sites (TSS) (Fig. 3b, c). Moreover, the binding sites of OGT, H2B Ser 112 GlcNAc and TET2 have the highest density around TSS (Fig. 3c). Knockdown of TET2 markedly suppressed the recruitment of OGT to tested target genes and reduced the level of H2B Ser 112 GlcNAc at those loci (Fig. 3d). By contrast, knockdown of OGT had little effect on TET2 at these loci, suggesting that OGT does not contribute to the recruitment of TET2 to these loci (Fig. 3d). Next, we asked whether there was a correlation between TET2-mediated OGT targeting and the targeted gene transcription levels. In wild-type ES cells, we found that genes that were occupied by OGT and TET2 and enriched with H2B Ser 112 GlcNAc were associated with high levels of transcription in ES cells (Fig. 3e and Supplementary Fig. 16). Moreover, we examined alterations to the gene transcription profile in TET2 knockdown ES cells. The percentage of downregulated genes that were occupied by OGT, H2B Ser 112 GlcNAc and TET2 was significantly higher than that of genes not occupied by OGT, H2B Ser 112 GlcNAc and TET2 (Supplementary Fig. 17). To validate these results, we performed qPCR to confirm the downregulation of a number of OGT, H2B Ser 112 GlcNAc and TET2 target genes (Fig. 3f and Supplementary Fig. 18). In addition, we reconstituted TET2-depleted ES cells with the His1382Tyr/Asp1384Ala mutant that abolishes the enzymatic activity of TET2. The His1382Tyr/Asp1384Ala mutant could not restore 5hmC at the TSS of a set of the TET2 and OGT common target genes. However, the His1382Tyr/Asp1384Ala mutant restored H2B Ser 112 GlcNAc and partially rescued the TET2 and OGT target gene expression (Supplementary Fig. 19). These results indicate that OGT-dependent histone O-GlcNAcylation contributes to TET2-dependent gene transcription.

Taken together, here we have shown that TET2 and OGT form a complex, which might regulate gene transcription (Supplementary Fig. 20). Besides TET2, TET3 also interacts with OGT. Like TET2, TET3 might also target OGT to chromatin for gene transcription regulation. Owing to the different tissue distribution, it is TET2 that targets OGT to the chromatin and regulates H2B O-GlcNAcylation in ES cells. Recently, it has been reported that H2B O-GlcNAcylation is associated with active TSS and positively regulates transcription. However, 5hmC-enriched regions are associated with both transcriptional activation and repression. Because TET1 is associated with the transcriptional repression complex Sin3A, it is likely that TET1-dependent 5hmC is associated with transcriptional repression. By contrast, TET2 exists in a totally different complex with OGT but not Sin3A. Thus, the TET2–OGT complex might be involved in transcriptional activation in ES cells. Further analysis on each individual 5hmC-enriched locus could distinguish the identity of 5hmC in transcription regulation. Moreover, OGT also affects other histone modifications during mitosis. It is possible that chromatin remodelling regulated by histone GlycNAcylation could be a general phenomenon in other biological processes. In addition to the transcription activation,
OGT is also involved in transcription repression\(^{19,29,30}\). Thus, TET2 is only one of the functional partners of OGT. OGT in other complexes may have an important role in gene silencing\(^{19,29,30}\).

**METHODS SUMMARY**

Details of cell cultures, plasmids, RNA interference and antibodies used, as well as descriptions of methods for purification of TET enzymes associated proteins, western blotting, immunoprecipitation, mass spectrometry analysis, in vitro 5hmC assay, dot blotting assay, silver staining, in vitro O-GlcNAcylation assay, histone fraction, electrophoretic mobility shift assay, chromatin immunoprecipitation assay, DNA immunoprecipitation assay, ChIP sequencing, messenger RNA analysis and statistical analyses are provided in Methods.

**Full Methods** and any associated references are available in the online version of the paper.

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**Author Information** The ChIP-seq and microarray data have been deposited in the Gene Expression Omnibus under accession number GSE41720. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to X.Y. (xiayu@umich.edu).
METHODS

Plasmids and antibodies. For protein affinity purification and other analyses, full-length complementary DNAs of human TET1, TET2 and TET3 were cloned into the p5-Flag-SBP (SBP) vector. For generating internal-deletion mutants, OGT cDNA fragments were PCR amplified and cloned into pCMV-Myc. For generating truncation mutants, TET2 cDNA was PCR amplified and cloned into the SBP vector. TET2CD (amino acids 916–2192) was cloned into the SBP vector and pFast-Bac vector. Internal-deletion mutants of OGT, enzymatic-dead mutant (Gly482Ser) of OGT and enzymatic-dead mutant (Hsl1382Tyr/Asp1384Ala) of TET2 were generated using the QuikChange site-directed mutagenesis kit (Stratagene).

Purification of TET-associated proteins. To search for binding partners of TET2 and TET3, we collected 1 l 293T cells stably expressing SBP–TET2 and SBP–TET3, respectively, and washed the cell pellets with PBS. Cells were lysed with 30 ml ice-cold NETN300 buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA and 300 mM NaCl). The soluble fraction was incubated with 0.5 ml streptavidin-conjugated agarose beads. The beads were washed with NETN100 buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA and 100 mM NaCl) three times. Associated proteins were eluted with 2 mM biotin (Sigma) in PBS, and further incubated with 50 ml S-protein–agarose beads (Novagen). The bound proteins were eluted with SDS sample buffer (20% glycerol, 120 mM Tris-HCl, pH 6.8, 4% SDS, 0.02% bromophenol blue and 2.5% beads). The beads were washed with NETN100 buffer (50 mM Tris-HCl, pH 7.4, cold NETN300 buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA and 300 mM NaCl). The insoluble pellets were collected, washed with Millipore. Twelve mononucleotide antibody against O-GlcNAc was purchased from Abcam. CTD110.6 mononucleotide antibody against O-GlcNAc was purchased from Covance. Mononuclear antibody against H2B Ser 112 GlcNAc was generated as described previously38.

Purification of TET-associated proteins. To search for binding partners of TET2 and TET3, we collected 1 l 293T cells stably expressing SBP–TET2 and SBP–TET3, respectively, and washed the cell pellets with PBS. Cells were lysed with 30 ml ice-cold NETN300 buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA and 300 mM NaCl). The soluble fraction was incubated with 0.5 ml streptavidin-conjugated agarose beads. The beads were washed with NETN100 buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA and 100 mM NaCl) three times. Associated proteins were eluted with 2 mM biotin (Sigma) in PBS, and further incubated with 50 ml S-protein–agarose beads (Novagen). The bound proteins were eluted with SDS sample buffer (20% glycerol, 120 mM Tris-HCl, pH 6.8, 4% SDS, 0.02% bromophenol blue and 2.5% β-mercaptoethanol), and analysed by 10% SDS–PAGE and mass spectrometry. Cells expressing empty vector were used as purification controls.

Silver staining. Purified materials were load onto 8% SDS–PAGE for electrophoresis. The gel was fixed with 50% methanol, 10% acetic acid for 15 min, and followed by further fixation with 10% ethanol, 5% acetic acid for 6 min. For staining, the gel was agitated in 50 ml 20 mg/mL Na2S2O4 for 30 min, then stained in 50 ml AgNO3 solution (100 mg AgNO3 in 0.1% formaldehyde) for 30 min. After washed with ddH2O, the gel was treated with 50 ml image developer (3% Na2CO3, 2.5% H2O2, 0.2% sodium thiosulphate). The reaction was stopped using stop solution (12.5 ml acetic acid and 25 g Tris in 500 ml ddH2O) when clear bands were observed.

Recombinant proteins. Recombinant proteins were purified from S9 insect cells. For generating baculovirus, DNA fragments containing full-length OGT, and deletion mutants of OGT, TET2CD and TET3CD were subcloned into the pFast-Bac vector with a GST or SBP tag. Baculoviruses were generated according to manufacturer’s instructions (Invitrogen). After S9 cells were infected with baculoviruses for 48 h, the cells were collected, washed with PBS and lysed with ice-cold NETN100 buffer. The soluble fraction was incubated with glutathione–sepharose beads (for GST–tag proteins) or streptavidin–conjugated beads (for SBP–tag proteins) and eluted with glutathione or biotin, respectively. To purify protein complex, S9 cells were co-infected with baculoviruses encoding GST–TET2CD and SBP–OGT for 48 h. The cells were then lysed and the soluble fraction was incubated with glutathione–sepharose beads or streptavidin–conjugated beads as indicated.

Cell lysate immunoprecipitation and western blotting. For immunoprecipitation, cells were lysed with ice-cold NETN300 buffer containing 10 mM NaF and 50 mM β-glycerophosphate. Supernatants were incubated with indicated antibodies and protein-G–conjugated sepharose beads (Amersham Pharmacia). Precipitates were washed five times with NETN100, subjected to SDS–PAGE and then subjected to autoradiography after incubation with EN3HANCE (PerkinElmer) and pVSVG (con). Tissue culture supernatants from 293T packaging cells transacted with the above plasmids were collected. Viral particles were centrifuged at 35,000 g for 3 h and then added to the culture media of ES cells. The virus-infected cells were selected by puromycin for 72 h and then used in the following experiments. Similar results were obtained from shRNA treatment with different sequences.

In vitro O-GlcNAcylation assay. Recombinant SBP–OGT protein (0.5 μg) was incubated with 2 μg substrates (histone octamers, mono-nucleosomes or 210-bp Smc-containing oligonucleotides with sequence listed in Supplementary Table 7) and 0.5 mM (0.5 μCi) UDP-[3H]GlcNAc (from ARC) in 25 μl reaction buffer (50 mM Tris-HCl, pH7.5, 12.5 mM MgCl2 and 1 mM dithiotreitol (DTT)) for 1 h at 37 °C. The reaction was stopped by 10 mM EDTA. To detect potential DNA glycosylation, DNA was released down by streptavidin beads. The beads were subjected to scintillation counter measurement.

β-glucosy tranferase (Zymo Research) was used as a positive control to produce GlcNAcylated Smc (5hmC) following the protocol of Zymo Research, with UDP-[3H]GlcNAc as the donor. For histone glycosylation analyses, the reaction was resolved with SDS–PAGE, and then subjected to autoradiography after incubation with EN3HANCE (from PerkinElmer). Nucleosome packaging was performed as described below with recombinant core histones purified from bacteria.

Electrophoretic mobility shift assay. The 157-bp DNA oligonucleotide (Supplementary Table 7) containing 5mC was produced by PCR using dATP, dGTP, dCTP and dTTP. The oligonucleotide was [32P]-labelled at the 5′ end by T4 DNA kinase and purified using a G50 spin column (Pharmacia Biotech). The labelled oligonucleotide (0.1 pmol) was incubated with GST (0.4 μg), GST–OGT (0.4 μg) or GST–TET2CD (0.1, 0.2 or 0.4 μg), in reaction buffer (50 mM HEPES, pH 7.5, 10 mM MgCl2, 50 mM NaCl, 75 mM KCl, 50 μM Fe(II)Cl2, and 1 mg/ml -1 bovine serum albumin (BSA)) for 30 min at room temperature. The samples were loaded onto 6% native polyacrylamide gel and run at 150 V in 0.5X Tris-borate-EDTA (TBE) buffer. The gels were then fixed with 30% methanol and 10% acetic acid and exposed to X-ray film.

For electrophoretic mobility shift assays using mono-nucleosomes, we generated oligonucleotides containing cytosine or 5mC by PCR. PCR products were end-labelled with [32P] and purified by gel electrophoresis. To generate mono-nucleosomes, labelled DNA oligonucleotides were incubated with core histones at a 1:1 molar ratio in a buffer containing 2 mM NaCl and 1 mM DTT to a total volume of 30 μl. Mixtures were then serially diluted against 0.4 M KCl and 0.2 mM NaCl in 10 mM Tris-HCl, pH7.5, 1 mM EDTA and 1 mM DTT for 4–10 h at each step. Mono-nucleosomes were separated from free DNA by ultra-centrifugation in 5–30% linear glycerol gradients. After centrifugation at 150,000 g in a SW50.1 rotor for 18 h at 4 °C, gradient fractions were collected. Fractions
containing mono-nucleosomes were pooled, and dialysed against 10 mM Tris-HCl, pH 8.0, and 1 mM DTT. For nucleosome-binding reactions, GST–OGT (0.2 µg) and GST–TET2CD (0.05, 0.1 or 0.2 µg) were incubated with 20 nM mono-nucleosomes in 20 µl, with final buffer conditions of 50 mM HEPES, pH 7.9, 10 mM MgCl2, 75 µM Fe(NH4)2, 50 mM NaCl, 1 mM DTT, 1% glycerol and 1 mM 3′-BSA. The reactions were incubated at room temperature for 1 h. Binding reactions were loaded onto 4% native polyacrylamide gels at 150 V in 0.5× TBE buffer. After electrophoresis, gels were dried and examined by autoradiography.

ChIP assay and DNA immunoprecipitation assay. ChIP assays were performed according to the protocol described by Upstate. ES cell DNA was sonicated to an average size between 300 and 600 bp. Solubilized chromatin was immunoprecipitated with antibodies against TET2, OGT, H2B Ser 112 GlcNAc, H3K4me3 and H3K27me3. Antibody–chromatin complexes were pulled-down using protein A–sepharose, washed and then eluted. After cross-link reversal and proteinase K treatment, immunoprecipitated DNA was extracted with phenol–chloroform, ethanol precipitated and treated with RNase. ChIP DNA was quantified using PicoGreen.

For hydroxymethylated DNA immunoprecipitation assay, genomic DNA was extracted using the Qiagen QIAamp DNA mini kit. The purified genomic DNA was sonicated using Diagenode Bioruptor 200. The DNA fragment size was determined by 2% agarose gel, and should be 200–800 bp. The samples were heat-denatured and mixed with 1 µg 5hmC antibody. The mixtures were incubated overnight at 4 °C and then pulled down by protein A–sepharose beads. The beads were extensively washed and digested by proteinase K. DNA was further purified by phenol–chloroform extraction and ethanol precipitation. 5hmC immunoprecipitated DNA was quantified using PicoGreen.

ChIP sequencing. DNA fragments isolated from ChIP were repaired to blunt ends by T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase using the END-IT kit (Epicentre). A single ‘A’ base was added to the 3′ end with Klenow. Double-stranded adapters (75 bp with a ‘T’ overhang) were ligated to the fragments with DNA ligase. Ligation products between 200 and 600 bp were gel-purified to remove unligated adapters and subjected to 20 PCR cycles. Completed libraries were quantified with PicoGreen. For ChIP-seq analyses, the DNA libraries were analysed by Solexa/Illumina high-throughput sequencing. The quality of each sample was determined by FastQC software. After prefiltering the raw data by removing sequence adapters and low-quality reads, the tags were mapped to the mouse genome (assembly mm9) by Bowtie software. Parameter settings were listed as follows: --v, 3 (reported alignments with at most three mismatches), --5, 3 and --3, 3 (trim3 bases from 5′ and 3′ end to remove low-quality bases). Peak detection was performed using MACS software from Galaxy browser (http://www.galaxy.psu.edu). Parameters settings were as follows: IgG ChIP-seq aligned reads were used as control file, tag size with 25 bp, band width with 300 bp. Venn diagram analysis was performed with Galaxy browser.

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Dot blotting assay. Genomic DNA isolated from 293T cells expressing TET2, OGT or both was denatured by 0.2 N NaOH and dotted on Hybond-N+ nitrocellulose membrane (Amersham Pharmacia Biotech). After ultraviolet cross-linking, membranes were blocked overnight with 10% non-fat milk and 1% BSA in TBST (150 mM NaCl, 10 mM Tris, pH 8.0, and 0.1% Tween20) at 4 °C followed by 1 incubation with either anti-5mC or anti-5hmC antibodies at room temperature. Membranes were washed four times with TBST, incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit antibodies (GE Healthcare), washed with TBST and developed using the ECL+ detection system (GE Healthcare).

Mass spectrometry analysis. For 5ghmC standard, β-glucosyltransferase was used to produce 5ghmC following the protocol of Zymo Research using UDP-GlcNac as the donor. The DNA was digested with DNA degradease plus (Zymo Research). The samples were subjected to liquid chromatography tandem mass spectrometry (LC–MS/MS). Liquid chromatography was performed using a 2.1 × 50 mm HSS T3 1.8 µm column (Waters), with gradient elution at a flow rate of 500 µl/min using 0.02% acetic acid in water as mobile phase A and methanol as mobile phase B. The gradient was (96% A + 4% B) to (70% A + 30% B) in 4.7 min. The elutes were directed to the mass spectrometer that was running in the product scan mode (60–500 m/z) selecting 461 as precursor ion (5ghmC). The collision energy used was 30 V.

For detecting 5ghmC or glucosylated-5hmC, genomic DNA from 293T cells expressing TET2 and OGT was digested as above. The digestes and products subjected to LC–MS/MS analysis using the same column and conditions as above. The mass spectrometer was running in multiple reaction monitoring mode, monitoring the transition of m/z 242.0 to 126.0 (5mC), m/z 258.0 to 142.0 (5hmC), 461.0 to 345.0 (5ghmC) and m/z 420.0 to 304.0 (glucosylated-5hmC), with the collision energy as 15 V.

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